

Suspension-cultured BY-2 tobacco cells produce and mature immunologically active house dust mite allergens

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Received 3 May 2006;

revised 1 September 2006;

accepted 9 September 2006.

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Summary

The replacement of crude allergen extracts by selected allergens currently represents a major goal for the improvement of allergy diagnosis and immunotherapy. Indeed, the development of molecularly defined vaccines would facilitate both standardization and enhance batch-to-batch reproducibility as well as treatment specificity. In this study, we have investigated the potential of tobacco plant cells to produce biologically active forms of the two major allergens from the house dust mite. A detailed characterization of these plant-made allergens has shown similar proteolytic maturation and folding as well as comparable immunoreactivity to their natural counterparts. Altogether, our results exemplify that suspension-cultured BY-2 tobacco cells represent a low cost and environmentally safe expression system suitable to produce recombinant allergens from *Dermatophagoides pteronyssinus* under a form appropriate for diagnostic and therapeutic purposes.

Keywords: Allergens, secretory pathway, glycosylation, immunogenicity, allergenicity, BY-2 tobacco cells.

Introduction

Currently, specific immunotherapy for allergy is performed with crude allergen extracts and is based on systemic or mucosal application of increasing doses of the allergens to which the patient is sensitized, in order to reduce allergic reactions to those allergens (Bousquet *et al.*, 1998). However, crude protein extracts used for desensitization are poorly characterized, difficult to standardize, and contain other non-allergenic or allergenic components that could stimulate additional sensitization (Van Ree *et al.*, 1997; Niederberger and Valenta, 2004). Therefore, immunotherapy specificity needs to be improved using selected highly purified allergen(s), based on patient's immunoglobulin E (IgE) reactivity profile.

Thanks to the cloning of a large number of allergen cDNAs from various organisms over the last decade, a great variety of recombinant allergens has been produced in bacteria, yeast or insect expression systems. Recombinant allergens

produced in these heterologous hosts were tested *in vitro* and *in vivo*, but some limitations often appeared when considering their biological activity as compared to the one of their natural counterparts (Heiss *et al.*, 1996; Shoji *et al.*, 1996; Vrtala *et al.*, 1996; Soldatova *et al.*, 1998). This is particularly true for complex allergens such as group I allergens. These molecules when they are produced as a recombinant form are often very heterogeneous due to their aberrant N- and/or O-glycosylation and inefficient proteolytic maturation (Greene *et al.*, 1991; Scobie *et al.*, 1994; Nishiyama *et al.*, 1995; Jacquet *et al.*, 2002; van Oort *et al.*, 2002). Moreover, some of these recombinant allergens are expressed in the heterologous expression system mainly under an insoluble form and very often they display a weaker IgE-binding reactivity than the native allergen.

Mimicking the wild-type configuration of natural allergens is critical to applying recombinant allergens to diagnostic and therapeutic purposes. Together with grass pollen allergens,

house dust mite allergens are the most important inhalant allergens. Type I respiratory allergies against common house dust mite species such as *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae* are extremely frequent worldwide. Allergy desensitization, also referred to as specific immunotherapy, is broadly recognized as the only aetiological treatment for such IgE-mediated diseases. Hence, there is a need for safe, efficacious and well-characterized vaccines against house dust mite allergy. With the aim to develop a recombinant house dust mite vaccine, group 1 and group 2 allergens are the most critical target allergens, since they are recognized by 70%–100% of house dust mite-sensitized patients. In addition, each of these allergens binds on average 30%–40% of anti-mite IgEs in patients' sera.

Here, we have investigated the possibility to use suspension-cultured BY-2 tobacco cells for the production of the two major allergens from *D. pteronyssinus*, Der p 1 and Der p 2, with a higher reliability than previously obtained with other heterologous expression systems. Der p 2 cDNA encodes a very simple protein of 129 amino acid residues and with a molecular mass of 14 kDa. It is devoided of N-glycosylation sites and shows strong homology with the other group 2 allergens (Chua et al., 1990a,b). Der p 2 biological activity and function are unknown. Der p 1 allergen is the strongest IgE-mediated immune response inducer. In contrast to Der p 2, this cysteine proteinase is a complex protein synthesized as a preproprotein precursor of 320 amino acid residues including an 18-amino acid signal peptide, an 80-amino acid N-terminal propeptide, and two potential N-glycosylation sites on Asn 34 and Asn 150 of the preproprotein. This enzymatically inactive form is subsequently processed into a 222-amino acid mature and biologically active protease.

In the present study, Der p 1 and Der p 2 were produced in suspension-cultured BY-2 tobacco cells. After purification, their post-translational maturations, folding and immuno-reactivity were compared to their natural homologues. Both analytical and biological data demonstrated that BY-2-cells are a well-adapted expression system for the production of fully functional recombinant allergens.

Results and discussion

Post-translational maturation is very similar for natural and plant-made Der p 2

The house dust mite allergen Der p 2 (nDer p 2) was chosen as a first model in this study, particularly on the basis of its extensive analysis in regard of biological and immunological activity when expressed in bacteria or yeast systems (Chua

et al., 1990a; Lynch et al., 1994; Hakkaart et al., 1998a,b). Indeed, recombinant Der p 2 (rDer p 2) produced in *Escherichia coli* as a fusion protein with Glutathion-S-Transferase (GST) was found to be almost as active as nDer p 2, although out of the 24 patient sera tested, two did not show any IgE reactivity (Lynch et al., 1994). A non-fused rDer p 2 was also expressed in the baker's yeast, *Saccharomyces cerevisiae*. This recombinant allergen was shown to be very similar to its natural counterpart regarding its biological activity, even if 5 out of 19 nDer p 2-specific monoclonal antibodies failed to react with the recombinant (Hakkaart et al., 1998a).

In our study, the rDer p 2 and an rDer p 2-His form C-terminally fused with a hexahistidine purification tag were produced in transgenic suspension-cultured tobacco cells stably transformed with the constructs presented in Figure 1A. As illustrated in Figure 1B (lanes 2 and 4), rDer p 2 has the same electrophoretic mobility as nDer p 2, corresponding to an apparent molecular weight of approximately 14 kDa. This is also the case when rDer p 2 is expressed in tobacco plants (Figure 1B, lane 3). Interestingly, when Der p 2 is expressed in BY-2 tobacco cells as a fusion with the (His)₆ tag, we have observed that in average rDer p 2-His is about five times more abundant than Der p 2 in the crude protein extract, suggesting that the presence of this C-terminal extension stabilizes the recombinant allergen (Figure 1B, lane 5).

rDer p 2-His was purified to homogeneity from the culture medium, via a three-steps purification procedure, including differential precipitation with ammonium sulphate (50%–90% saturation) and immobilized metal affinity chromatography followed by size-exclusion chromatography (Figure 2A). The molecular masses of purified rDer p 2-His and nDer p 2 were determined by the MALDI-TOF (Matrix-Assisted Laser Desorption Ionization–Time of Flight) mass spectrometry analysis. A molecular weight of $14\,080 \pm 150$ Da was obtained for nDer p 2, which is consistent with the expected calculated molecular weight (14149.4 Da) (data not shown). The estimated molecular weight of rDer p 2-His, including the six histidine residues of the tag, is 14972.2 Da. Consistent with this estimation, the MALDI-TOF mass spectrum of rDer p 2-His showed an ion at 14931.7 ± 200 Da (Figure 2B). This result is also in perfect agreement with the molecular weight estimated from the electrophoretic mobility of rDer p 2-His after SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) (Figures 1B and 2A). A second ion at 29834.4 Da indicates that small amounts of an rDer p 2-His dimer are present in the preparation.

The analysis by circular dichroism (Figure 2D) demonstrated that the recombinant allergen expressed in tobacco cells was correctly folded compared to its natural counterpart

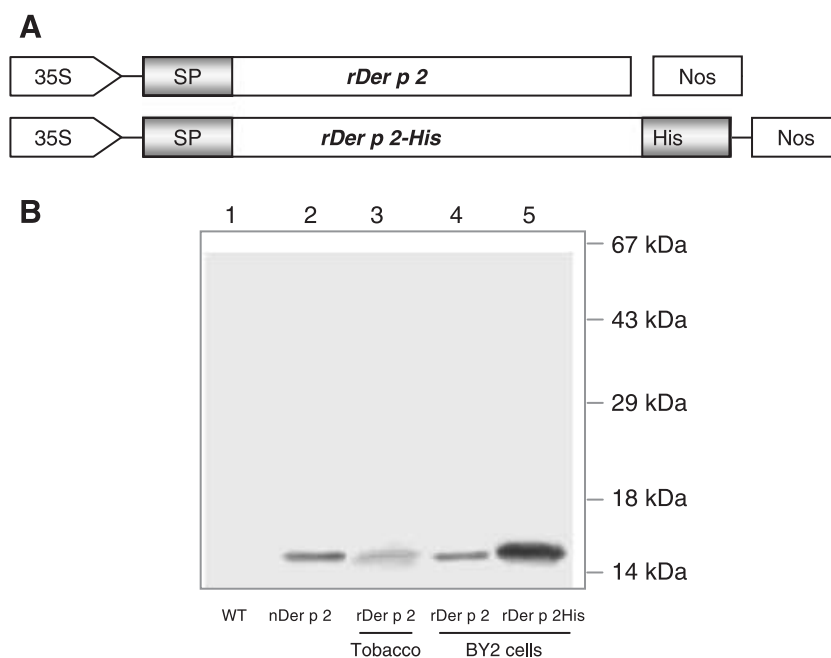


Figure 1 Production of mature Der p 2 in BY-2 tobacco cells. (A) Der p 2 cDNA allergen constructs used for transformation of BY-2 tobacco cells and tobacco plants via *Agrobacterium tumefaciens*. (B) Der p 2 expression was compared after SDS-PAGE and Western blotting in crude protein extracts from BY-2 cells expressing rDer p 2 (lane 4) or rDer p 2-His (lane 5) and a protein extract from tobacco plants expressing rDer p 2 (lane 3), (20 µg protein per lane). Controls are a crude protein extract from wild-type BY-2 cells (lane 1, 20 µg protein) and purified mite nDer p 2 (lane 2, 3 µg). Immunodetection was performed using a polyclonal serum directed against mite Der p 2.

purified from house dust mite extracts. More precisely, our data indicate that both nDer p 2 and rDer p 2-His comprise a high beta sheet content (53%), which is consistent with the two three-stranded antiparallel β sheets structure previously established by NMR studies of Der p 2 produced in *E. coli* (Mueller *et al.*, 1998). As a conclusion, the house dust mite allergen Der p 2 and the recombinant forms of this allergen expressed in BY-2 tobacco cells have similar physico-chemical properties. The only difference we have observed was in their N-terminal sequences with the two first amino acids missing in the mature form of the allergen produced in suspension-cultured tobacco cells (Figure 2C). Surprisingly, Der p 2 produced in tobacco plants has the same N-terminal sequence as the natural allergen from mites. This observation suggests that the two amino acids missing in the recombinant form produced in tobacco cell cultures probably do not result from incorrect signal sequence cleavage, but more probably from a proteolytic cleavage occurring in the culture media after secretion.

BY-2 tobacco cells produce the complex allergen Der p 1

The house dust mite allergen Der p 1 (nDer p 1) is a cysteine protease having a digestive function in the gut of *D. pteronyssinus*. Recombinant expression of Der p 1 proved to be a difficult task. Indeed, nDer p 1 is synthesized as a preproprotein and requires a two-step proteolytic maturation, i.e. cotranslational cleavage of the signal peptide and post-

translational cleavage of the propeptide to reach the biologically active mature form. Several expression systems have been used to produce a recombinant version of Der p 1 (rDer p 1) or its *D. farinae* Der f 1 homologue (Best *et al.*, 2000; Jacquet *et al.*, 2000; Takahashi *et al.*, 2000; Yasuhara *et al.*, 2001). Expression of Der p 1 in *E. coli* or *S. cerevisiae* using the cDNA deleted from the sequence coding the propeptide has failed, or at the best resulted in a recombinant allergen with either a poor IgE reactivity or a poor solubility (Greene *et al.*, 1991; Chua *et al.*, 1992). As observed in other expression systems, our attempts to express the mature form of Der p 1 (Δ proDer p 1) in BY-2 tobacco cells stably transformed with the Δ proDer p 1 construct (Figure 3A) have failed (Figure 3B, lane 5). In contrast, high expression levels and IgE reactivity were observed when Der p 1 was expressed in *Pichia pastoris*, mammalian cells and *Drosophila* using the full-length cDNA sequence encoding the preproprotein (Jacquet *et al.*, 2000; Massaer *et al.*, 2001; Jacquet *et al.*, 2002; van Oort *et al.*, 2002). However, pro-Der p 1 is not or inefficiently matured in these expression systems and lacks enzyme activity. For instance, in a recent attempt to produce Der p 1 in *P. pastoris*, the prosequence was not cleaved, resulting in a pro-allergen with a decreased IgE reactivity, and as illustrated in Figure 3B (lane 4), rDer p 1 expressed in *P. pastoris* migrates as a larger smear from 32 kDa to 55 kDa due to hyperglycosylation and absence of propeptide cleavage in the yeast expression system (van Oort *et al.*, 2002).

Interestingly, BY-2 tobacco cells transformed with the full-length cDNA sequence encoding the preproDer p 1 protein

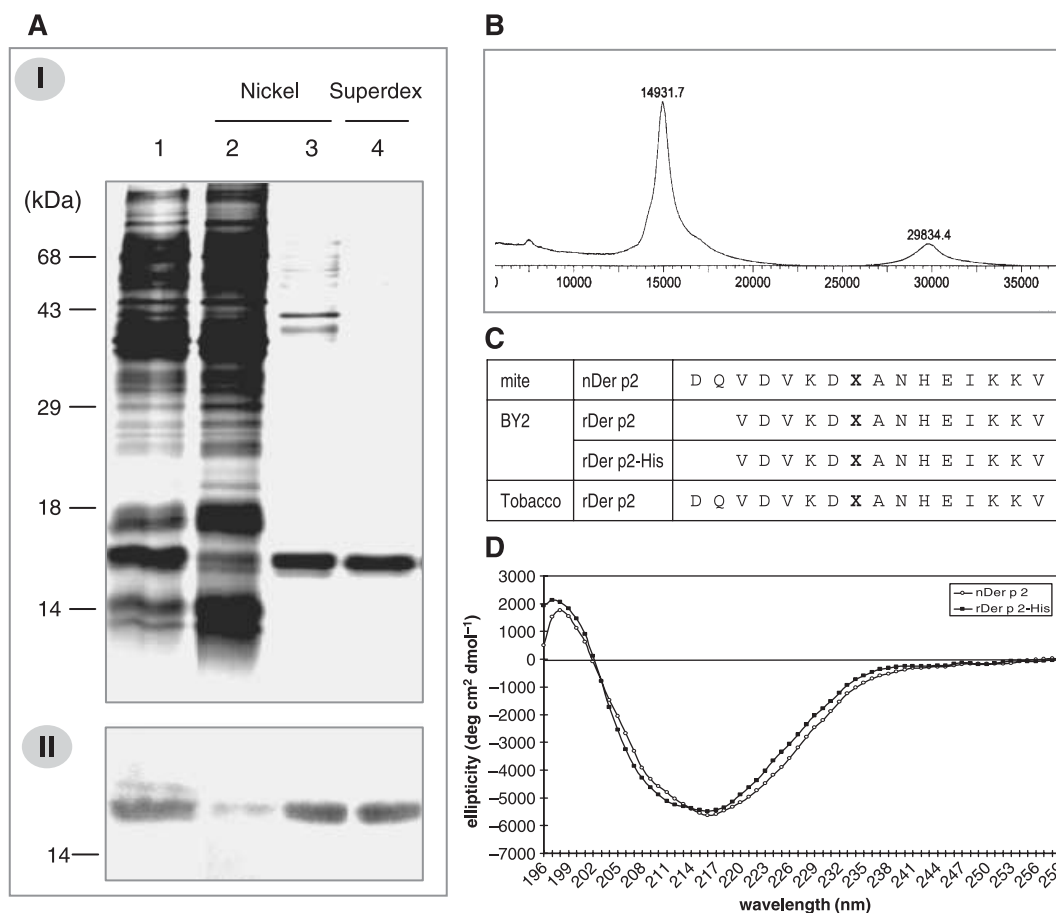


Figure 2 Purification and post-translational maturation of natural and plant-made Der p 2. The physicochemical properties of the natural nDer p 2 and plant-made rDer p 2-His were compared by mass spectrometry (B), N-terminal sequencing (C) and circular dichroism (D) after purification (A). (A) Purification of rDer p 2-His produced in BY-2 tobacco cells by nickel affinity chromatography (Nickel) and size exclusion chromatography (Superdex). Total proteins from culture medium of BY-2 cells expressing rDer p 2-His (lane 1), non-retained protein fraction (lane 2), retained protein fraction after nickel affinity chromatography (lane 3) and purified rDer p 2-His obtained after size exclusion chromatography of the retained protein fraction are analysed by SDS-PAGE (I) and Western blotting with an immuniserum directed against nDer p 2 (II). (B) Mass spectrometry analysis of rDer p 2-His purified from the culture medium of BY-2 cells. (C) N-terminal sequence of nDer p 2 from mite, rDer p 2 and rDer p 2-His from BY-2 tobacco cells (BY-2) and of rDer p 2 from tobacco plants (tobacco). (D) Circular dichroism profiles of nDer p 2 and rDer p 2-His produced in BY-2 cells.

accumulate three polypeptides antigenically related to Der p 1 of 27, 30 and 32 kDa, respectively (Figure 3B, lane 3). In order to define more precisely the origin of these different forms, rDer p1 maturation was further characterized using a pulse-chase experiment in rDer p1 suspension-cultured BY-2 cells. Proteins were pulse-labelled for 1 hour with [³⁵S] methionine and cysteine and chased for 0, 30 min, 3 h, 6 h and 24 h (Figure 3C). Polypeptides antigenically related to Der p 1 were then immunoprecipitated from intra- and extracellular proteins and analysed by SDS-PAGE and fluorography. As illustrated in Figure 3C, after 1 hour of pulse, two polypeptides are detected in the intracellular protein extract (Figure 3C, lane 1). These polypeptides are secreted in the culture medium where they are matured during the chase to a form of similar electrophoretic mobility as mature

nDer p1, which is the most abundant after 24 h of chase (Figure 3C, lane 10). Maturation of rDer p1 in the culture medium is probably due to the presence of extracellular proteases secreted by BY-2 cells (Schiermeyer *et al.*, 2005). Results obtained after rDer p 1 retention in the endoplasmic reticulum are consistent with this hypothesis. Indeed, rDer p 1 is not matured and accumulates in the endoplasmic reticulum (ER) as a proform after fusion of an HDEL signal at the C-terminal end of the protein (Figure 3D, compare lanes 3 and 4).

A second hypothesis that rDer p 1 itself is responsible for its proteolytic maturation after secretion could be excluded based on two observations. First, we have observed that the pH of the extracellular medium is not compatible with the proteolytic activity of Der p 1 during the culture. Second, we

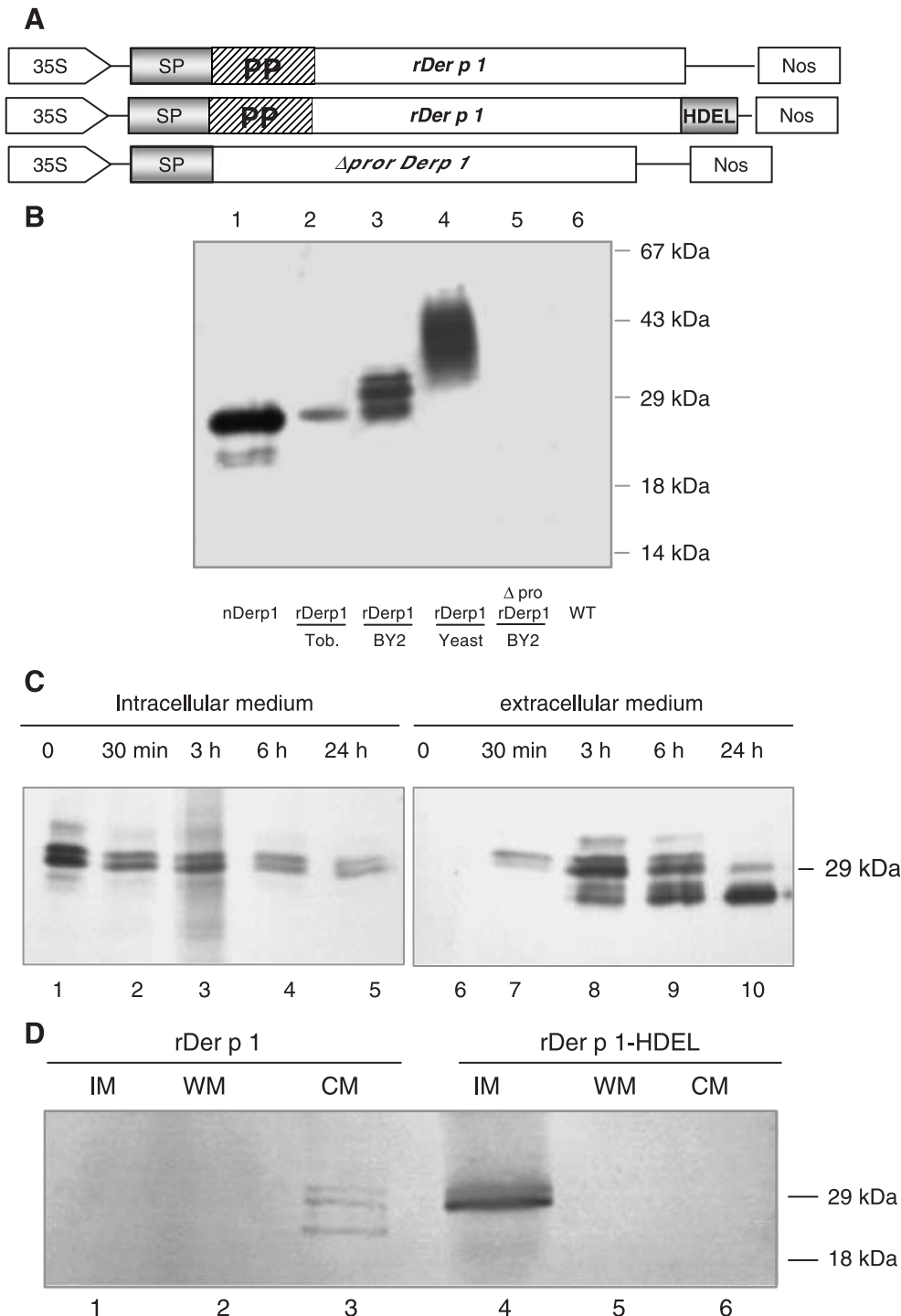


Figure 3 Production and post-translational maturation of Der p 1 in BY-2 tobacco cells. (A) Der p 1 cDNA allergen constructs used for transformation of BY-2 tobacco cells and tobacco plants via *Agrobacterium tumefaciens*. SP, signal peptide; PP, propeptide. (B) Western blot analysis of Der p 1 from *D. pteronyssinus* (lane 1), or transgenic tobacco plants (Tob., lane 2), transgenic BY-2 tobacco cells (BY-2, lanes 3 and 5) or transgenic yeast (Yeast, lane 4) expressing rDer p 1 (lanes 2–4) or Δ pro-rDer p 1 (lane 5). Lane 6 was loaded with a crude protein extract from wild-type tobacco cells. Each lane was loaded with 20 μ g of crude protein extract, except for lane 1 loaded with 5 μ g of purified nDer p 1. After SDS-PAGE and transfer on a nitrocellulose membrane, immunodetection was performed with a polyclonal serum directed against nDer p 1. (C) A pulse-chase experiment illustrates that rDer p 1 is matured after secretion in the culture medium of suspension-cultured tobacco cells. (D) Proteins from intracellular medium (IM), washing medium (WM) or culture medium (CM) of BY-2 cells expressing rDer p 1 (lanes 1–3) or rDer p 1-HDEL (lanes 4–6) are separated by SDS-PAGE and analysed by immunodetection with immunoserum specific for nDer p 1. Twenty micrograms of proteins are loaded on the gel for each lane.

have observed a similar proteolytic maturation for an inactive Der p 1 form produced after substitution of a cysteine 132 for a valine (data not shown).

Altogether these results clearly indicate that BY-2 tobacco cells produce and secrete the complex allergen Der p 1. However, the production of Der p 1 in this plant expression system requires the presence of the propeptide sequence (Patent PCT/FR2003/002085), which is consistent with a role recently proposed for this propeptide in facilitating post-translational folding and probably preventing ER-associated degradation of the misfolded allergen. Interestingly, our results also illustrate that in contrast with other heterologous expression systems, BY-2 tobacco cells have the capacity to mature rDer p 1, after secretion in their culture media, into a form showing an electrophoretic mobility similar to nDer p 1.

rDer p1 is proteolytically matured, N-glycosylated and correctly folded in BY-2 tobacco cells

The reduced IgE binding capacity observed for many recombinant allergens produced in heterologous hosts such as *E. coli* or *S. cerevisiae* was generally explained through incorrect folding or differences in the post-translational modifications of the recombinant protein. This is why we have further investigated the capacity of tobacco cells to reproduce the complex maturation of Der p 1.

When produced in BY-2 tobacco cells, three allergen forms are made during the proteolytic maturation of the proallergen. These forms were purified as illustrated in Figure 4A for N-terminal sequencing. As shown on Figure 4B, these forms result from a two-step proteolytic maturation occurring at the N-terminal end of the proallergen. The final product slightly differs from nDer p 1 and contains five additional amino acids (DLNAE) at its N-terminal end. Interestingly, this form is the only one detected when rDer p 1 is produced in tobacco plants (Figure 3B, lane 2).

The amino acid sequence Der p 1 preproprotein deduced from its cDNA contains two consensus N-glycosylation sites (*asterisks in Figure 4B), one in the propeptide (Asn34) and the other in the mature allergen (Asn150). Before this study there was still some controversy as to whether the natural allergen was glycosylated or not (Chapman and Platts-Mills, 1980; Jacquet *et al.*, 2000). In the present study, we have first investigated whether N-glycosylation was part of the complex maturation of the natural allergen. As illustrated in Figure 5A, the electrophoretic mobility and lectin reactivity of nDer p 1 were compared before (lanes 1) and after (lanes 2) deglycosylation with PNGaseF. nDer p 1 reacts with two different lectin probes specific for glycoproteins, ConA and RCA; in

contrast, after deglycosylation with PNGaseF, nDer p 1 shows a decreased molecular weight (left panel compare lanes 1 and 2) and does not react with glycan-specific probes (lanes 2, middle and right panels). These results clearly indicate that nDer p 1 is a glycoprotein containing high-mannose-type or complex N-glycans on Asn 150. Based on these data, we further investigated the glycosylation of Der p 1 produced in the plant expression system. As illustrated Figure 5B, the three rDer p 1 forms react with concanavalin A (ConA), a lectin specific for high-mannose-type N-glycans. The reactivity of the mature allergen form produced in BY-2 cells with this probe specific for N-glycans excludes that N-glycosylation of rDer p 1 occurs exclusively on the glycosylation site located in the propeptide. The major rDer p 1 forms react not only with ConA, but also with antibodies specific for plant complex N-glycans containing α 1,3fucose, β 1,2xylose and Lewis a (Le^a) antennae (Faye *et al.*, 1993; Fitchette-Lainé *et al.*, 1997). These structural features are specific for plant complex N-glycans (Gomord *et al.*, 2004).

MALDI-TOF mass spectroscopy (MALDI-TOF-MS) analysis of oligosaccharides isolated from the rDer p 1 has shown predominantly complex-type N-glycans such as GlcNAc(X)Man₃(F)GlcNAc₂ and GlcNAc₂(X)Man₃(F)GlcNAc₂ on the mass spectra (Figure 5C, glycans a and b), and a minor proportion of the same glycans without α 1,3-Fuc or with the Lewis a epitope (Figure 5C, glycan c). These results are in accordance with the immunoblot analysis and suggest that glycans N-linked to rDer p 1 are processed into complex N-glycans in the Golgi during the secretion of this allergen. In addition, the presence of Le^a epitopes, typical for plant extracellular glycoproteins (Fitchette *et al.*, 1999), is consistent with rDer p 1 secretion in the culture medium of BY-2 tobacco cells.

Der p 1 is a cysteine protease and we have taken advantage of this enzymatic activity to investigate whether rDer p 1 was correctly folded. Two fluorogenic substrates, Cbz-Phe-Arg-AMC and Boc-Gln-Ala-Arg-AMC, were used to estimate the proteolytic activity of Der p 1 (Jacquet *et al.*, 2002). As illustrated in Figure 4C, rDer p 1 is biologically active and the specific activity of the recombinant allergen looks similar to the natural one, indicating that rDer p 1 is correctly folded.

Altogether our results show that Der p 1 expressed in BY-2 tobacco cells is proteolytically matured and that the cleavage of the propeptide and the folding of this allergen in the plant expression system preserve its cysteine protease activity. In addition, Der p 1 is N-glycosylated with either high-mannose-type or complex-type N-glycans in both the natural and the heterologous expression system.

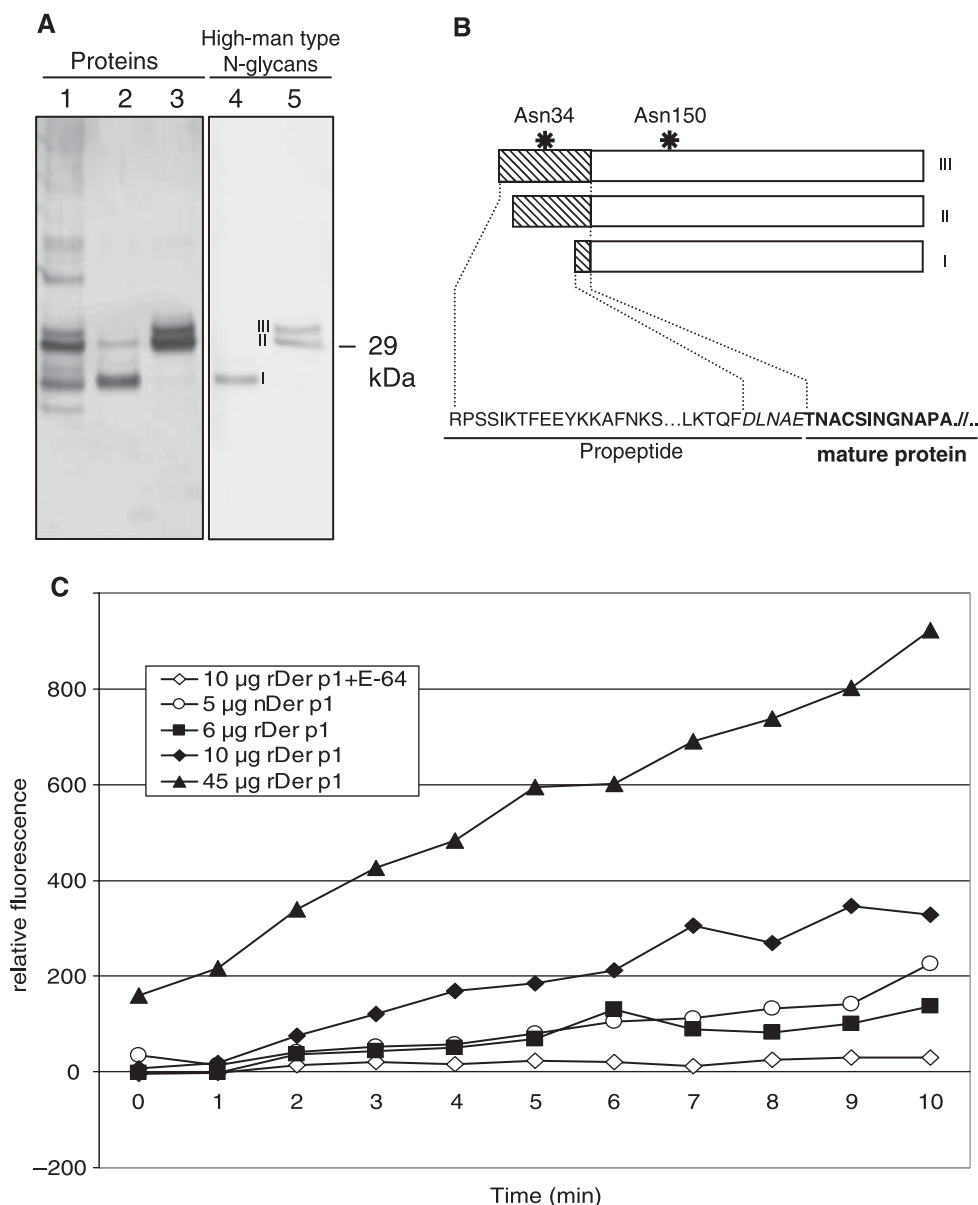


Figure 4 Purification and structural characterization of rDer p1. Mature (A, lanes 2 and 4) and intermediate (A, lanes 3 and 5) forms of rDer p1 were separated by size exclusion chromatography from rDer p1 immunopurified from the culture medium of BY-2 tobacco cells (A, lane 1). The different forms were separated by SDS-PAGE and either stained in the gel (proteins, A, lanes 1–3) or transferred on nitrocellulose and analysed on blot for reactivity with a lectin specific for high-mannose-type N-glycans (A, lanes 4–5) or N-terminal sequencing (B). Stars on Figure 4b correspond to the two N-glycosylation site identified on the Der p1 protein sequence (Asn 34; Asn 150). Cysteine protease activity of nDer p1 and rDer p1 are compared for different amounts of recombinant protein (C). In some experiments, cysteine proteinase inhibitor E-64 was incubated with recombinant allergen as a negative control.

Plant-made mite allergens are immunologically active

Immunoreactivity of rDer p 2-His and nDer p 2 with IgE were compared using sera from 14 patients allergic to *D. pteronyssinus* in a radio-allergosorbent (RAST) analysis. As shown in Figure 6A, the correlation between IgE reactivities was analysed by Spearman's rank correlation coefficient and was found to be excellent (R Spearman = 0.99; $P < 0.00001$).

These results indicate that rDer p 2-His possesses all the antigenic determinants responsible for reactivity with human IgE. This result is a further indication that the recombinant allergen produced in tobacco cells has the same conformation as the natural mite allergen. In the same way, immunogenicity of nDer p 1 and rDer p 1 were compared in a competitive RIA or in mAb-based sandwich ELISA. As illustrated in Figure 6B, no significant differences were observed between the two

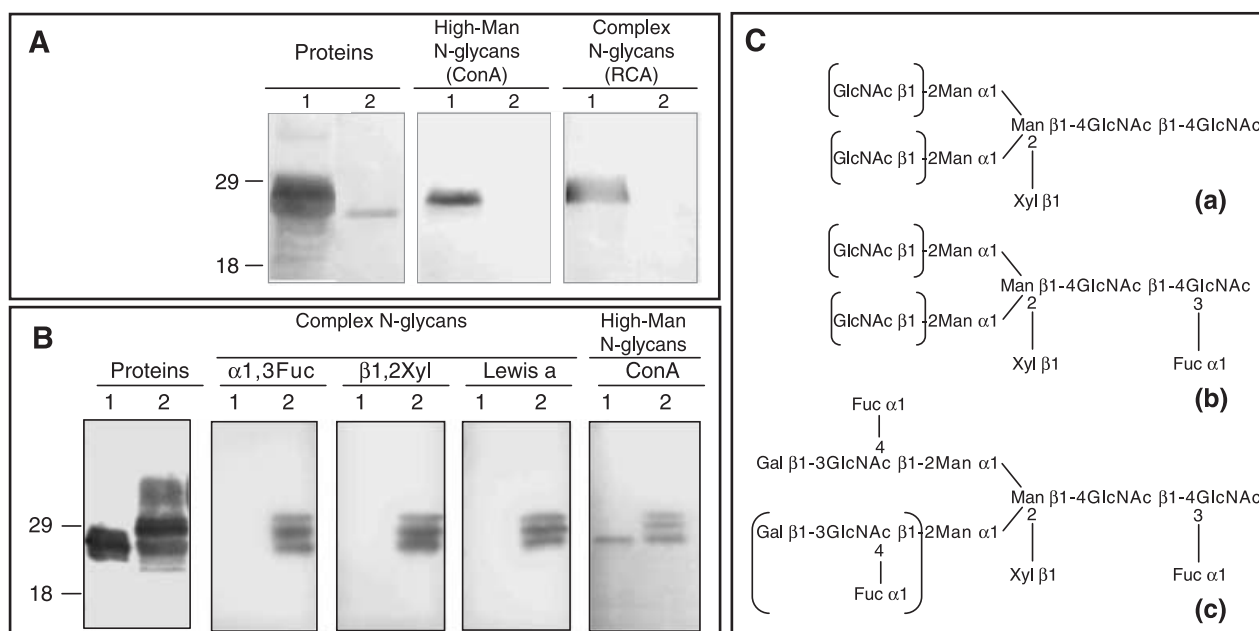


Figure 5 N-glycosylation of natural and recombinant forms of Der p 1. (A) Immunopurified nDer p 1 shows an increased electrophoretic mobility after deglycosylation with PNGase F (compare lanes 1 and 2 on the left panel, showing a gel stained for proteins). Consistent with this result, the natural allergen reacts with ConA, a lectin specific for high mannose N-glycans (lane 1, middle panel) and RCA, a lectin specific for N-acetylglucosamine motifs of complex N-glycans (lane 1, right panel). This reactivity is lost after deglycosylation of nDer p 1 with PNGase F (lanes 2 on middle and right panels). (B) Immunopurified rDer p 1 (lanes 2 on each panel) reacts with antibodies specific for plant complex N-glycans containing $\alpha 1,3\text{Fuc}$, $\beta 1,2\text{Xyl}$ and Lewis a glycotopes and with ConA, a lectin specific with high mannose type N-glycans. In contrast, nDer p 1 (lanes 1 on each panel) reacts exclusively with ConA as previously illustrated on panel A. (C). Major complex N-glycan structures identified on rDer p 1 using mass spectrometry analysis.

allergen forms and interestingly, rDer p 1 seems to be recognized even more efficiently than nDer p 1 as calculated by parallel-line analysis. However, we cannot exclude that this is due to an IgE cross-reactivity with either contaminant plant glycoproteins or complex N-glycans on rDer p 1. Indeed, the presence of IgE that are specific for plant N-glycans in the sera of allergic human patients is well documented (Aalberse *et al.*, 1981; Bencurova *et al.*, 2004; Manduzio *et al.*, in preparation).

Histamine release tests were then performed to compare the ability of natural and recombinant allergens (Der p 1, Der p 2 and Der p 2-His) to stimulate histamine release from basophils. For this comparison the natural and recombinant allergen forms were incubated for 45 min with basophils sensitized with IgE from two different Der p 2-positive sera or five different Der p 1-positive sera. As above for IgE reactivity, natural and recombinant allergens were indistinguishable in their histamine-releasing capacity (Figure 7). In particular, this result shows that the His-tag do not influence the capacity of rDer p 2 to release histamine.

Both rDer p 1 and rDer p 2 were also compared to their natural counterparts in a basophil activation assay, based on the measurement of the activation marker CD203c by

cytofluorometry. As shown in Figure 8, both recombinant Der p 1 and Der p 2 allergens were as potent as natural purified ones in stimulating basophils from a house dust mite-allergic patient. No quantitative differences were found when comparing recombinant and natural proteins in concentrations between 5 ng/mL (data not shown) to 500 ng/mL, suggesting that rDer p 1 and rDer p 2 have a well-preserved IgE reactivity.

Finally, T lymphocyte proliferation assays were conducted with carboxyl fluorescein succinimidyl ester (CFSE)-labelled T-cell lines specific for either Der p 1 or Der p 2 isolated from peripheral blood mononuclear cells from a house dust mite allergic patient. As shown in Figure 9, both rDer p 1 and rDer p 2-His (as well as rDer p 2, not shown) produced in plant cells induced the proliferation of mite-specific T lymphocytes to a level comparable to their natural purified counterparts. No proliferation was induced when cells were stimulated with medium alone (RPMI) or nDer p 1 or nDer p 2 used as negative controls, as appropriate. Collectively, these results establish that both plant-made allergens exhibit immunogenic properties comparable to their natural counterparts. These properties make such proteins suitable for diagnostic, but also for immunotherapy, most particularly through mucosal

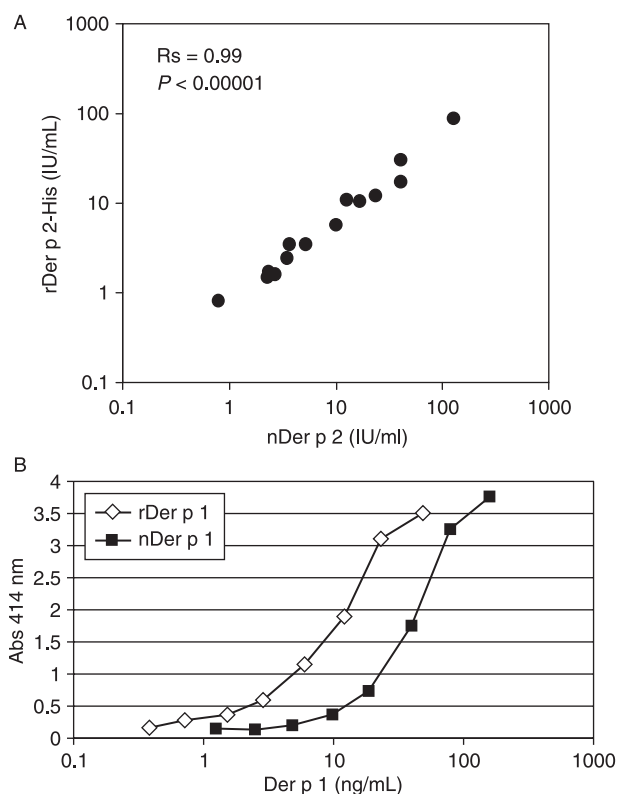


Figure 6 Immunogenicity of recombinant allergens produced in BY-2 cells. (A) Reactivity of nDer p 2 and rDer p 2-His with human IgE. For 14 patients, a RAST was carried out on both allergens. Results are expressed in International Units (IU) of IgE 1 IU = 2.4 ng. (B) IgE reactivity observed with IgE from patient allergic to Der p 1.

(e.g. sublingual) routes of administration. IgE recognition allows to target allergens to oral Langerhans cells which express constitutively both high (FcεRI) and low (CD23) affinity Fc receptors for IgEs. Such cells can then present allergens to naïve T cells to induce allergen-specific tolerance (Moingeon *et al.*, 2006).

Conclusion

The low cost correlated to a well-established biosafety makes the plant expression system very attractive for molecular farming. This was illustrated over the last decade, when transgenic plants have been used with success for production of dozens of recombinant proteins of commercial interest such as antibodies, industrial proteins or human pharmaceuticals (<http://www.molecularfarming.com/PMPs-and-PMIPs.html>). However, to our knowledge, only two industrial proteins – avidin and β-glucuronidase – have been commercialized up to now (Fischer *et al.*, 1999; Hood and Jilka, 1999) and the world's first registration for a plant made pharmaceutical

(PMP) was obtained in January 2006 for a vaccine against Newcastle disease in chickens (<http://www.dowagro.com>). Indeed, although plant cells are able to carry most post-translational maturation steps required for the biological activity of many proteins from eukaryotes (as illustrated here for the first time with two proteins from house dust mites), there is still a limited use of recombinant glycoproteins produced in a plant expression system for *in vivo* therapy in mammals (Gomord *et al.*, 2005). Plant N-glycan immunogenicity and allergenicity are not a limitation as far as the production of the non-glycosylated Der p 2 allergen is concerned. Our results demonstrate the possibility to use suspension-cultured tobacco cells for production of a soluble, correctly folded and highly immunoreactive recombinant Der p 2 allergen that could be used for applications in diagnostic and immunotherapy. In contrast, the presence of N-glycans containing α1,3Fuc and/or β1,2Xyl on rDer p 1 might significantly decrease the specificity of diagnostic test performed with this recombinant allergen. Indeed, sera from around 20% to 30% of grass pollen allergic patients contain IgE antibodies against plant complex N-glycans, which cause *in vitro* cross-reactivity of such sera (Aalberse *et al.*, 1981; Bencurova *et al.*, 2004; Manduzio *et al.* in preparation). These difficulties related to differences in glycan structures on recombinant glycoproteins are not specific to the plant expression system; indeed, the same limitation is observed for Der p 1 produced in *P. pastoris* (van Oort *et al.*, 2002) since the sera of about 20% of allergic patients contain mannan-specific IgE antibodies. Fortunately, strategies are under investigation to engineer glycosylation in all eukaryotic expression systems including plants, so that the expression host will be suitable for the production of recombinant therapeutic glycoproteins harbouring humanized non-immunogenic N-glycans (Gomord and Faye, 2004; Gomord *et al.*, 2004).

Beside these limitations in the specificity of plant-made glycoallergens used as tools for diagnostic, conversely, it could be an advantage for immunotherapy to have a plant N-glycan associated to a non-plant glycoallergen with the aim to target antigen presenting cells, most particularly through lectins or mannose/fucose receptors expressed on the cell surface of dendritic cells (Condaminet *et al.*, 1998).

This work exemplifies the high potential of suspension-cultured BY-2 tobacco cells as bioreactors for the production under controlled and environmentally safe conditions of recombinant allergens that could be used for diagnostic or immunotherapy of type I allergies. In this respect plant-suspension-cultured cells now emerge as complementary to other expression systems, such as yeast or *E. coli* for animal or plant allergens production.

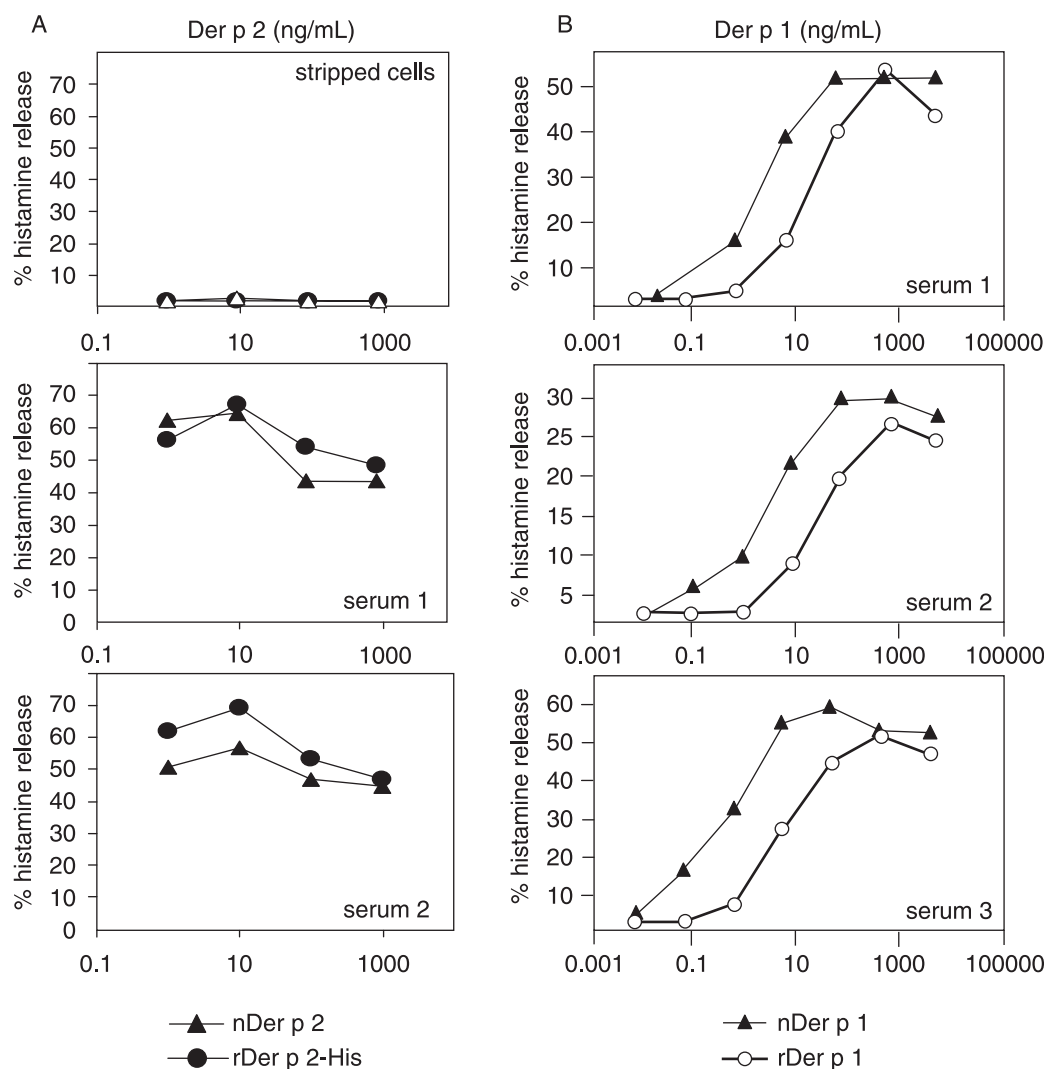


Figure 7 Histamine-release assays. Both recombinant allergens were compared to their natural counterpart for histamine-releasing capabilities with 2 (Der p 2, A) or 3 (Der p 1, B) positive sera. The stripped cells (A) are used here as a negative control. (A) In both case (A and B), concentrations of allergens ranged from 0.1 ng/mL to 10 µg/mL.

Experimental procedures

Construction of plant transformation vectors

Der p 2 cDNA was provided by Dr Wayne Thomas (Perth, Australia) in a pUC19 vector, and this isoform contained an amino acid substitution (R→A at the position 17) compared to the sequence published by Chua *et al.* (1996). Der p 2 cDNA was used as a matrice to insert a histidine tag at its C-terminus by PCR mutagenesis. cDNA encoding Der p 2 was amplified with the following primers 5'-ATCCCACTATCCTTCGC-3' (complementary to nucleotides 769–812 in the 35S promoter referred to pBI221) and 5'-GGACTAGTGGATCCTCAGTGATGGTGATGGTGATGATCGATCTT-**GTCATCGTCATCGCGAATTTAGCATGAGT**-3' (in bold complementary to nts 418–438 in Der p 2 sequence; underlined are the six histidine residues), and digested by *Xba*I and *Bam*HI endonucleases. The use of two primers of different sizes was described previously

(Gomord *et al.*, 1997; Pagny *et al.*, 2000; Pagny *et al.*, 2003). This cDNA was inserted in pBLT1221 previously digested by *Xba*I and *Bam*HI restriction enzymes; the presence of the histidine tag was confirmed by sequencing. cDNA encoding Der p 2 with or without His-tag was excised by *Eco*RI or *Eco*RI/*Hind*III endonucleases, respectively, and inserted in pBLT1121 binary vector (Pagny *et al.*, 2003) previously digested by *Sma*I or *Eco*RI/*Hind*III endonucleases.

Der p 1 cDNA was provided by Dr Ronald van Ree (Sanquin, Amsterdam, The Netherlands) in pPICZαA vector (van Oort *et al.*, 2002). The cDNA encoding Der p 1 preproprotein was digested by *Xho*I and *Not*I endonucleases and subcloned in pBLT1221 vector (Pagny *et al.*, 2003) previously digested at *Sma*I/*Not*I restriction sites. The *Kpn*I/*Sac*I fragment corresponding to the Der p 1 preprosequence downstream the 35S promoter and upstream the Nos terminator, was then inserted in the pBLT1121 binary vector, previously digested by *Kpn*I/*Sac*I in order to express Der p 1 in plant cell via *Agrobacterium tumefaciens*. The mutant Der p 1 with carboxy-terminal HDEL was made by PCR mutagenesis at the 3' end using a

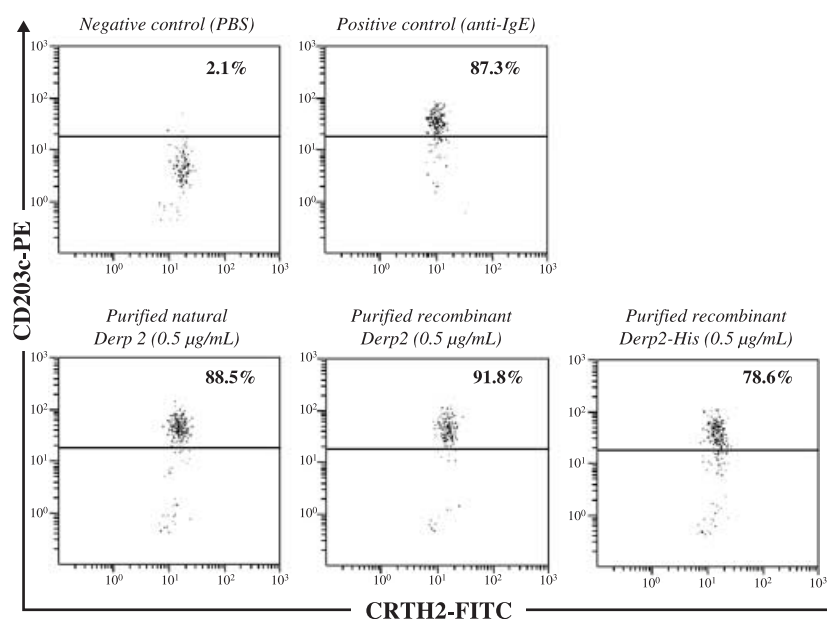
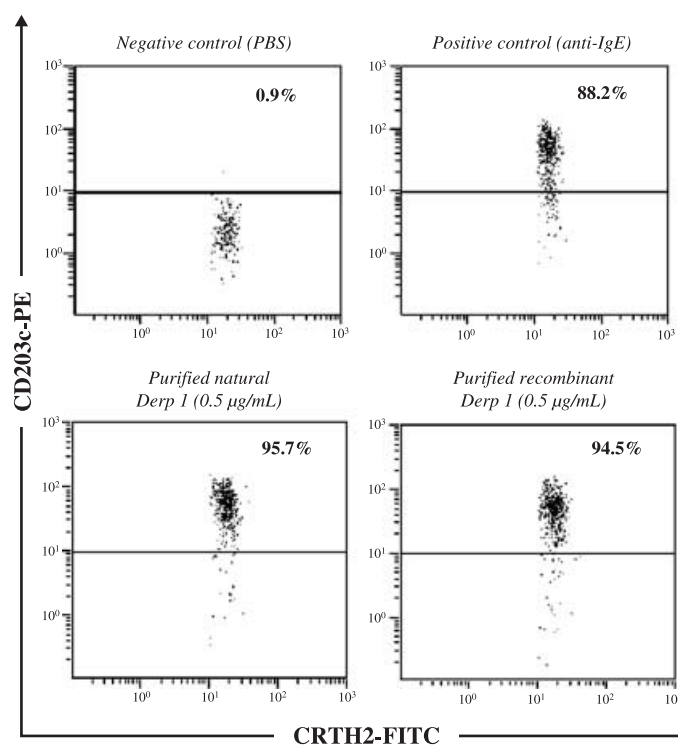
A- Basophil activation by nDer p 2 and rDer p 2**B- Basophil activation by nDer p 1 and rDerp 1**

Figure 8 Basophil activation by natural and recombinant Der p 1 (B) and Der p 2 (A). After excluding T lymphocytes (CD3 positive cells), basophils are analysed by cytofluorometry for CD203c and CRTH2 expression: without stimulation (negative control), after anti-IgE stimulation (positive control), in presence of either purified natural or recombinant Der p 1 or Der p 2, respectively, used at the same concentrations (0.5 µg/mL). Results are expressed as percentage basophils positive for the activation marker CD203c.

forward primer (5'-ATATCTAGAATGAAAATTGTTTTGCC-3') and a reverse primer (5'-TATGAGCTCTTAAAGCTCATCATGGTGATGGT-GATGGTGATGGAGAATGACAACATATGGAT-3'). For this PCR, we used the pBLTI121-Der p 1 vector described above. PCR products were digested by *Xba*I/*Sac*I endonucleases and ligated in the *Xba*I/*Sac*I sites of the plant expression cassette in pBLTI121.

Stable transformation of plant and suspension-cultured tobacco cells and culture conditions

The pBLTI121/Der p 2, pBLTI121/Der p 2-His, pBLTI121/Der p 1, and pBLTI121/proDer p 1 constructs were transferred into *A. tumefaciens*

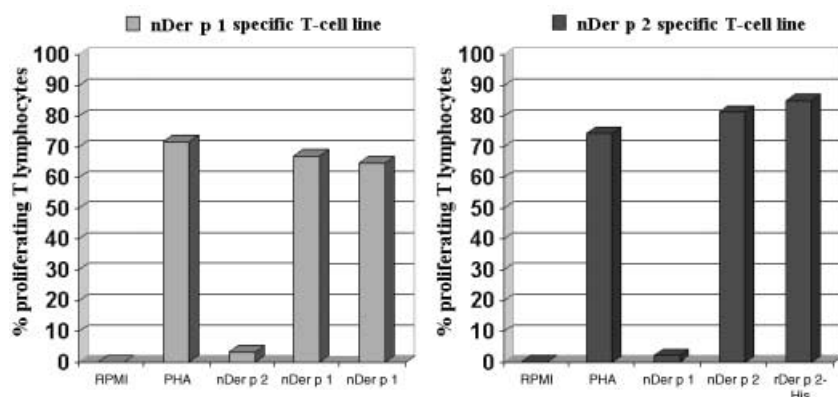


Figure 9 Proliferative response of nDer p 1 or nDer p 2 specific T-cell line with various stimulations. T-cell lines specific for either nDer p 1 or nDer p 2 were generated from PBMCs of a house dust mite allergic patient following *in vitro* stimulation with natural purified allergens. Cells were stained with CFSE and stimulated for 12 days with natural or recombinant proteins at a concentration of 20 µg/mL in the presence of irradiated autologous PBMCs. Medium alone (RPMI) was used as a negative control.

(LBA4404) (Höfgen and Willmitzer, 1988). Transformed bacteria were used to transform suspension-cultured cells of *Nicotiana tabacum* L. (cv. Bright Yellow 2 – BY-2) as described previously by Gomord *et al.* (1998). Transformants were selected in presence of kanamycin at 100 µg/mL. Transformed calli were screened for Der p 2 or Der p 1 expression by Western blot analysis. Selected transgenic calli expressing the protein of interest were then used to initiate transgenic cell suspension cultures, as described by Gomord *et al.* (1998). Batch cultures were maintained in 500 mL Erlenmeyers flask containing 150 mL of BY-2 medium on an orbital shaker at 140 r.p.m. at 25 °C in the dark. Transformation of tobacco plants (*N. tabacum* L. cv. PB D6) was achieved as described in Boulis *et al.* (2003).

Protein extraction, SDS-PAGE and Western blot analysis

Crude protein extracts were obtained from transgenic calli ground with two volumes of prewarmed denaturation buffer (Tris 60 mM pH 6.8, SDS 1%, glycerol 10%, β-mercaptoethanol 2%), boiled for 5 min, and centrifuged 15 min at 8000 *g*.

For cell fractionation, the culture medium was collected by filtration of the suspension culture. Tobacco cells were then washed for 1 h at 4 °C with 1 volume fresh culture medium containing 0.5 M NaCl (washing medium). The mixture of culture medium and washing medium obtained from one suspension culture is considered as representative of extracellular compartment. After this incubation with 0.5 M NaCl, the tobacco cells were washed with fresh medium and homogenized in 2 mL extraction buffer (Tris-HCl, 0.5 M; pH 6.8; HCl 30 mM; KCl, 0.1 M; β-mercaptoethanol, 2%) per gram of cells (fresh weight). The homogenate was centrifuged for 15 min at 10 000 *g* and the supernatant obtained was analysed as representative of intracellular compartment.

These extracts were analysed by SDS-PAGE electrophoresis in 15% polyacrylamide gels. After SDS-PAGE, proteins were transferred from the gel on to a nitrocellulose membrane (Schleicher & Schuell Bioscience, Inc., NH, USA) and immunodetected using polyclonal rabbit antisera directed either against nDer p 2 or nDer p 1 at a 1 : 1000 dilution as it has been described previously in Faye *et al.* (1993). Polyclonal rabbit antisera directed either against nDer p 2 or nDer p 1 were provided by Sanquin, Amsterdam, The Netherlands (van der Zee *et al.*, 1988). Goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad, Hercules, CA, USA) was used at 1 : 3000 as a secondary antibody. rDer p 2 expressed in *E. coli* (Mueller *et al.*, 1998) or immunopurified nDer p 1 were used as

positive controls. Affinodetection with concanavalin A or immunodetection with antibodies directed against β1,3 xylose or α1,3 fucose or lewis a epitopes were performed as described in Faye *et al.* (1993).

Pulse-chase experiments

After filtration, tobacco BY-2 cells (300 mg) were resuspended in 1 mL of fresh culture medium. For each experiment, suspension-cultured cells were pulse-labelled in the presence of 80 µCi of the [³⁵S] protein labelling mix (PerkinElmer, Wellesley, MA, USA) at 25 °C under gentle shaking (140 r.p.m.) for 30 min. At the end of the pulse, cells were chased for various periods of time by adding 110 µL of culture medium containing methionine (50 mM) and cysteine (50 mM). At the end of the chase, cells were incubated for 30 min, at 5 °C in the same media adjusted to 0.5 M NaCl. After a 5-min centrifugation the supernatant was collected (extracellular media) and the cell pellet was sonicated in a lysis buffer (Tris-HCl 50 mM, pH 7.5 containing 150 mM NaCl, SDS 1%, Triton ×100 1%, methionine 100 mM, EDTA 1 mM, sodium ascorbate 1 mM, leupeptine 100 µM, PMSF 1 mM) to obtain the intracellular protein extract.

Immunoprecipitation of Der p 1-related or Der p 2-related polypeptides was performed as described in Gomord *et al.* (1997) using 5 µL of the anti-Der p 1 or anti-Der p 2 immunoserum, respectively.

Purification of recombinant allergens

rDer p 2-His was purified from the culture medium collected by filtration of the suspension culture. A first purification step was realized by precipitation at 50% saturation in ammonium sulphate. After precipitation and centrifugation at 3000 *g* for 30 min, the protein pellet obtained from 750 mL of culture medium was resuspended in 5 mL of buffer A (NaCl 0.5 M, Tris-HCl 25 mM, pH 7.0). The protein solution was mixed with 3 mL of affinity gel charged with NiSO₄ (Ni-NTA agarose; Qiagen, Hilden, Germany) for 1 h at 4 °C in a rotary shaker. Subsequently, the affinity gel was transferred in a column and five successive washes were done with 5 mL of buffer A for 15 min at 4 °C. Proteins with high affinity for the resin were eluted with 5 mL of buffer A supplemented by 250 mM of imidazole. Eluted proteins were then desalted on a PD-10 gel filtration column (GE Healthcare UK Ltd, Amersham, Buckinghamshire, UK), and analysed by SDS-PAGE and immunoblotting. High molecular

weight contaminants were then separated from rDer p 2-His on a Superdex 75 HR 10/30 column (GE Healthcare UK Ltd), equilibrated with buffer B (NaCl 0.15 mM, Na_2HPO_4 50 mM, pH 7), using a BioCad chromatography system (Perspective Biosystems, Hertford, UK) at a flow rate of 0.4 mL/min. Fractions of 0.5 mL were collected and analysed for protein content by SDS-PAGE electrophoresis and Western blotting. Fractions containing rDer p 2-His were pooled, desalted on nanosep column (Pall Filtron, East Hills, NY, USA) and assessed for purity by SDS-PAGE and protein silver staining. Protein concentration was determined by absorbance at 280 nm using extinction coefficient of 9890 (M^{-1} , cm^{-1}).

nDer p 2 used in this study was purified from whole body mite extract by affinity purification using Sepharose-coupled Der p 2 specific monoclonal antibody (mAb alphaDpx) as described in Hakkaart *et al.* (1998b).

Recombinant Der p 1 and Der p2 without Tag were purified from the culture medium of BY-2 tobacco cells by immunoaffinity chromatography with Sepharose-coupled either to a monoclonal antibody against nDer p 1 or to polyclonal antibodies directed against Der p 2 (van der Zee *et al.*, 1988). Extracellular medium of the suspension-culture of BY-2 tobacco cells expressing recombinant allergens was collected by filtration and concentrated by using a Prep/Scale™ Spiral Wound Ultrafiltration Module (Millipore, Billerica, MA, USA) with a Prep/Scale™-TFF Cartridges of 10KDa (Millipore). Ten litres of extracellular medium was reduced in one. After allowing the recombinant allergen to bind the immunoaffinity matrix, the column was washed with PBS (phosphate-buffered saline, pH 7.4) and subsequently eluted with 50% ethylene glycol and 5 mM lysine pH 11.0. Purity was assessed by SDS-PAGE/silver staining.

N-terminal amino acid sequencing

The N-terminal amino acid sequence of the purified proteins (diluted at 70 pmol/mL) was determined using an Applied Biosystems 492 automated protein sequencer (Applied Biosystems, Foster City, CA, USA). The classical Edman degradation procedure was carried out with 18 cycles of pulsed-liquid chemistry.

Circular dichroism

In circular dichroism experiments, ellipticity measurements were performed either with nDer p 2 (100 mg/mL) or rDer p 2-His (150 mg/mL) both dissolved in water. Spectra were recorded on CD6 dichrograph (Horiba Jobin Yvon, Edison, NJ, USA) in 1 mm path length quartz cells (Hellma). The instrument was calibrated using d(+)-10-camphorsulphonic acid. Experimental data were averaged from 10 cycles that were subtracted from blank before fitting to estimate the conformational contents (Chang *et al.*, 1978; Sreerama and Woody, 1998).

MALDI-TOF mass spectrometry

MALDI-TOF mass spectra of the proteins were recorded on a micro-mass ToF spec E mass spectrometer (Micromass, Manchester, UK). This instrument was operated at an accelerating voltage of 25 kV in linear mode. The apparatus was operated at a pressure of approximately 10–7 mbar in the source and 10–6 mbar in the analyser. The

nitrogen laser wavelength was set at 337 nm, having a pulse width of 4 ns. The MALDI-TOF mass spectra performed in positive ion mode were obtained using a post-acceleration detector of 15 kV. They were smoothed once and externally calibrated with apomyoglobin (16 952 Da) and cytochrome c (12 361 Da). The laser shots were summed for each mass spectrum in order to have an acceptable signal to noise ratio. Two microlitre of the sample at a concentration around 10 pmol/mL were dissolved in a same volume of matrix solution containing 2 mg of sinapinic acid diluted in acetonitrile/water-trifluoroacetic acid (TFA) 0.1% (40/60 v/v). The sample-matrix mixture was homogenized and 1 μL was deposited on to the target and allowed to dry.

N-glycan analysis by MALDI-TOF mass spectrometry

N-glycans were released from 1 mg of purified rDer p 1 by successive treatments with pepsin and PNGase A as previously described in Bakker *et al.* (2001). Then, according to this study, free N-glycans were desalted prior to MALDI-TOF analysis. The MALDI-TOF mass spectra of the N-glycans were acquired on a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems) equipped with a 337-nm nitrogen laser. Mass spectra were performed in the reflector, delayed extraction mode using 2,5-dihydroxybenzoic acid (Sigma, St Louis, MO, USA) as matrix. The matrix, freshly dissolved at 5 mg/mL in a 70 : 30% acetonitrile/0.1% TFA, was mixed with the solubilized oligosaccharides in a ratio 1 : 1 (v/v). These spectra were recorded in a positive mode, using an acceleration voltage of 20 000 V with an extraction delay time of 100 ns. They were externally calibrated using commercially available mixtures of peptides (Applied Biosystems) such as des-Arg1-Bradykinin (904.4681 Da), Angiotensin I (1296.6853 Da), Glu1-Fibrinopeptide B (1570.6774 Da), ACTH clip 18–39 (2465.1989 Da). Laser shots were accumulated for each spectrum in order to obtain an acceptable signal to noise ratio.

Cystein protease activity assay

As previously described by Jacquet *et al.* (2002), the proteolytic activity of Der p 1 was assayed in Tris-HCl (pH 7, 50 mM) containing EDTA (1 mM) and L-cysteine (20 mM) at 25 °C in a final volume of 1 mL. Hydrolysis of Cbz-Phe-Arg-7-amino-4-methylcoumarin (Cbz-Phe-Arg-AMC, B9521, Sigma) and Boc-Gln-Ala-Arg-7-amino-4-methylcoumarin (Boc-Gln-Ala-Arg-AMC, B4153, Sigma) (both substrates at a final concentration of 100 μM) was monitored using a FL600 Microplate Reader (Bio Tek Instruments, Winooski, VT) with λ_{ex} = 380 nm and λ_{em} = 460 nm. Papain (P4762, Sigma) was used as an internal control for this assay. In some experiments, Der p 1 was incubated with the cysteine proteinase inhibitor E-64 at the final concentration of 10 μM before the enzymatic assay.

Measurement of IgE binding activity (RAST)

IgE binding capacities of allergens were compared by RAST analysis (Radio Allergo Sorbent Test) as described previously by Aalberse *et al.* (1981). Briefly, both natural and recombinant proteins were coupled to cyanogen bromide-activated Sepharose 4B (250 μg /100 mg Sepharose). The Sepharose was resuspended to 2 mg/mL in PBS-AT (phosphate-buffered saline, bovine serum albumin 0.3%, Tween 20 0.1%), of which 250 μL were incubated

with 50 µL of human serum. After overnight incubation, unbound material was washed away and 50 µL of ^{125}I -labelled sheep anti-human IgE antibodies (CLB, Amsterdam, The Netherlands) were added. After an overnight incubation and washing steps, bound radioactivity was measured in a gamma-counter. The results were expressed as IU/mL (international units/mL) calculated from a serial dilution of a 2% (w/v) house dust mite extract with known Der p 1 or Der p 2 content. Der p 1 and Der p 2 content in unknown samples were calculated by parallel-line analysis.

Der p 2 two-site ELISA and RAST

The immunogenicity of rDer p 2 was analysed by two-site ELISA and RAST experiments according to a protocol described in Hakkaart et al. (1998a).

Histamine release assay

Basophils were partially purified from a buffy-coat of a non-allergic donor by centrifugation over Percoll. The cells were stripped from their IgE by lactic acid treatment, and subsequently re-sensitized by addition of Der p 2- or Der p 1-positive sera. Cells were then incubated with native or recombinant allergens at 37 °C for 45 min. Cells were centrifuged, and histamine release in the supernatant was measured by a fluorometric method (Siraganian). Results are expressed as percentage release of the total histamine content of the cells.

Basophil activation assay

Measurement of activated basophils by allergen stimulation was performed using the Allergen kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instruction. The rise in CD203c expression before and after allergen challenge reflects basophil activation in response to an allergen. Heparinized whole blood cells from house dust mite allergic subject were incubated at 37 °C with allergens for 15 min. The reaction was stopped on ice, followed by a 30-min staining with antibodies directed against CRTH2 (mAb BM16) or CD203c (mAb 97A6). After lysis of erythrocytes and fixation, basophils were identified by flow cytometry on the basis of CRTH2 surface expression. The threshold for positivity was determined with a negative control (i.e., whole blood and vehicle without allergen). Basophils were analysed for CD203c and CRTH2 expression: without stimulation (negative control), after anti-IgE stimulation (positive control), or in presence of either an house dust mite extract, purified nDer p 1 or nDer p 2, rDer p 1 or rDer p 2 at a concentration of 10 µg/mL. Results are expressed as percentage of basophils expressing the activation marker CD203c.

T cell *in vitro* stimulation assay

Peripheral blood mononuclear cells (PBMC) from a house dust mite allergic patient were separated from heparinized venous blood by gradient centrifugation. Cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM β -mercaptoethanol, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal calf serum (FCS) at a density of 5×10^6 /mL and cultured

in the presence of either nDer p 1 or nDer p 2 at a concentration of 20 µg/mL for 6 weeks, yielding separate T-cell lines specific for either Der p 1 or Der p 2. Cells were fed with 20 U/mL interleukin 2 starting at day 5 and every 3–4 days afterwards. Fourteen days after *in vitro* stimulation, cells were starved for 5 days, prior to being labelled with CFSE to detect proliferating specific T cells. To this aim, cells were resuspended at a concentration of 10×10^6 cells/mL in serum-free PBS and incubated with CFSE (0.8 µM, final concentration) for 10 min at 37 °C. CFSE labelling was stopped by adding an equal volume of heat-inactivated FCS. After several washes, 0.5×10^6 cells per well were plated in a 48-well plate in the presence of 0.5×10^6 irradiated autologous PBMCs as presenting cells and cultured with the various natural or recombinant proteins at a concentration of 20 µg/mL in RPMI medium containing 10% FCS. No interleukin 2 was added. After being cultured for 7 days, cells were harvested, washed and PerCP-conjugated anti-CD4 antibodies were added for 15 min at 4 °C. Proliferating CD4⁺ T lymphocytes were detected by cytofluorometry as cells exhibiting a diminished CFSE fluorescence.

Acknowledgements

This work was supported by a grant from Stallergenes SA, SEITA-ALTADIS and ANVAR. The authors acknowledged the stimulating participation of Dr A.R. Schoofs as a coordinator of this project. The authors would like to thank Dr Wayne Thomas for providing the Der p 2 cDNA.

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