Molecular and immunological characterization of a novel timothy grass (*Phleum pratense*) pollen allergen, Phl p 11

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Summary

Background Allergy to grass pollen is typically associated with serum IgE antibodies to group 1 and/or group 5 allergens, and additionally often to one or several less prominent allergens. Most of the grass pollen allergens identified to date have been characterized in detail by molecular, biochemical and immunological methods, timothy grass being one of the most thoroughly studied species. However, a 20-kDa allergen frequently recognized by IgE antibodies from grass pollen allergics has so far escaped cloning and molecular characterization.

Objective To clone and characterize the 20 kDa timothy grass pollen allergen Phl p 11.

Methods Phl p 11 cDNA was cloned by PCR techniques, utilizing N-terminal amino acid sequence obtained from the natural allergen. Phl p 11 was expressed as a soluble fusion protein in *Escherichia coli*, purified to homogeneity and used for serological analysis and to study Phl p 11 specific induction of histamine release from basophils and skin reactivity in sensitized and control subjects.

Results Phl p 11 cDNA defined an acidic polypeptide of 15.8 kDa with homology to pollen proteins from a variety of plant species and to soybean trypsin inhibitor. The sequence contained one potential site for N-linked glycosylation. Serological analysis revealed that recombinant Phl p 11 shared epitopes for human IgE antibodies with the natural protein and bound serum IgE from 32% of grass pollen-sensitized subjects ($n = 184$). Purified recombinant Phl p 11 elicited skin reactions and dose-dependent histamine release from basophils of sensitized subjects, but not in non-allergic controls.

Conclusion As the first representative of group 11 grass pollen allergens, Phl p 11 has been cloned and produced as a recombinant protein showing allergenic activity. One-third of grass pollen-sensitized subjects showed specific IgE reactivity to recombinant Phl p 11, corresponding in magnitude to a significant proportion of specific IgE to grass pollen extract.

Keywords allergen, allergy, grass pollen, IgE antibody, recombinant protein
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Introduction

A hallmark of atopic allergy is the formation of IgE antibodies to proteins present in the sensitizing biological material. Upon contact with the allergen source, these proteins will act to cross-link IgE antibodies present on the surface of mast cells, thereby eliciting the release of inflammation mediators such as histamine. As a result, an allergic reaction occurs [1].

In the industrialized world, up to 10% of the human population shows allergic sensitization to grass pollen, making this one of the most important airborne allergen sources [2]. Considerable efforts have been made towards the characterization of pollen allergens from a variety of grass species using biochemical and immunological methods. A number of IgE

binding proteins have thus been identified that exhibit conserved structure and serological cross-reactivity between species. Based on these criteria, such immunologically related grass pollen allergens have been assigned to groups designated by numbers. These include group 1, group 2/3, group 4 and group 5 allergens, which are represented in pollen of most grass species [3].

With the application of molecular biology to this area of research, the primary structure of a significant number of important allergens from different sources has been established during recent years. To date, seven different allergens from timothy grass (*Phleum pratense*) pollen have been cloned: Phl p 1 [4, 5], Phl p 2 [6], Phl p 5 [7–9], Phl p 6 [10, 11], Phl p 7 [12], Phl p 12 (profilin) [13] and Phl p 13 [14]. These allergens have all been produced as recombinant proteins that, by different *in vitro* and *in vivo* activity assays, have been shown to share immunological and allergenic properties with their native counterparts.

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Using a panel of four recombinant allergens (rPhl p 1, rPhl p 2, rPhl p 5 and profilin) in serological and skin testing procedures, positive results were obtained in 95% of a large population of grass pollen-allergic individuals [15]. Sensitization to allergens such as Phl p 7 (a calcium-binding, two-EF-hand protein) and Phl p 12 occurs in a smaller proportion of grass pollen allergics, but they share IgE epitopes with homologous proteins present in pollen of trees and weeds and can therefore cause immediate-type symptoms in sensitized individuals upon contact with these unrelated allergen sources [12, 16, 17].

In the present study we describe the identification and cloning of a cDNA coding for a new timothy grass pollen allergen, Phl p 11, with sequence homology to pollen proteins of other grasses as well as unrelated plant species, including known allergens, and to soybean trypsin inhibitor. We further report the high-level expression in *Escherichia coli* and purification of recombinant Phl p 11 and its immunological characterization. The IgE binding properties of rPhl p 11 were evaluated using a large panel of sera from grass pollen-sensitized subjects and we demonstrate the capacity of the recombinant allergen to induce histamine release from human basophils and to elicit immediate type skin reactions. The potential utility of recombinant Phl p 11 for diagnostic and therapeutic purposes is discussed.

Materials and methods

General reagents, plasmids, oligonucleotides, bacterial strains and antibodies

Salts and buffers were purchased from Sigma (St Louis, MO, USA) and Fluka (Buchs, Switzerland). Pollen from timothy grass (*Phleum pratense*) was obtained from Pharmacia Allergon AB (Välinge, Sweden). Protein analysis by SDS-PAGE was performed using 4–20% gradient Tris-glycine gels (Novex, San Diego, CA, USA) and for electroblotting Hybond-C Extra membrane (Amersham Life Science, Amersham, UK) was used. For immunoblot analysis of IgE binding, rabbit anti-IgE antiserum (MIAB, Uppsala, Sweden) and horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Life Science) were used, followed by ECL detection (Amersham Life Science). Preparation of polyadenylated RNA from total RNA and subsequent synthesis of cDNA for RT-PCR were performed using the mRNA Purification Kit and the First-strand cDNA Synthesis Kit, both from Amersham Pharmacia Biotech (Uppsala, Sweden). Plasmids pET-23a(+) and pMAL-c2 were purchased from Novagen (Madison, WI, USA) and New England Biolabs (Beverly, MA, USA), respectively. Restriction endonucleases *Eco*RI, *Hin*dIII, *Nde*I and *Xho*I, as well as *Taq* DNA polmerase and deoxynucleotides were from Amersham Pharmacia Biotech. *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA, USA). DNA from PCR and other enzyme reactions was purified using appropriate Wizard kits from Promega (Madison, WI, USA). For solid phase capture of biotinylated PCR products, streptavidin-modified magnetic beads (M-280) from Dynal AS (Skøyen, Norway) were used.

For large-scale plasmid preparation, the Plasmid Maxi Kit from Qiagen (Düsseldorf, Germany) was used. Oligonucleotides were obtained from Scandinavian Gene Synthesis (Köping, Sweden). DNA sequencing was performed using the T7 Sequencing Kit from Amersham Pharmacia Biotech and

[α-^{35}S]dATP from Amersham Life Science. The *E. coli* strains used were XL1-Blue MR (Stratagene) for cloning purposes and BL21 (Novagen) harbouring plasmid pT7POL23 [18] for expression. HiTrap Chelating columns (Amersham Pharmacia Biotech) were used for immobilized metal ion affinity chromatography (IMAC). Buffer exchange and size exclusion chromatography of protein preparations were performed using an FPLC system and columns packed with Sephadex G-25 and Superdex 75, respectively (Amersham Pharmacia Biotech). Quantitative serology for the recombinant allergen was established using Pharmacia CAP System (Pharmacia Diagnostics, Uppsala, Sweden), employing reagents and procedures as recommended by the supplier. For IgE detection in immunoblot inhibition experiments, an ^{125}I-labelled anti-human IgE antibody from Pharmacia Diagnostics was used. Histamine release from isolated granulocytes of allergic and healthy individuals was measured by a radioimmunoassay (Immunotech, Marseille, France). As a positive control for histamine release capacity of cells, the monoclonal anti-IgE antibody E124.2.8 Dε2 (Immunotech) was used. Histamine and sodium chloride solution for skin prick tests were obtained from ALK (Hørsholm, Denmark).

Patient samples

A total of 188 grass pollen-allergic subjects or serum samples were examined in this study. One hundred and fifty serum samples were from an in-house collection at Pharmacia Diagnostics, selected on the basis of IgE sensitization to *P. pratense*. Thirty-eight subjects were from a Vienna clinic and were characterized by case history indicative of grass pollen allergy, positive radioallergosorbent test (RAST) result for timothy grass pollen, and positive skin prick test to grass pollen extract. The allergen sensitization profiles of these subjects were established with natural and recombinant timothy grass pollen allergens as described [19]. Serum samples from two non-allergic individuals were included for control purposes.

Protein extracts, SDS-PAGE and immunoblot analysis

Phleum pratense pollen was extracted at room temperature for 2 h in 5 mL of distilled water per gram of pollen. After centrifugation for 5 min at 13 000 *g*, the clear supernatant was divided into small aliquots and stored at − 20 °C until use. The pollen extract was subjected to reducing SDS-PAGE and either stained with Coomassie Brilliant Blue or electroblotted onto nitrocellulose membrane. Protein blots were blocked for 1 h at room temperature using either 1% (v/v) Tween-20 in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$) or 5% (w/v) defatted dry milk in PBS and then incubated overnight with patient serum diluted fivefold in PBS containing 0.1% Tween-20. After washing in the same buffer, bound IgE was visualized using a rabbit anti-IgE antiserum followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG and ECL detection.

Protein sequencing

An IgE-binding protein band corresponding to Phl p 11 was identified by immunoblotting using an essentially monoreactive serum sample. The band was excised from a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel, homogenized and extracted in 6 M guanidinium hydrochloride. After removal of

polyacrylamide fragments by centrifugation, the extracted protein was subjected to 20 cycles of sequencing from the N-terminus using a Hewlett-Packard G1000A instrument (Hewlett-Packard Co., Palo Alto, CA, USA).

Cloning and characterization of Phl p 11 cDNA

Polyadenylated RNA was isolated from total RNA of *Phleum pratense* pollen, prepared by the guanidinium isothiocyanate method of Chirgwin et al. [20]. Phl p 11 cDNA was generated by 3′-RACE, performed essentially according to Frohman [21], and RT-PCR, using cloned *Pfu* DNA polymerase throughout the experiment. All thermocycler reactions were carried out in the following reagent conditions: 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 mg/mL BSA, 0.1% Triton X-100, 10% DMSO, 0.4 mM dNTP and primers at 0.5 μM each.

First-strand cDNA was synthesized from purified poly-A^+ RNA using the primer 5′-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GC(T)$_{18}$–3′ (Q_T). For 3′-RACE, the two nested specific forward primers GSP-1 (5′-CAT TAC ATA TGG ACA AGG GCC CSG GCT TCG TSG TSA C−3′) and GSP-2 (5′-CAT GAA TTC GGA CGC GTC TAC TGC GAC-3′) were used, together with the two nested universal reverse primers Q_O (5′-CCA GTG AGC AGA GTG ACG-3′) and Q_I (5′-GAG GAC TCG AGC TCA AGC-3′). Primers GSP-1 and GSP-2 were designed from the N-terminal amino acid sequence of the Phl p 11 protein while primers Q_O and Q_I were identical to adjacent parts of cDNA synthesis primer Q_T.

To generate a Phl p 11-enriched template for 3′-RACE, second-strand cDNA was synthesized by 40 cycles of primer extension of biotinylated GSP-1 using first-strand cDNA as template. The cycling profile used was: 95 °C/5 min, followed by 40 cycles of 95 °C/60 s, 58 °C/60 s, 72 °C/90 s. The product of this reaction was then immobilized on streptavidin-modified magnetic beads and washed with 0.1 M NaOH and TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). In the first round of 3′-RACE, a sample of immobilized second-strand cDNA and primers GSP-1 and Q_O were used in the cycling profile: 95 °C/5 min, followed by 40 cycles of 95 °C/1 min, 72 °C/2 min. One μL of a 20-fold dilution of this reaction was used as template together with primers GSP-2 and Q_I in the second round of 3′-RACE, with the cycling profile: 95 °C/5 min, followed by 30 cycles of 95 °C/60 s, 58 °C/60 s, 72 °C/90 s. The GSP-2 and Q_I primers were designed to incorporate *Eco*RI and *Hin*dIII sites, respectively, at the ends of the amplification product. After purification and cleavage with these enzymes, the product was cloned between the *Eco*RI and *Hin*dIII sites of pBR322 [22]. Five candidate clones were subjected to DNA sequencing, revealing a single open reading frame corresponding to Phl p 11.

Amplification of full-length Phl p 11 coding sequence from immobilized second-strand cDNA was performed using the GSP-1 primer and the reverse primer PP11/R-X (5′-AGT CAC TCG AGT GGC GTC TCG GGG GCG TC-3′), which was based on the 3′ end of the Phl p 11 open reading frame. These two primers were designed to incorporate terminal *Nde*I and *Xho*I sites, respectively, in the PCR product. The thermocycling profile used in this reaction was the same as that in round two of the 3′-RACE experiment. The amplification product, purified and digested with *Nde*I and *Xho*I, was cloned between the *Nde*I and *Xho*I sites of a pET-23a(+)-

derivative designed for expression of the gene of interest as a fusion to the maltose binding protein (MBP) of *E. coli*. The resulting full-length construct for expression was verified by DNA sequencing.

DNA and amino acid sequence analyses, including translations, protein property predictions and sequence comparisons, were performed using programs of the Wisconsin Package (Genetics Computer Group, Madison, WI, USA).

Phl p 11-homologous amino acid sequences were retrieved from publicly available databases. Selected sequences were aligned using CLUSTAL W [23]. The GDE sequence editor (S. Smith, Harvard University) and COLORMASK (J. Thompson, EMBL Heidelberg) were used to colour conserved residues with related properties [23]. Protein secondary structure and solvent accessibility predictions were made using the PHD program on the EMBL PredictProtein server [24, 25].

Expression and purification of rPhl p 11

The *P. pratense* allergen was expressed in *E. coli* as a fusion to MBP. Plasmid DNA from one selected clone was introduced into strain BL21 harbouring plasmid pT7POL23, which provides T7 RNA polymerase in a stringently controlled, temperature-dependent manner [18]. LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, adjusted to pH 7.0 using 1 M NaOH) was inoculated 1:500 with an overnight culture and first grown at 30 °C to mid-log phase. The incubation temperature was then raised to 42 °C for 1 h, followed by 4 h at 30 °C before harvest. Cells were collected by centrifugation at 10 000 *g* for 10 min at 4 °C and resuspended in 5 mL of buffer A (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 100 mM β-mercaptoethanol, 5 mM imidazole) per gram (fresh weight) of cells. The resuspended cells were ruptured by sonication while kept on ice, followed by centrifugation to remove solid material. Following exchange to buffer B (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM β-mercaptoethanol) containing 5 mM imidazole using Sephadex G-25, the supernatant was loaded onto a Ni^{2+}-charged 5 mL HiTrap Chelating column for IMAC. The column was washed with 20 mM imidazole in buffer B and elution was performed with a 20 to 250 mM gradient of imidazole in buffer B. Fractions containing the eluted fusion protein were pooled and subjected to a final step of size exclusion chromatography through Superdex 75, equilibrated and eluted with non-reducing buffer, to obtain a homogeneous, unaggregated preparation without visible contamination by *E. coli* proteins. To serve as a negative control in functional studies, MBP alone was expressed from BL21 [pT7POL23] cells harbouring the expression vector without insert and the protein purified as above, except that buffer B containing 5 mM imidazole was used in place of buffer A at the stage of cell homogenization. The concentration of MBP-Phl p 11 and MBP in the final preparations was determined from their absorbance at 280 nm, using calculated extinction coefficients of 1.30 and 1.47 per mg/mL, respectively.

Assessment of IgE-binding activity of rPhl p 11 using Pharmacia CAP System

In vitro IgE-binding activity of the purified recombinant allergen was examined using Pharmacia CAP System, an immunoassay system used for IgE antibody detection in clinical diagnosis of atopic allergy. Experimental ImmunoCAP tests were prepared by covalent immobilization of the purified

allergen onto activated cellulose at a concentration chosen to achieve an adequate linear measuring range and a background for negative sera well below the conventional cut-off value of $0.35\,kU_A/L$. Negative control tests carrying MBP alone were prepared using the same protein concentration at immobilization. For determination of specific IgE to the whole complement of natural *P. pratense* pollen proteins, the regular pollen extract-based ImmunoCAP test was used. For the purpose of comparison with a previously established recombinant allergen, all serum assays were run in parallel with rPhl p 2 ImmunoCAP tests. Assay controls and calculation of statistical parameters attesting to the quality of the assays were performed using standard assay system routines and software (Pharmacia Diagnostics).

Immunoblot analysis of IgE binding properties of rPhl p 11

The proportions of timothy grass pollen-specific IgE directed against rPhl p 11 and rPhl p 5 were investigated by a RAST inhibition-based experiment. Serum samples from 10 rPhl p 11-reactive subjects were diluted 1:10 in buffer C (50 mм sodium phosphate pH 7.5, 0.5% (v/v) Tween 20, 0.5% (w/v) BSA, 0.05% (w/v) NaN_3) and pre-adsorbed overnight at $4\,°C$ with either rPhl p 11, MBP (negative control) or rPhl p 5 (positive control), all at a final concentration of $10\,µg/mL$. To ensure conditions of antigen excess on the solid phase, approximately $0.2\,mg$ of natural timothy grass pollen protein extract was immobilized on nitrocellulose strips of exactly the same size ($0.6 \times 3\,cm$). Strips were blocked by pre-incubation with buffer C (once for 1 h and twice for 5 min) and then exposed to the pre-adsorbed sera at $4\,°C$ overnight. The following day, strips were washed four times in buffer C and probed with ^{125}I-labelled anti-human IgE antibody at room temperature overnight. Strips were then washed again four times in buffer C and dried. The amount of ^{125}I-labelled anti-human IgE antibody was determined using a gamma counter (Wallac, Turku, Finland). The percentage inhibition of IgE binding after pre-incubation of sera with rPhl p 5 or rPhl p 11 was calculated as follows: % inhibition = 100 − 100 × (cpm rPhl p 5/cpm MBP or rPhl p 11/cpm MBP).

The capacity of the recombinant allergen to bind Phl p 11-specific IgE antibodies was studied by IgE immunoblot inhibition experiments [17]. Sera from two grass pollen-allergic subjects with IgE reactivity to rPhl p 11 were pre-adsorbed with purified rPhl p 11 at $10\,µg/mL$ serum, or, for control purposes, with an equal concentration of MBP or BSA. Pre-adsorbed sera were exposed to nitrocellulose-blotted timothy grass pollen proteins separated by SDS-PAGE and bound IgE was detected as described [17].

Histamine release experiments

Granulocytes were isolated by dextran sedimentation of heparinized blood samples [26, 27] from two grass pollen-allergic subjects and one non-allergic subject. Aliquots of washed cells were incubated with a range of concentrations ($0.001\,µg/mL$, $0.01\,µg/mL$, $0.1\,µg/mL$, $1\,µg/mL$) of purified rPhl p 11, MBP, and a monoclonal anti-IgE antibody. Histamine released in the supernatant was measured by radioimmunoassay. Total histamine was determined after freeze-thawing of cells. Results were displayed as mean values of triplicate determinations and represent the percentage of total histamine.
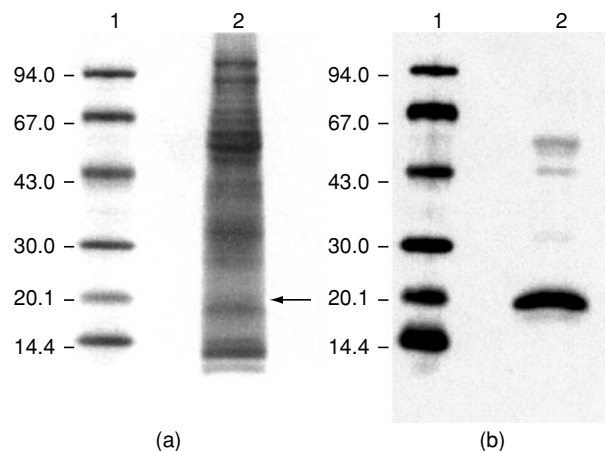
Skin testing

After informed consent was obtained from two grass pollen-allergic and four non-allergic individuals, skin prick tests were performed on their forearms as described [28]. Individuals were pricked with $20\,µL$ aliquots of solutions containing different concentrations ($0.1\,µg/mL$, $1\,µg/mL$, $10\,µg/mL$, $100\,µg/mL$) of purified rPhl p 11 and MBP, and with timothy grass pollen extract, histamine and sodium chloride. The skin reactions were recorded 20 min after sample application by photography and by transferring a ballpoint pen-tracing of the weal area to paper using adhesive tape. Mean weal diameters (Dm) were determined as follows: $Dm = 0.5 \times (D1 + D2)$ where D1 and D2 represent the largest longitudinal and transverse diameters in mm, respectively.

Results

Immunochemical detection, isolation and protein sequencing of natural Phl p 11

Immunoblot analysis of serum from a grass pollen-allergic subject, which lacked IgE antibodies to all purified or recombinant allergens from *P. pratense* currently available for serological testing (rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7 and rPhl p 12), revealed predominant IgE-binding to a single protein band at approximately 20 kDa. One faint band in Coomassie-stained SDS-PAGE aligned perfectly with the IgE-reactive band in the immunoblot analysis (Fig. 1), although a more abundant protein of slightly smaller size could not unambiguously be ruled out. Protein from both these bands was extracted separately and a portion of each applied to nitrocellulose membrane for dot-blot analysis. Incubation with the reactive serum and subsequent IgE detection allowed a positive identification of the band of slightly higher MW as the target for IgE antibodies present in the serum sample (not shown). The extracted protein was subjected to N-terminal sequencing and the following 20-residue determination was obtained: DKGPGFVVTGRVYCDPCRAG. A database search for

Fig. 1. Identification of Phl p 11 using SDS-PAGE and immunoblot analysis. (a): Pollen extract was reduced and separated by SDS-PAGE, followed by Coomassie Brilliant Blue staining. A faint band identified as Phl p 11 is marked with an arrow. (b): Immunoblot analysis of a duplicate gel, showing the binding of one patient's serum IgE antibodies. Lane 1: MW markers. Lane 2: *P. pratense* pollen extract.

homologous sequences revealed an exact match to the rye grass allergen Lol p 11, previously purified and amino acid sequenced by van Ree et al. [29].

cDNA cloning and sequence analysis of Phl p 11

After back-translation of the N-terminal amino acid sequence into DNA sequence, using the codon preference seen in other genes expressed in *P. pratense* pollen, two nested forward PCR primers (GSP-1 and GSP-2) were designed for use in 3'-RACE and RT-PCR. First-strand cDNA was synthesized from a poly-A^+ RNA preparation, using a universal oligo-dT primer carrying terminal target sequence for two nested reverse PCR primers, Q_O and Q_T, to be used in subsequent steps of amplification. Specifically enriched second-strand cDNA, generated by 40 cycles of primer extension of GSP-1 on first-strand cDNA, was used as template in the first round of 3'-RACE, carried out with primers GSP-1 and Q_O. In a second round, 1/1000 of the first round reaction was used as template together with primers GSP-2 and Q_I. Analysis of this reaction by agarose gel electrophoresis revealed two distinct bands of similar intensity, approximately 700 and 800 bp in size (not shown). The use of raised annealing temperature did not change the appearance of the second round 3'-RACE product. The double-band product was therefore tentatively considered genuine and specific. The product was cloned and transformants harbouring inserts matching both fragment sizes were identified and analysed by DNA sequencing.

All five clones examined contained inserts of nearly identical sequence and it appeared that the difference in size between the two bands seen after the second round of 3'-RACE was due to alternative sites for priming of cDNA synthesis (Fig. 2), possibly as a result of heterogeneity in the site of transcript polyadenylation. All clones contained an identical open reading frame with a codon usage that agreed well with that of previously known genes expressed in *P. pratense* pollen. Beyond the observed stop codon, none of the three forward reading frames displayed codons that fulfilled this criterion. In order to obtain a cDNA encoding the full-length polypeptide, an RT-PCR reaction was performed using forward primer GSP-1 and reverse primer PP11/R-X, the latter designed from the 3' end of the open reading frame. The product of this reaction, which appeared as a single band in agarose gel electrophoresis, was cloned in an expression vector and its sequence confirmed.

The open reading frame of the cDNA defined a polypeptide of 143 amino acid residues with a calculated isoelectric point of 4.8, a molecular mass of 15.8 kDa and one potential site for N-linked glycosylation (Fig. 2). A similarity search through the databases available at NCBI (http://www.ncbi.nlm.nih.gov) identified pollen proteins from a range of mono- and dicotyledonous plant species with sequence homology to the polypeptide deduced from the cDNA sequence. These included *Lolium perenne* (rye grass), *Phalaris coerulescens* (canary grass), *Oryza sativa* (rice), *Zea mays* (maize), *Betula pendula* (birch), *Arabidopsis thaliana*, *Lycopersicon esculentum* (tomato), *Olea europaea* (olive), *Syringa vulgaris* (lilac) and *Ligustrum vulgare* (privet). The level of amino acid sequence identity within this family of pollen proteins ranged from 32 to 95% (Table 1) and an alignment, displaying secondary structure predictions and conserved features, is shown in Fig. 3. From the sequence comparisons it is clear that the *P. pratense* allergen is

a counterpart of the *L. perenne* allergen Lol p 11 and should therefore be designated Phl p 11.

The most prominent difference in primary structure observed between Phl p 11 and Lol p 11 (sequence accession No. A54002) was a stretch of nine additional amino acid residues (–DLRDAPETP) at the C-terminus of Phl p 11, equivalent to a 1.0-kDa increment in molecular mass. In comparison with the *L. perenne* homologue, the Phl p 11 sequence contained a total of six amino acid substitutions, four of which were non-conservative (D42N, K56G, D57L, K83T). At position 103, which was not determined in the case of Lol p 11, an asparagine residue was present in the Phl p 11 sequence. The two homologues showed conservation of one potential site for N-linked glycosylation (residue 24) and six cysteine residues.

As previously shown by van Ree et al. [29], group 11 grass pollen allergens are structurally related to the soybean trypsin inhibitor and may therefore present antigenic structures similar to proteins belonging to this family. Very recently, structurally related allergens from English plantain, *Plantago lanceolata* (Pla 1 1), and goosefoot, *Chenopodium album* (Che a 1), were reported [31, 32].

The discrepancy between the observed apparent MW of the native Phl p 11 allergen by SDS-PAGE and the MW calculated from the deduced amino acid sequence is presumably explained by post-translational modification of the native allergen. In support of this is the report by van Ree et al. [29], where the homologous *L. perenne* protein was shown to carry N-linked glycosylation amounting to approximately 8% of the total molecular mass, and the conservation of the corresponding glycan attachment site in the amino acid sequence of Phl p 11.

Expression in Escherichia coli *and purification of rPhl p 11*

With the aim of allergenic and serological characterization of the Phl p 11 allergen, the protein was expressed in *E. coli* and purified to homogeneity. Because of poor solubility when the allergen was initially expressed with an N-terminal hexahistidine tag as the only engineered addition, we chose instead to produce it as a fusion to the *E. coli* maltose binding protein as a means to aid solubility. After preparing a construct where transcription of the fusion was under control of the T7 promoter, using *E. coli* XL1-Blue as a cloning host, the plasmid was transferred to strain BL21 harbouring plasmid pT7POL23. In this binary system the construct is quiescent at 30 °C and recombinant protein expression induced by a temperature shift to 42 °C (Fig. 4).

Using this strain for expression, accumulation of MBP-Phl p 11 to approximately 10% of total cellular protein was obtained, as estimated from Coomassie-stained SDS-PAGE (Fig. 4). Analysis of fractionated cellular material revealed that approximately half of the fusion protein was present in the soluble phase (not shown). The proportion of soluble protein tended to be higher when the culture had been returned to 30 °C after a period of induction at 42 °C, as opposed to being kept at 42 °C until harvest (not shown). In order to minimize aggregation of the soluble fusion protein, post-harvest processing was performed under reducing conditions. After buffer exchange to lower the concentration of reductant in the cleared cell extract, the protein was subjected to a first step of purification by IMAC. While the eluted material appeared as a single distinct band of the expected size on reducing

SDS-PAGE, analytical gel filtration indicated the presence of different aggregation forms in addition to the monomer. A step of size exclusion chromatography using Superdex 75 was therefore added to the purification process. The final preparation appeared monomeric by analytical gel filtration and free of contaminating bacterial proteins by SDS-PAGE. It appeared stable and no formation of aggregates was observed upon storage at − 20 °C. The final yield of purified protein was 12 mg per litre of bacterial culture, or 1.7 mg per gram of cell pellet (fresh weight).

Analysis of antibody recognition of rPhl p 11

To examine the IgE antibody binding capacity of the recombinant allergen and investigate the frequency and magnitude of Phl p 11-specific IgE sensitization among grass pollen-allergics, serological tests were prepared for use with Pharmacia CAP System. As a control for antibody binding to the MBP part of the fusion protein, tests carrying MBP alone were prepared and used in parallel. Upon analysis of serum samples of 184 grass pollen-sensitized subjects using these tests, 59 (32%) of them were found to contain specific IgE reactivity to the recombinant allergen (Table 2). The average level of IgE to rPhl p 11 in the specifically reactive sera was 16 kU$_A$/L, as compared to 79 kU$_A$/L of IgE to natural extract of *P. pratense* pollen. Thus, it appears that on average among these subjects, approximately 20% of the IgE reactivity to *P. pratense* pollen allergens was directed to rPhl p11.

In two of the sera that showed a positive result with the rPhl p 11 test, there was also an apparent binding of IgE to MBP alone. For one of these sera the IgE determination was in fact higher with the MBP test, and this serum was therefore regarded as lacking IgE to rPhl p 11. For the other serum, the contribution by MBP to the IgE binding by the fusion protein was only

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Asp Lys Gly Pro Gly Phe Val Val Thr Gly Arg Val Tyr Cys Asp    15
GAC AAG GGC CCG GGC TTC GTG GTG ACG GGA CGC GTC TAC TGC GAC    45

Pro Cys Arg Ala Gly Phe Glu Thr Asn Val Ser His Asn Val Gln    30
CCC TGC CGC GCC GGC TTC GAG ACC AAC GTC TCC CAC AAC GTC CAA    90

Gly Ala Thr Val Ala Val Asp Cys Arg Pro Phe Asn Gly Gly Glu    45
GGG GCG ACC GTG GCG GTG GAC TGC CGG CCG TTC AAC GGC GGC GAG    135

Ser Lys Leu Lys Ala Glu Ala Thr Thr Asp Gly Leu Gly Trp Tyr    60
AGC AAG CTC AAG GCG GAG GCG ACG ACG GAC GGT CTG GGC TGG TAC    180

Lys Ile Glu Ile Asp Gln Asp His Gln Glu Glu Ile Cys Glu Val    75
AAG ATC GAG ATC GAC CAG GAC CAC CAG GAG GAG ATC TGC GAG GTG    225

Val Leu Ala Lys Ser Pro Asp Thr Thr Cys Ser Glu Ile Glu Glu    90
GTG CTG GCC AAG AGC CCC GAC ACG ACG TGC TCC GAG ATC GAG GAG    270

Phe Arg Asp Arg Ala Arg Val Pro Leu Thr Ser Asn Asn Gly Ile   105
TTC CGC GAC CGC GCC CGC GTC CCG CTC ACC AGC AAC AAC GGC ATC    315

Lys Gln Gln Gly Ile Arg Tyr Ala Asn Pro Ile Ala Phe Phe Arg   120
AAG CAG CAG GGC ATC CGC TAC GCC AAC CCC ATC GCA TTC TTC CGC    360

Lys Glu Pro Leu Lys Glu Cys Gly Gly Ile Leu Gln Ala Tyr Asp   135
AAG GAG CCG CTC AAG GAG TGC GGC GGG ATC CTC CAG GCC TAC GAC    405

Leu Arg Asp Ala Pro Glu Thr Pro *                             143
CTC AGG GAC GCC CCC GAG ACG CCA TGA AGC CCC ACA CCA GCA CGA    450

CGT ACC ACC TAT AGT TAC TTG CCG CCG GCC GAG ACG ATG TTA CCT    495
                            ▽
CTG CGA GCC GCT GCC GGA GAG GAR ATG ACA ACC TTT TAA TGG GCC    540
                            ▽
TCA CGT GCG CCT TAA TAT TCR CGT CCT GCT TTC TCT TTT ATT CAT    585
                ▽
GTT ATT GTC TTC CTG TYC TCT AAT TAT TTA CGT GTT GAC CTA TAT    630
                                        ▼
GTG AGC TAG TTC CAA GGA TCT GTT CTA TGT GTA ATA AGA GAA CAC    675
        ▽     ▼
AAA TAT TTS GTA CGT GCA TAT CCG ATG TAT ATC CTC TTT TCG GGG    720
    ▽  ▽                                              ▽
AAA AAA AAW AYT CTG ATG TAT ATC CTC TGG ACA CAA ATT AAR TGG    765
    ▼       ▽  ▼
CCA GCT AAT GAA TTS AGT ACT (A)n                          786+n
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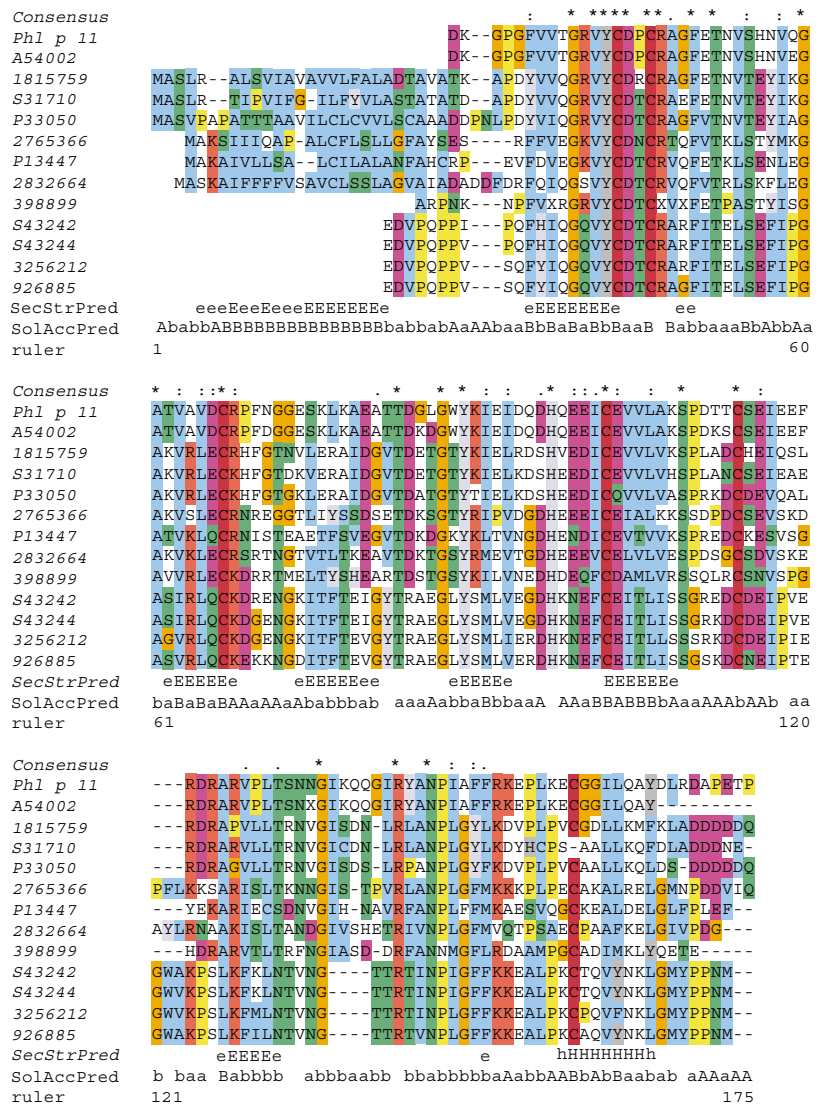
Fig. 2. cDNA and deduced amino acid sequence of Phl p 11. An open reading frame identical to all clones conformed closely with the codon preference derived from all published *P. pratense* genes expressed in pollen. The underlined nucleotide sequences represent primers GSP-1 and GSP-2. Open arrowheads indicate nucleotide differences between the clones that were analysed; the standard nucleotide ambiguity codes are used at those positions. Homopolymer stretch length variation between the clones is indicated by shading. The sequence shown represents the longest of five analysed clones, while black arrowheads mark where the other cDNAs ended in a poly-A stretch. The amino acid sequence marked by solid underlining represents the 20 residues that were determined by N-terminal microsequencing of the natural pollen protein. A single site for potential N-linked glycosylation is indicated by dotted underlining. The cDNA sequence shown will be available under the Accession No. AF521563.

Table 1. Amino acid sequence identities among the group 11 grass pollen allergens and related pollen proteins*

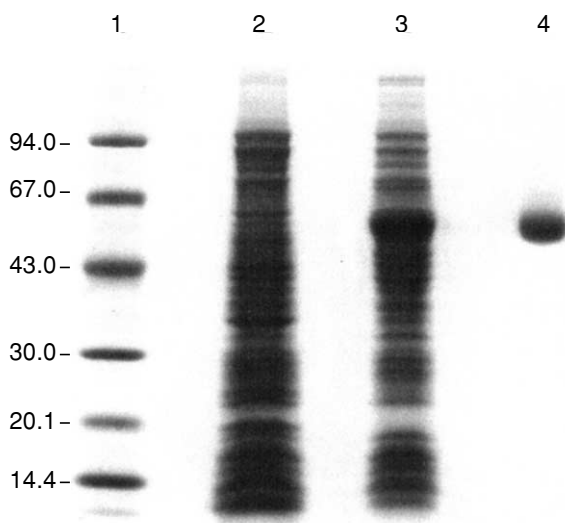| Lolium	Oryza	Phalaris	Zea	Birch	Arabidopsis/A	Arabidopsis/B	Lycopersicon	Olea	Syringa/A	Syringa/B	Ligustrum	
95%	48%	49%	45%	46%	43%	41%	40%	34%	33%	34%	34%	Phleum
	51%	49%	46%	46%	44%	40%	43%	35%	34%	34%	35%	Lolium
		76%	69%	43%	45%	46%	45%	35%	35%	35%	36%	Oryza
			79%	43%	39%	43%	40%	35%	34%	34%	36%	Phalaris
				46%	43%	44%	36%	38%	38%	38%	39%	Zea
					52%	42%	48%	40%	39%	40%	40%	Birch
						41%	46%	40%	38%	39%	40%	Arabidopsis/A
							38%	32%	32%	33%	32%	Arabidopsis/B
								39%	40%	40%	39%	Lycopersicon
									88%	87%	88%	Olea
										97%	90%	Syringa/S46
											88%	Syringa/S28

*Sequences were aligned pairwise using the Bestfit program of the Wisconsin Package and the resulting percentages of residue identity are shown. The plant species and corresponding sequence accession numbers are: *Phleum pratense*, this paper; *Lolium perenne*, A54002; *Oryza sativa*, S31710; *Phalaris coerulescens*, 1815759; *Zea mays*, P33050; *Betula pendula*, 2765366; *Arabidopsis thaliana*/A, 2832664; *Arabidopsis thaliana*/B, 398899; *Lycopersicon esculentum*, P13447; *Olea europaea*, 926885; *Syringa vulgaris*/A, S43244; *Syringa vulgaris*/B, S43242; *Ligustrum vulgare*, 3256212.

Fig. 3. Multiple sequence alignment, secondary structure and solvent accessibility prediction of Phl p 11 and structurally related proteins. Each sequence retrieved from the database is preceded by its accession number: A54002 (*Lolium perenne*), 1815759 (*Phalaris coerulescens*), S31710 (*Oryza sativa*), P33050 (*Zea mays*), 2765366 (*Betula pendula*), P13447 (*Lycopersicon esculentum*), 2832664 and 398899 (*Arabidopsis thaliana*), S43242 and S43244 (*Syringa vulgaris*), 3256212 (*Ligustrum vulgare*), and 926885 (*Olea europaea*). The consensus line derives from a Gonnet Pam250 matrix in which asterisks represent fully conserved residues. Colons and periods represent strongly and weakly conserved residue positions, respectively [30]. All entries are shown in full, except the *A. thaliana* sequence 2832664, which was truncated to show only the domain aligning with the protein family examined here. Positions marked × indicate unidentified or atypical residues. The third through to the eighth sequence include a putative N-terminal leader peptide. Hyphens indicate gaps introduced to maximize the number of aligning residues. Sequences are coloured to illustrate conservation of features additional to amino acid sequence identity. Glycine and proline residues are coloured orange and yellow, respectively, while conserved cysteine residues are shown in bright red. Other residues are coloured according to conservation of their physico-chemical properties (purple: acidic, blue: hydrophobic, grey: hydrophobic tendency, light red: basic, green: hydrophilic). The two lines below the sequences indicate predicted secondary structure (SecStrPred) and solvent accessibility (SolAccPred), both according to the PHD programme, with uppercase letters indicating predictions of high certainty. E/e and H/h indicate residues predicted to be part of β-strand and α-helix structures, respectively. A/a and B/b indicate residues predicted to be exposed to solvent or buried, respectively.

about 1%, which was considered insignificant. In total, only four sera of all 184 tested (2%) showed detectable IgE binding to MBP alone, indicating that MBP may be a suitable fusion partner for recombinant allergen production in instances when a soluble non-fusion protein cannot be efficiently produced in *E. coli*.

For the purpose of comparison, the 184 serum samples were also tested with an assay specific for a previously established major grass pollen allergen, rPhl p 2. IgE antibody reactivity directed to this allergen was found in 103 (56%) of all tested subjects, with an average IgE level of $11.4\,kU_A/L$. Binding to rPhl p 2 would thereby account for approximately 15% of the total level of IgE to whole, natural extract of *P. pratense* pollen in this subset of sera, which was $74\,kU_A/L$ on average. In summary, the serological analysis shows that the *E. coli*-expressed rPhl p 11 has significant and specific IgE antibody binding capacity, comparable in frequency and magnitude with that of rPhl p 2.

Fig. 4. SDS-PAGE analysis of recombinant Phl p 11 expression in *E. coli*. A strain prepared for expression of a MBP-Phl p 11 fusion protein was grown to mid-log phase and then subjected to a temperature shift in order to de-repress the expression system. Samples were prepared by boiling pelleted cells in loading buffer containing SDS and β-mercaptoethanol. Lane 1: MW markers. Lane 2: pre-induction sample. Lane 3: post-induction harvest. Lane 4: purified protein. Proteins were visualized by Coomassie Brilliant Blue staining.

Inhibition of IgE binding to natural grass pollen extract by soluble rPhl p 11

To compare in a more direct way the IgE binding characteristics of recombinant and natural Phl p 11, an immunoblot inhibition experiment was performed. In this analysis, competition for IgE binding to immobilized natural allergen by soluble rPhl p 11 would be visualized as attenuation of IgE binding to immobilized natural Phl p 11 after pre-incubation of patient serum with the recombinant allergen. As a control for unspecific inhibition, both serum samples used were pre-incubated with BSA and MBP in parallel with the rPhl p 11 pre-treatment. While the control proteins had no visible effect on IgE binding to extract proteins, as compared with pre-incubation with buffer (not shown), pre-treatment of the serum samples with rPhl p 11 almost completely abolished the autoradiography signal at 20 kDa molecular weight (Fig. 5). The result demonstrated that the recombinant protein shared epitopes for human IgE antibodies with natural Phl p 11.

The contribution of Phl p 11 to the total IgE binding activity of pollen proteins was further examined by dot blot inhibition experiments in which rPhl p 5, an allergen known for its high IgE binding capacity [7], was used for comparison. Equal amounts of pollen protein extract were spotted onto identical pieces of nitrocellulose membrane and exposed to patients' sera that had been pre-incubated with either rPhl p 11, rPhl p 5 or MBP. From serological analyses, these sera were known to contain IgE to both Phl p 11 and Phl p 5, but not to MBP. As controls, buffer incubation and serum from one non-allergic individual were used. After washing, membrane-bound IgE was determined radiometrically and the inhibition effects of rPhl p 11 and rPhl p 5 were calculated in relation to the MBP-pre-treated samples. The results of the experiment are shown in Table 3. On average rPhl p 11 was found to inhibit 25% of the IgE binding to pollen extract, which corresponds to the quantitative serological data shown in Table 2, while rPhl p 5 caused an average inhibition of 55%. We conclude that Phl p 11 accounts for a relevant proportion of timothy grass pollen-specific IgE antibodies, although smaller than Phl p 5.

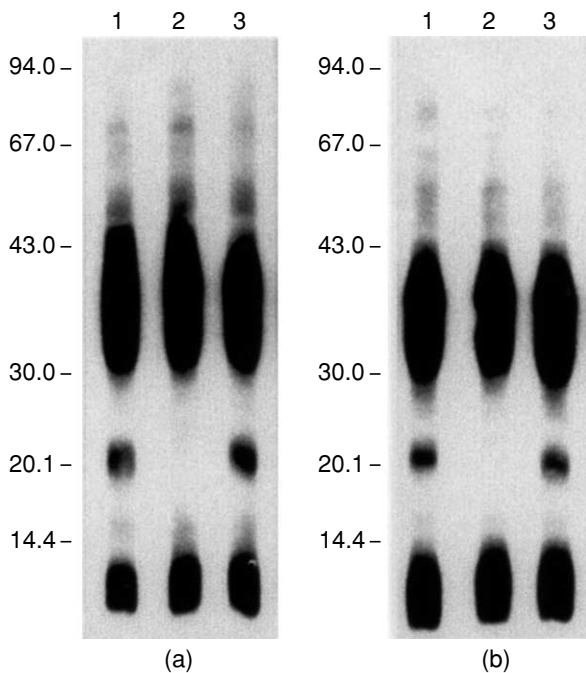## rPhl p 11 induces basophil histamine release and immediate skin reaction

Biological activity of rPhl p 11, i.e. allergenic activity in reactive tissues or on effector cells, was examined by skin tests and histamine release experiments.

Table 2. Summary of the specific IgE serology established using Pharmacia CAP System*

	Grass pollen-allergics (n = 184)				Non-allergic controls (n = 2)			
	PPE	rPhl p 11	MBP	rPhl p 2	PPE	rPhl p 11	MBP	rPhl p 2
Average (IgE) in all sera (kU_A/L)	48.6	5.27	0.18	6.40	0.16	0.18	0.13	0.12
No of reactive	184	59	4	103	0	0	0	0
% reactive	100%	32%	2%	56%	0%	0%	0%	0%
Average (IgE) in reactive subgroup (kU_A/L)	NA	16.1	2.1	11.4	NA	NA	NA	NA
Average (IgE) to PPE in reactive subgroup (kU_A/L)	NA	78.8	235	74.5	NA	NA	NA	NA
% of (IgE) to PPE	NA	20%	0.9%	15%	NA	NA	NA	NA

*Serum samples from 184 grass pollen allergics (left section) and two non-allergic individuals (right section) were analysed for specific IgE to *Phleum pratense* pollen extract (PPE), MBP-Phl p 11 (rPhl p 11), MBP alone, and rPhl p 2. Mean values of duplicate determinations are shown.
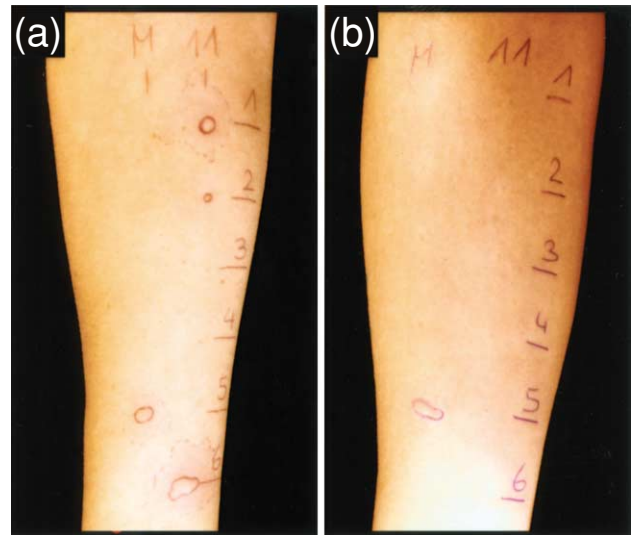
Fig. 5. Immunoblot inhibition of IgE binding to immobilized *P. pratense* extract proteins by soluble rPhl p 11. Pollen extract was separated by reducing SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was incubated with serum samples from two Phl p 11-sensitized grass pollen-allergics (a and b), after pre-adsorption with either BSA (lane 1), MBP-Phl p 11 (lane 2) or MBP (lane 3).

Fig. 6. Specific induction of immediate skin reactions by rPhl p 11. One allergic patient with IgE reactivity to rPhl p 11 (a) and one non-allergic individual (b) were skin prick tested with different concentrations (1: 100 μg/mL; 2: 10 μg/mL; 3: 1 μg/mL; 4: 0.1 μg/mL) of rPhl p 11 (11) and MBP (M), with histamine (5, left), sodium chloride (5, right) and timothy grass pollen extract (6).

Table 3. Inhibition of IgE binding to immobilized *Phleum pratense* pollen extract by soluble rPhl p 11 and rPhl p 5*

Serum ID	MBP cpm	rPhl p 11		rPhl p 5	
		cpm	% inhibition	cpm	% inhibition
155	8846	7504	15	2619	70
173	1885	960	49	779	59
174	5001	3584	28	1995	60
179	3473	2880	17	1704	51
181	12778	9694	24	5808	55
184	1446	1354	6	705	51
185	6131	4557	26	4103	33
187	759	568	25	437	42
188	12530	7760	38	3470	72
Non-allergic control	127	56	NA	66	NA
Buffer control	146	146	NA	66	NA
Mean inhibition			25%		55%

*Nitrocellulose strips carrying equal amounts of immobilized pollen protein extract were exposed to serum samples pre-incubated with soluble rPhl p 11, rPhl p 5 or MBP. After washing, membrane-bound IgE was determined radiometrically and inhibition by rPhl p 11 and rPhl p 5 was calculated in relation to the value obtained from the MBP-pre-treated serum sample. Mean values of duplicate determinations are shown.

In the skin test experiments, a grass pollen-allergic subject with specific IgE antibody reactivity to rPhl p 11 showed distinct skin reactions upon challenge with rPhl p 11 at the two highest concentrations used (10 and 100 μg/mL). In contrast, none of four non-allergic individuals tested showed a visible weal reaction at any of the allergen concentrations used, suggesting the absence of any unspecific irritational effect of the recombinant allergen in this form of challenge. Photographs of the skin tests in the allergic and one of the four non-allergic individuals are shown in Fig. 6. Another allergic subject, multi-sensitized and suffering from atopic dermatitis, was also skin tested with rPhl p 11. This subject carried a high level of IgE specific to rPhl p 11 and produced skin reactions at the three highest concentrations of rPhl p 11. Both allergics showed skin reactivity to the grass pollen extract while no reaction to MBP occurred in any of the subjects tested. All weal reactions scored in the experiment are shown in Table 4.

In the histamine release experiments, basophil-enriched cells from the blood of two allergic subjects and one non-allergic subject were used. Cells of one of the allergics showed strong and dose-dependent release of histamine, similar to that obtained with the cross-linking anti-IgE antibody. From cells of the other allergic subject, a low level of histamine release was seen, reaching saturation at 0.1 μg/mL of rPhl p 11. No induction of histamine release occurred from cells of the non-allergic individual upon challenge with rPhl p 11 (Figs 7a–c). MBP alone induced no histamine release in any of the cases.
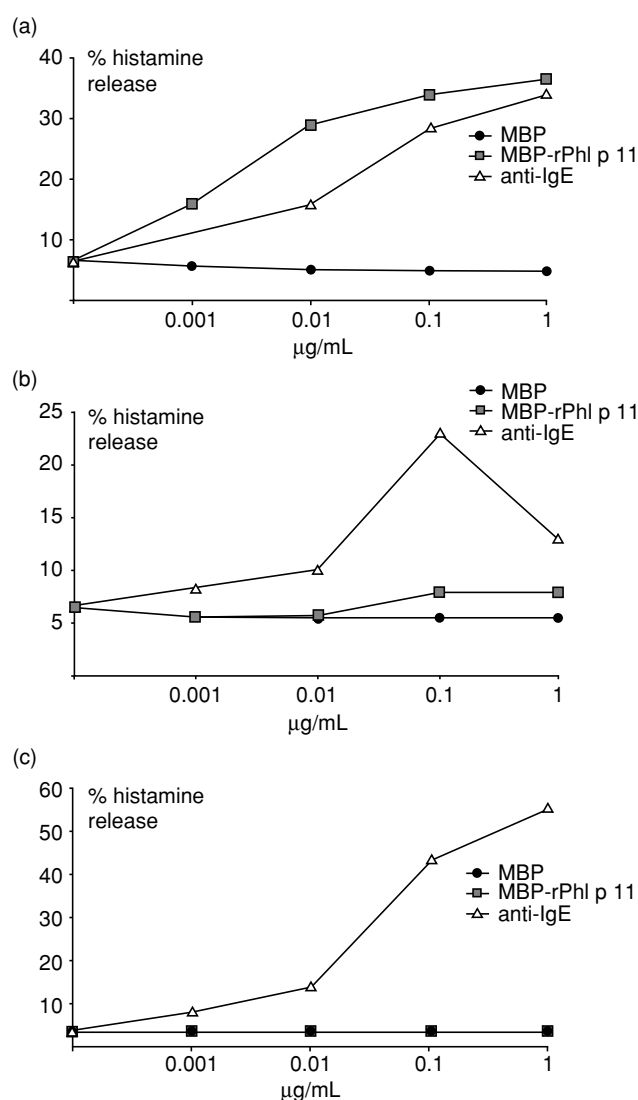
Discussion

Grass pollens belong to the most frequently sensitizing and potent allergen sources. They contain a number of allergenic molecules, several of which have been identified and characterized in the recent past [3]. In this paper we report the identification, cloning and recombinant production of a novel *P. pratense* pollen allergen, which adds new important epitopes to the growing panel of recombinant grass pollen allergens [33]. We suggest the designation Phl p 11 for this allergen, in keeping with its similarity in amino acid sequence to the rye grass

Table 4. Skin prick tests with rPhl p 11*

Subject	Mean weal diameter (mm)						
	rPhl p 11				MBP all concentrations	Pollen extract	Histamine
	100 µg/mL	10 µg/mL	1 µg/mL	0.1 µg/mL			
Allergic no. 1	5.5	2.5	–	–	–	9.0	3.6
Allergic no. 2	8.0	5.5	4.0	–	–	10.5	7.0
Non-allergic no. 1	–	–	–	–	–	–	8.0
Non-allergic no. 2	–	–	–	–	–	–	7.5
Non-allergic no. 3	–	–	–	–	–	–	4.9
Non-allergic no. 4	–	–	–	–	–	–	8.5

*Two allergics and four non-allergics were skin tested with rPhl p 11 at different concentrations, with timothy grass pollen extract and histamine. The diameters of the resulting weal reactions were determined and are presented in mm.

Fig. 7. Specific induction of histamine release from basophils by rPhl p 11. Cells from one allergic patient with high (a) and one with low (b) level of IgE reactivity to rPhl p 11, and from a non-allergic individual (c), were exposed to different concentrations of purified MBP-Phl p 11, MBP alone or anti-IgE antibody (X-axis). The top concentrations of the three proteins are equivalent to 17.1, 23.5 and 6.7 nM, respectively. Release of histamine, expressed as percentage of total histamine, is shown on the Y-axis.

allergen Lol p 11, previously characterized by protein chemical techniques [29].

The amino acid sequence deduced from the Phl p 11 cDNA was 95% identical to that of Lol p 11 and showed a lower but significant homology to a group of allergens identified in a variety of other plant species (e.g. rice, maize, birch, tomato, olive, lilac and privet). Members of this allergen group show sequence similarity with soybean trypsin inhibitor-related proteins, which is interesting in view of the notion that molecular functions of allergens relating to protein turnover or stability may in direct or indirect ways contribute to allergenicity [34].

Despite the fact that the group 11 grass pollen allergens are glycoproteins [29] and contain several cysteine residues, we were able to produce soluble, monomeric and immunologically active rPhl p 11 allergen by utilizing MBP as a fusion partner for expression in *E. coli*. The ability of MBP to promote solubility of a wide variety of 'passenger' proteins, including allergens, has been documented in numerous reports and specifically examined in several recent studies [35–38]. Different mechanisms by which MBP could prevent aggregation and inclusion body formation have been invoked, including facilitation of productive folding and blockage of non-native folding pathways by a chaperone-like activity of MBP, or sequestration of otherwise aggregation-prone folding intermediates by way of intramolecular hydrophobic interaction with surface structures on MBP. In the case of intracellular expression of recombinant single chain antibodies (scFv), fusion to MBP gave rise to a soluble and stable product with full antigen binding activity, strongly suggesting proper folding of the 'passenger' scFv such that disulphide bonds known to otherwise be critical to activity could either form spontaneously upon post-lysis air exposure or be structurally substituted by stabilizing contact with MBP [37]. Although the study reported here did not directly examine the folding state of rPhl p 11, the results demonstrate that the protein, at least in part, presents native-like determinants able to compete with the natural allergen for IgE binding.

Extensive serological characterization of IgE reactivity to rPhl p 11 was carried out using a quantitative assay system where allergen is covalently immobilized on the activated cellulose. Using the rPhl p 11-specific tests, we found that about one-third of all grass pollen-sensitized subjects analysed ($n = 184$) contained serum IgE antibodies binding to rPhl p 11 and that the magnitude of binding corresponded to a significant proportion of grass pollen-specific IgE antibodies in these

subjects. The prevalence of IgE antibodies binding to *E. coli*-expressed rPhl p 11 found in this study agrees closely with that reported by van Ree et al. [29] for chemically deglycosylated natural Lol p 11, supporting their notion that about half of the cases of IgE recognition of the natural allergen is due to its glycan moiety.

Evidence supporting the authenticity of epitope presentation by rPhl p 11 was obtained from immunoblot inhibition experiments, where natural grass pollen proteins were attached on solid phase and rPhl p 11 used as fluid-phase inhibitor. Specific and extensive inhibition of IgE binding to the natural allergen occurred in both of two patient sera examined, demonstrating that rPhl p 11 could compete with natural Phl p 11 for IgE antibody binding. Taken together, the serological data show that immunoreactive rPhl p 11 can be produced using *E. coli* expression and that the recombinant protein shares epitopes for IgE antibodies with the natural allergen. Based on the results obtained, we believe that rPhl p 11 represents an important addition to the panel of recombinant grass pollen allergens useful for *in vitro* diagnosis of grass pollen allergy.

The immunological activity of the recombinant Phl p 11 allergen, as shown by its function as solid phase reagent in immunoassay and its capacity to compete with natural Phl p 11 for IgE binding, was further analysed with respect to biological activity. In an experiment on basophils from a high-level Phl p 11-sensitized allergic individual, rPhl p 11 induced dose-dependent release of histamine, demonstrating its capacity to productively cross-link cell surface-bound IgE antibodies. Limited histamine release occurred from cells of a low-grade Phl p 11-sensitized subject and none from cells of a non-allergic upon incubation with rPhl p 11. Evidence of specific biological activity of rPhl p 11 *in vivo* was obtained from skin test experiments. In two sensitized subjects, dose-dependent weal reactions resulted from challenge with a dilution series of the allergen, while no reaction occurred in four non-allergic controls tested. The rPhl p 11 fusion partner MBP alone gave rise to no reaction in either of these experiments. Hence, rPhl p 11 exhibited biological activity that fulfilled criteria of specificity.

While the analyses performed in this study demonstrate immunological activity and specificity of bacterially expressed rPhl p 11, it is very likely that the natural allergen carries post-translational modification missing from the recombinant molecule. Although a number of examples have accumulated where bacterial expression has been successfully used to produce allergens active by different criteria, it appears that in some instances carbohydrate moieties of glycosylated allergens may facilitate the formation of IgE binding structures or even participate directly in antibody binding [29, 39–41]. Relevant to this discussion is the immunological analysis of chemically deglycosylated natural Lol p 11 reported by van Ree et al. [29], which suggested the involvement of carbohydrate structures in the IgE binding properties of this allergen. Thus, we cannot exclude that a qualitative difference in allergenic properties exists between natural Phl p 11 and the recombinant molecule described in this paper and that expression of Phl p 11 in a glycosylated form, using a eukaryotic host, could yield a recombinant allergen with different IgE binding characteristics. On the other hand, in view of recent notions that glycan epitopes may not be efficient elicitors of IgE-mediated reactions or informative in relation to clinical allergy manifestation [42–46], it is possible

that an unmodified recombinant allergen expressed in *E. coli* is more useful for diagnostic purposes.

Despite the significant sequence homology among the members of the widely represented (grasses, trees and weeds) group of allergens exemplified by Phl p 11 in timothy grass and Ole e 1 in olive tree pollen, little cross-reactivity for IgE antibodies appears to exist between them. In a preliminary analysis, we have been unable to detect cross-reaction between rPhl p 11 and Ole e 1 (Niederberger et al., unpublished data) and the results of recent studies on other members of this allergen family [31, 32] are in agreement with this observation.

One interesting implication of the apparent lack of significant cross-reactivity between rPhl p 11 and other members of this allergen family is that they may be useful as diagnostic markers to more precisely identify the primary sensitizer of allergic individuals, as compared with natural extracts or cross-reactive components such as profilin, two-EF-hand allergens or Bet v 1 homologues. Thus, a preferential IgE recognition of Phl p 11, in relation to other members of this allergen family, may suggest a primary sensitization by grass pollen rather than another allergen source containing cross-reactive components. The use of selected recombinant allergens may in this way provide information useful for advice on allergen avoidance and adequate selection of allergen extract for specific immunotherapy treatment.

In conclusion, this study is the first report on cDNA cloning and recombinant production of an IgE-reactive and biologically active group 11 grass pollen allergen. Recombinant Phl p 11 may be used to identify group 11 allergen sensitization in patients and, perhaps, in future forms of specific immunotherapy.

Acknowledgements

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