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Involvement of the N-Terminus of Der p 2 in IgE and Monoclonal Antibody Binding

Abstract

The major house dust mite allergen Der p 2 was expressed as a recombinant fusion protein in *Escherichia coli* either with glutathione-S-transferase as fusion partner or with a poly-histidine tag. Both recombinant fusion proteins failed to react with 3/14 Der p 2-specific monoclonal antibodies (mAbs). When Der p 2 was expressed in yeast with one alanine linked N-terminally to the allergen, no reactivity was observed. When expressed without any fusion partner, all 14 mAbs showed reactivity. The addition of a single N-terminal alanine also disrupted an important epitope for IgE. In RAST inhibitions, an average decrease in inhibitory potency of 72–32% was observed (n = 16) with a maximum decrease of 91%. These observations suggest that the N-terminus of Der p 2 is involved in an important epitope for IgE that is disrupted by the addition of one single amino acid. Recombinant Der p 2 molecules should therefore preferably lack any fusion peptide.

Key Words

Baker's yeast
IgE
Monoclonal antibodies
Recombinant Der p 2
Saccharomyces cerevisiae
Fusion proteins

Introduction

The isolation of a large number of allergen cDNA clones from various organisms has been reported over the last decade. Allergens are often expressed in the bacterium *Escherichia coli*. For their expression many different vectors are available. In some of these systems the bacterial allergen is produced as a fusion protein, facilitating purification. In some cases, fusion partners can be cleaved off, frequently leaving a number of additional amino acid residues.

In our study, we focussed on the house dust mite allergen Der p 2. The cDNA coding for this major allergen was isolated [1], and expressed as a glutathione-S-transferase fusion protein (GST-Der p 2) derived from the expression construct pGEX-p2 [2]. The recombinant allergen was reported to show a good overall reactivity with IgE. Minor discrepancies, however, were detected. From the 24 patients tested

2 did not show IgE reactivity with GST-Der p 2, while being reactive with the natural group 2 allergen (nat Der p 2) [2]. A possible explanation for the reported decreased IgE reactivity might be the presence of the fusion partner attached to the N-terminus of the allergen.

In this study the influence of N-terminally linked fusion partners on IgE and monoclonal antibody (mAb) binding was investigated. Der p 2 was expressed in *E. coli* as fusion protein with a poly-histidine tag attached to the N-terminus. Two different Der p 2 forms were also expressed in yeast, one with a single alanine residue as fusion partner (attached to the N-terminus) and the second as a nonfusion protein, containing amino acid residues 1–129 as deduced from the published cDNA [1]. We demonstrate that the addition of one single alanine residue to the N-terminus disturbs an epitope for a mAb, and leads to a decrease in IgE binding for most of the patients tested (14/16).

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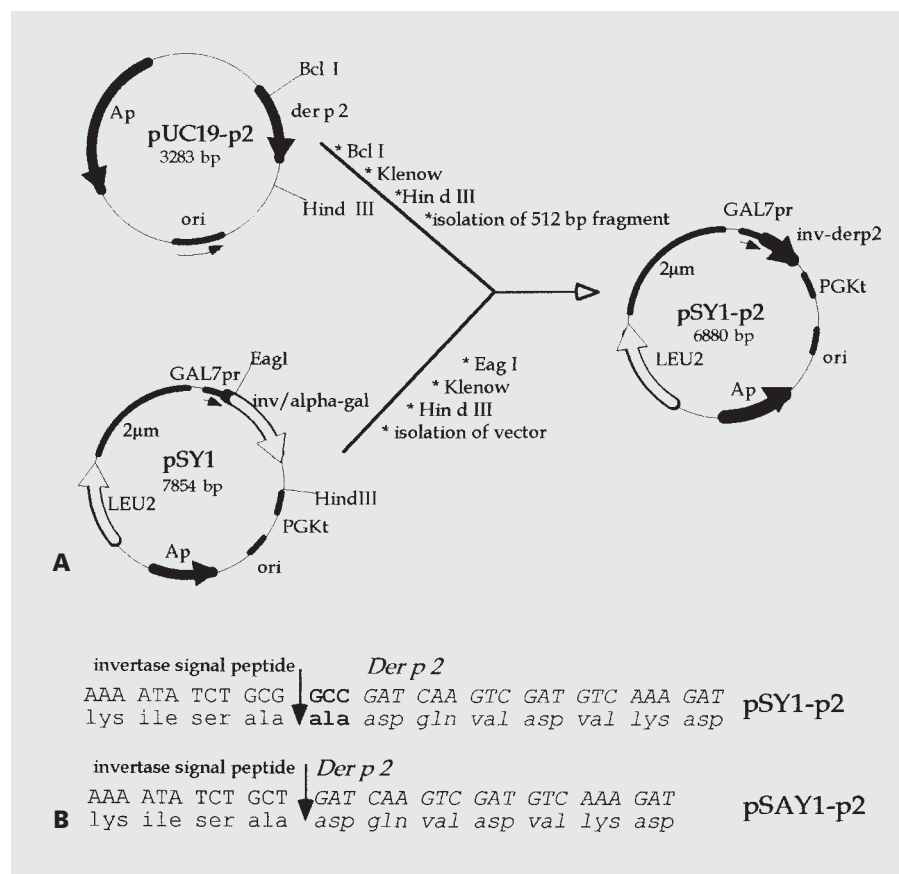


Fig. 1 The construction of pSY1-p2. **A** Der p 2 cDNA was isolated as a 512-bp *Bcl I* (filled in)-*HindIII* fragment, and ligated into pSY1, digested with *EagI* (filled in) and *HindIII*, resulting in the construct pSY1-p2. **B** The DNA coding the fusion of invertase signal sequence with Der p 2 is shown in case of pSY1-p2 and pSAY1-p2. Also the resulting amino acid residues are presented.

Materials and Methods

Sera

Sera of house dust mite-allergic patients ($n = 20$), showing reactivity with the group 2 allergen, with RAST values ≥ 0.7 RAST units (RU) IgE were obtained from our diagnostic department.

Monoclonal Antibodies

The anti-Der p 2 mAbs used were α DpX [3], 10E11, 5H7, 6H5, 8E5, 8H7, 7D6, 10G7, 10H7, obtained after immunization of BALB/c mice with a fraction from mite whole body extract 60 kD [Van Leeuwen and Aalberse, unpubl. data]. Furthermore, 2B12B3, 3G5A2 [4], 6D6, 4E5 and 7A1 [5] were kindly provided by Dr. M.D. Chapman (Charlottesville, Va., USA).

Natural Allergen Source

As natural source of Der p 2 for RAST analysis, a 2% *Dermatophagoides pteronyssinus* whole body extract from Commonwealth Serum Laboratories (CSL, Parkville, Australia) was prepared as described elsewhere [3].

Construction of Plasmids

The Der p 2 cDNA-containing plasmid pUC9-p2 [1] was kindly provided by Dr. W.R. Thomas (Subiaco, Australia). DNA-modifying enzymes were purchased from Pharmacia Biotech Uppsala, Sweden) or Gibco (Paisley, UK). For PCR Vent polymerase (New England Bio-

labs, Beverly, Mass., USA) was used. DNA oligonucleotides were purchased from Pharmacia (Roosendaal, The Netherlands). *E. coli* strains DH5 α [6] and JM110 [7] were used for amplification of plasmids. They were cultivated in 2YT medium (1.6% trypton, 1% yeast extract, 0.5% NaCl), if necessary supplemented with 100 μ g ampicillin/ml at 37°C. General recombinant DNA techniques were performed as described by Maniatis et al. [8]. Transformants were selected on 2YT plates (1.7% agar) containing 100 μ g/ml ampicillin. Recombinant clones were identified by restriction enzyme analyses. Sequence analysis was performed by the chain termination method [9].

From pUC9-p2 [1] the 596-base pair (bp) *EcoRI* fragment was isolated. This fragment was cloned into pUC19 (not shown). From the resulting construct pUC19-p2 the coding region of the Der p 2 cDNA was isolated as a 512-bp *BclI*-*HindIII* fragment.

pQE10-p2. The 512-bp *BclI*-*HindIII* fragment was ligated into the expression vector pQE10 [10].

pSY1-p2. The *BclI* end was filled in using Klenow's DNA polymerase and ligated into the pSY1 restricted with *EagI* (filled in with Klenow's DNA polymerase) and *HindIII* (fig. 1A). This ligation resulted in a hybrid gene encoding the invertase leader signal peptide fused to the Der p 2 cDNA separated by one single alanine residue (fig. 1B).

pSAY1-p2. The construction of this plasmid has been described [14]. In this construct the invertase signal peptide is fused to the mature allergen (fig. 1B).

Figure 2 summarizes the constructs used and their resulting recombinant Der p 2 molecules.

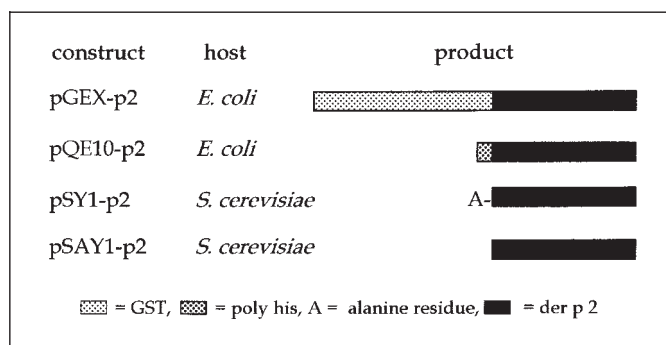


Fig. 2. Recombinant Der p 2 from various vectors. The translation products of four expression plasmids containing Der p 2 cDNA are schematically represented. The two yeast allergens are presented as the mature secreted proteins, thus minus their signal sequences, in case of correct processing as shown in figure 1B.

Expression of Recombinant Der p 2 in *E. coli*

Induction of the pQE10-p2 transformants, encoding poly-his-Der p 2, and isolation of the recombinant protein was carried out according to the producer's manual [10]. Poly-his-Der p 2 possesses an 11-amino acid tag attached to the N-terminus. This tag contains a histidine sextet for purification by means of nickel-chelate chromatography [10]. The allergen was purified under denaturing conditions (8 M urea) and was resolubilized in PBS after dialysis.

In addition to pQE10-p2, poly-his-Mim44 was produced from pQE9-mpil transformants [11]. This recombinant form of Mim44, a mitochondrial inner membrane protein from *Saccharomyces cerevisiae*, was used as a negative control in the binding assays with mAbs.

Expression of Recombinant Der p 2 in Yeast and Purification

The yeast strain *S. cerevisiae* BJ 1991 (*MATa*, *leu2-1*, *trp1-1*, *ura3-52*, *prb1-1122*, *pep4-3*) [12] was transformed [13] with pSY1, pSY1-p2, pSAY1-p2 (resulting in the secretion of α -galactosidase, ala-Der p 2 and recombinant (rec) Der p 2, respectively). The production of recombinant Der p 2 in yeast and its purification by means of gel filtration is described elsewhere [14]. A purity >95% as judged by SDS-PAGE/silver staining was observed (not shown). Of the purified allergen, the protein concentration was measured with Pierce BCA Protein Assay Reagent (Pierce, Rockford, Ill., USA) at 37°C, following the manufacturer's instructions. BSA was used as protein standard.

By means of automatic Edman degradation (Applied Biosystems/Perkin Elmer Procise 494), the predicted sequences – as represented in figure 1B – were confirmed by analysis of the first 7 amino acid residues: DQVDVKD and ADQVDVK for rec Der p 2 and ala-Der p 2, respectively.

Der p 2 Two-Site ELISA

mAbs 10E11 (culture supernatant) and α DpX (ascites) were protein A-purified. α DpX was biotinylated, 0.8 mg NHS-LC-biotin (Pierce, Rockford, Ill., USA)/1.2 mg mAb. ELISA plates (Nunc, Roskilde, Denmark) were coated with either mAb 10E11 (1 μ g/ml) or 2B12B3 (3 μ g/ml) overnight in 0.1 M NaHCO₃. Next the plates were blocked with 0.1% BSA in 0.1 M NaHCO₃ for 30 min. Samples were incubated for 1 h in PBS/0.3% BSA/0.1% Tween 20 (PBS-AT). Incubation with biotinylated mAb- α DpX (3 μ g/ml in PBS/0.2% gelatine/0.02% Tween

20/0.1% BSA) for 1 h was followed by incubation with streptavidin-HRP (Amersham, UK) in PBS 1:1,000, after which staining took place with TMB (0.1 mg/ml in 0.1 M NaAc; pH = 5.5; 0.003% H₂O₂). Between the incubation steps the plates were washed with PBS/0.01% Tween 20. For quantification purposes standards were prepared from purified recombinant or natural Der p 2.

RAST and RAST Inhibition

To 100 mg of CNBr-activated Sepharose-4B (Pharmacia Biotech, Uppsala, Sweden) was coupled, according to the producer's manual: 295 μ l (50 $\times$) concentrated pSY1-p2 and 325 μ l (50 $\times$) concentrated pSAY1-p2 growth medium containing 50 μ g allergen (ala-Der p 2 and rec Der p 2, respectively) and 350 μ l (50 $\times$) concentrated growth medium from pSY1; 1 ml 2% CSL mite whole body extract, containing 30 μ g Der p 2; bacterial lysate containing 300 μ g poly-his-Der p 2 and an equal volume of poly-his-Mim44 containing lysate. Sepharose was suspended in 50 ml of PBS-AT and 500 μ l were used for testing.

In a RAST, 500 μ l (1 mg) Sepharose in PBS-AT was incubated with 50 μ l human serum or mAb overnight end-over-end at room temperature. After washing 5 times with PBS + 0.1% (v/v) Tween 20 (PBS-T) a ¹²⁵I-radiolabelled antibody directed to either mouse IgG or human IgE was added. After incubation overnight end-over-end at room temperature, the Sepharose was washed 4 times with PBS-T and the bound radioactivity was measured, and percentage binding of total counts was calculated. Subsequently, results were expressed as arbitrary RU/ml. RUs were calculated by means of a standard curve composed of several dilutions of a serum with a known total IgE content incubated with Sepharose-coupled sheep antibodies against human IgE: RUs were calculated by using Slide Write Plus 6.0. They were only used for the human IgE assay.

For RAST inhibition 50 μ l of patients serum or mAb (diluted when necessary, to achieve nonsaturating conditions) was preincubated with 50 μ l inhibitor. After 2 h of incubation at room temperature, 500 μ l of Sepharose was added, and the RAST was further carried out as described above.

As a measure for the relative inhibitory potency of ala-Der p 2 the ratio of the amounts of allergen (rec Der p 2/ala-Der p 2) required for 50% binding (in some cases 25% binding) was chosen.

Results

Reactivity of the Recombinant Allergens with mAbs

Poly-his-Der p 2 was tested in a RAST for its reactivity with mAbs directed to nat Der p 2. Three of the 14 mAbs tested (10E11, 4E5 and 3G5A2) did not show reactivity with the recombinant allergen, even though poly-his-Der p 2 was coupled to Sepharose at a 10-fold higher concentration (fig. 3A). Ala-Der p 2 and rec Der p 2 were expressed and screened for reactivity with the 14 mAbs. Like the poly-his-allergen, ala-Der p 2 lacked reactivity with the mAbs 10E11, 4E5 and 3G5A2 (fig. 3B), but the 3 mAbs showed reactivity with rec Der p 2 (fig. 3B). The reactivity of both allergen molecules was further evaluated in two-site ELISAs with mAb 10E11 or 2B12B3 as catching antibody, and mAb α DpX for detection (fig. 4). In this assay 10E11 also failed

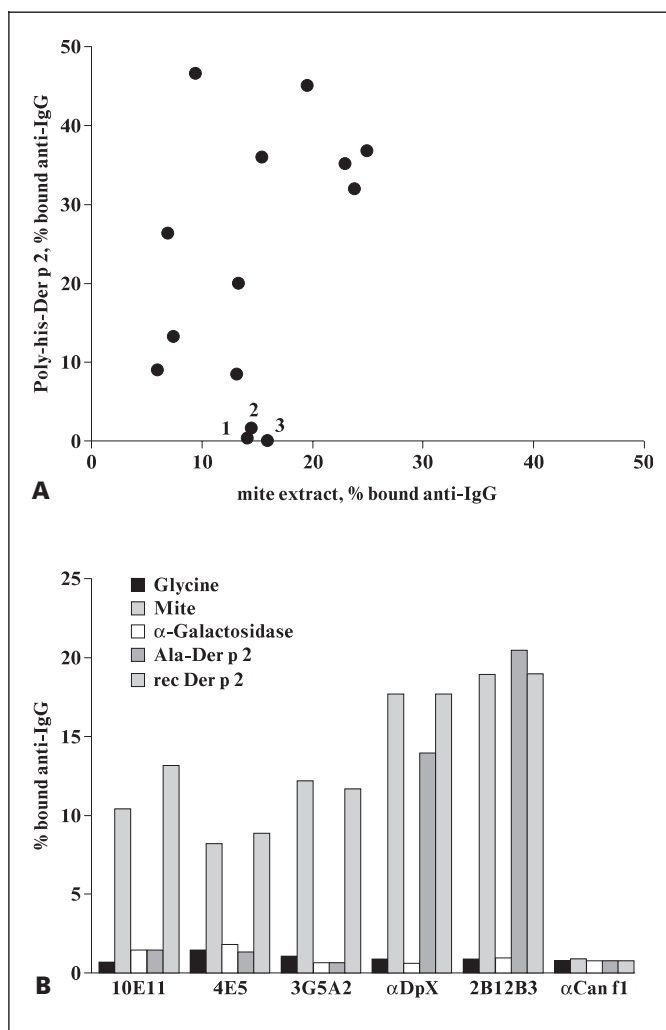


Fig. 3. Reactivity of different recombinant Der p 2 forms with mAbs. **A** Reactivity of poly-his-Der p 2 with 14 anti-Der p 2 mAbs. Poly-his-Der p 2 and mite extract were coupled to Sepharose and tested for reactivity with the mAbs. As negative control for the allergens glycine Sepharose and recombinant poly-his-Mim 44 were used. As a negative control for the used mAbs, a mAb directed against Can f 1 (α Can f 1) was also tested. All these control experiments resulted in binding percentages below 2%; these values are not represented. Dilutions of mAbs were for culture supernatants 1:50 (10E11 and α Can f 1), 1:1,000 (5H7, 6H5, 8E5, 8H7, 7D6, 10G7, 10H7), 1:10,000 (α DpX and 2B12B3) and for ascites 1:1,000 (3G5A2, 6D6, 4E5 and 7A1). Unreactive mAbs: 1 = 10E11, 2 = 3G5A2, 3 = 4E5. **B** Reactivity of ala-Der p 2 and rec Der p 2 with different mAbs. Five anti-Der p 2 mAbs (and 1 mAb directed against Can f 1: α Can f 1) were tested on the different group 2 allergens. As a negative control for nat Der p 2, glycine Sepharose was tested; as a negative control for both yeast-derived group 2 allergens growth culture containing α -galactosidase was tested. Results are given as percent counts added. Dilutions of mAbs were the same as in **A**.

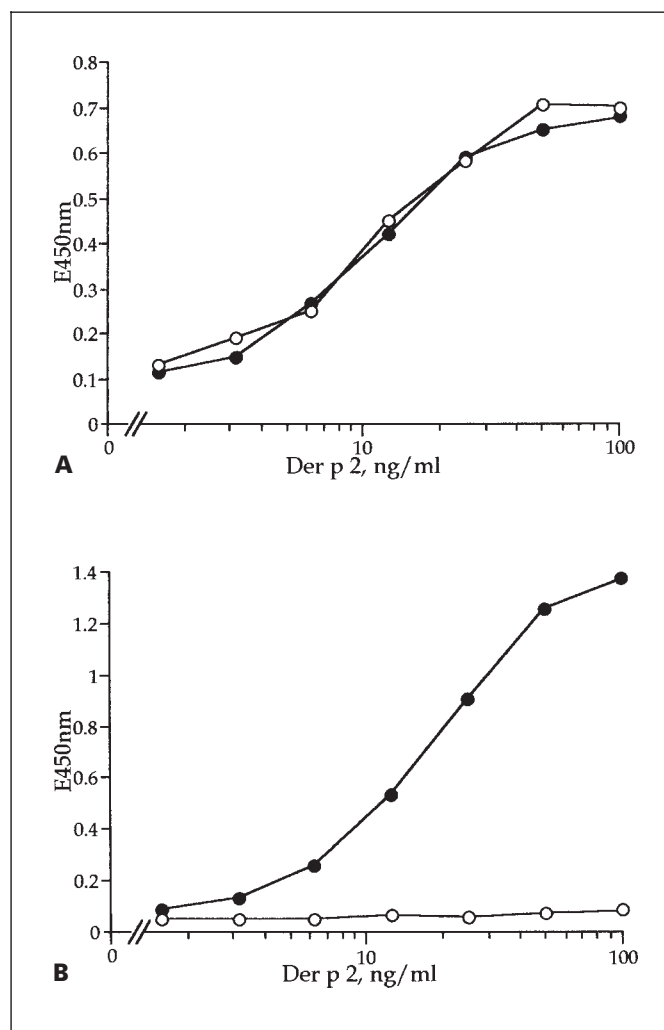


Fig. 4. Reactivity of rec Der p 2 and ala-Der p 2 in two-site ELISAs. Two-site ELISAs were carried out with mAbs 2B12B3 (**A**) and 10E11 (**B**) as catching antibody. Detection of the recombinant allergen took place with mAb α DpX. ● = rec Der p 2; ○ = ala-Der p 2.

to bind ala-Der p 2. Furthermore, the capacity of ala-Der p 2 to inhibit a rec Der p 2 RAST was studied. The rec Der p 2 RASTs for 10E11, 2B12B3 and α DpX were tested for inhibition with rec Der p 2 and ala-Der p 2 (fig. 5A–C). Ala-Der p 2 did not significantly inhibit the RAST for 10E11.

Reactivity of Ala-Der p 2 and rec Der p 2 with IgE

For 20 patients a RAST on rec Der p 2 and ala-Der p 2 was carried out (fig. 6). The mean anti-ala-Der p 2/anti-rec Der p 2 IgE ratio observed was 0.64 ± 0.24 . Inhibition of the rec Der p 2 RAST was investigated for 16 sera with ala-Der p 2 and rec Der p 2. The four sera that were not tested had ratios of 1.0, 0.4, 0.59 and 0.56. The results of two sera are

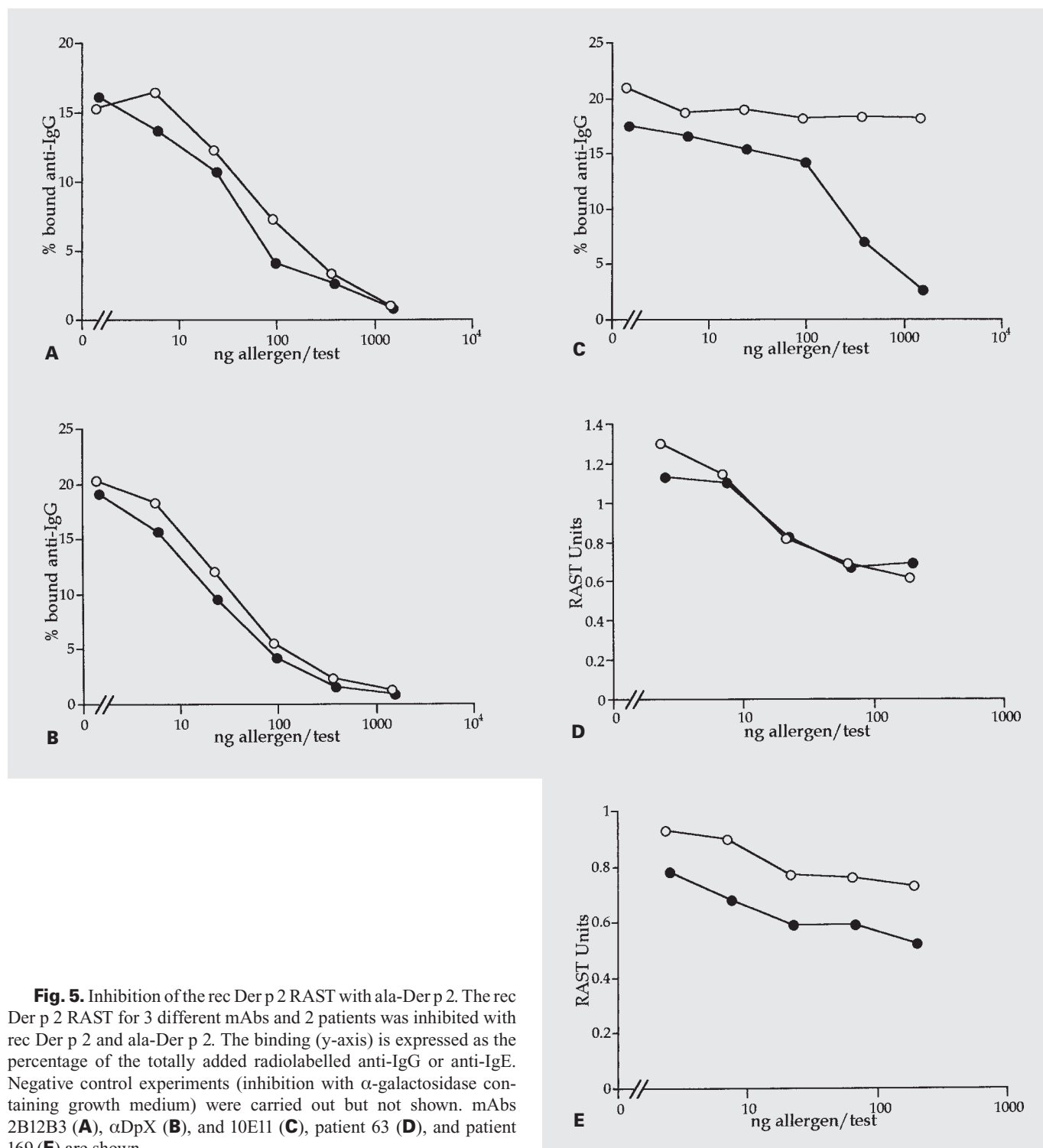


Fig. 5. Inhibition of the rec Der p 2 RAST with ala-Der p 2. The rec Der p 2 RAST for 3 different mAbs and 2 patients was inhibited with rec Der p 2 and ala-Der p 2. The binding (y-axis) is expressed as the percentage of the totally added radiolabelled anti-IgG or anti-IgE. Negative control experiments (inhibition with α -galactosidase containing growth medium) were carried out but not shown. mAbs 2B12B3 (**A**), α DpX (**B**), and 10E11 (**C**), patient 63 (**D**), and patient 169 (**E**) are shown.

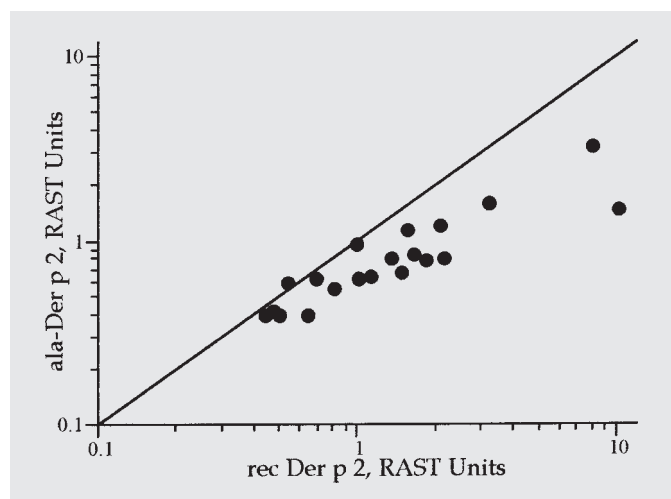


Fig. 6. Rast on ala-Der p 2 and rec Der p 2. Twenty sera were tested for IgE reactivity with ala-Der p 2 and rec Der p 2. Both allergens were coupled to Sepharose. The sera used (with their dilutions) were 20, 49, 63, 76, 102, 140, 143, 165, 169, 170 (1/1); CV, 136, 2, 32, 66, 46 (1/3); 3, 179 (1/9); 135 and 186 (1/27). Negative control RAST with pSY1 growth medium was carried out for all sera below 0.3 RU (results not shown).

represented, one serum showing a substantial reactivity with this epitope (fig. 5E), while the other did not discriminate between these two allergen molecules (fig. 5D) indicating that this patient has no detectable IgE binding to this epitope. Table 1 summarizes the decrease in inhibitory potency for the 16 sera. The mean decrease in inhibitory potency was $72 \pm 32\%$ with a maximum decrease of 91%.

Discussion

Initially recombinant GST-Der p 2 derived from pGEX-p2 [2] was the available tool in our studies. In order to obtain a rec Der p 2 with a smaller fusion partner, poly-his-Der p 2 was produced from the expression plasmid pQE10-p2. This recombinant allergen lacked reactivity with three Der p 2-specific mAbs, even when poly-his-Der p 2 was coupled to Sepharose in a 10-fold higher concentration.

The reason for this epitope deficiency might be an incorrect tertiary structure of the recombinant protein. IgE binding studies with synthesized peptides [15] and large recombinant peptides [16] indicated that the conformation of the protein is important to express the antigenic determinants. Other studies, too, are indicating that the antigenic determinants on the group 2 allergen are dependent on the tertiary structure of the protein [17, 18]. It is possible that the poly-his-Der p 2 is not correctly folded and thus not all de-

Table 1. Inhibitory potency of ala-Der p 2

Serum or mAb	Allergen required for 50% inhibition, ng		IP ala-Der p 2
	rec Der p 2	ala-Der p 2	
CV	4.3	10.2	0.42
MV	20.4	138.2	0.15
2	0.4	41.5	0.01
3	32.2	107.5	0.30
32	19.3	104.4	0.18
46	77.0	1,061.0	0.07
63	18.9	19.6	0.96
66	6.5 ^a	87.2 ^a	0.07
102	27.5	146.0	0.19
135	5.5	13.4	0.41
136	1.7	15.3	0.11
143	48.0	42.0	1.12
169	6.3 ^a	230.0 ^a	0.03
179	25.5	241.0	0.11
178	16.3 ^a	80.4 ^a	0.20
186	18.3	173.0	0.11
αDpX	63.0	62.9	0.68
2B12B3	79.0	24.8	0.62
10E11	230.0	—	—

The rec Der p 2 RAST was inhibited by rec Der p 2 and ala-Der p 2. RAST inhibitions were carried out in two experiments. One patient was tested in both experiments. The results obtained from the second RAST inhibition were equal to the results from the first experiment. The inhibitory potency (IP) of ala-Der p 2 [ratio of the amounts of allergen (ala-Der p 2/rec Der p 2) required for 50% binding] is listed in the third column.

^a Amounts of allergen required for 25% binding are given.

terminants are fully expressed. This incorrect folding might be caused by the fusion to the poly-his tag.

In order to obtain a properly folded recombinant Der p 2 we expressed the group 2 allergen in the baker's yeast *S. cerevisiae* pSY1-p2 transformants secreted ala-Der p 2, which is sequence-identical to the mite Der p 2 with one additional (vector-encoded) single alanine residue attached to the N-terminus. Ala-Der p 2, however, also lacked reactivity with the three mAbs.

To investigate whether this additional N-terminal alanine residue disturbs a possible antigenic determinant, we constructed another expression plasmid, pSAY1-p2. These yeast transformants secreted a mature Der p 2 (rec Der p 2) [14], thus one that is sequence-identical to the cloned mite Der p 2 [1]. This recombinant molecule showed reactivity with the three nonreacting mAbs. The reactivity of ala-Der p 2 and rec Der p 2 was further studied. Ala-Der p 2 lacked reactivity with mAb 10E11 in different assays. Furthermore,

the addition of the alanine residue resulted in a decrease in IgE reactivity for 14/16 sera tested. Thus this particular antigenic determinant is also involved in IgE binding. For the allergen Der f 2 similar results were reported: deletion of 10 amino acids at the N terminus resulted in a decrease in IgE binding [19].

Besides the blocking of the N-terminus – in one way or another leading to a diminished antigenicity – another phenomenon with respect to the fusion partner has to be considered. In the pGEX-p2-derived recombinant allergen, Der p 2 is fused to GST from *Schistosoma japonicum*. A 26-kD allergen from *D. pteronyssinus* that is homologous with GST has been cloned recently [20]. Furthermore, experiments carried out in our laboratory are indicating that for several sera IgE to mite-derived GST was cross-reactive with pGEX-derived GST (unpubl. results). Therefore, in binding studies with human IgE the two effects – loss of binding caused by disturbance of the N-terminal epitope, and additional IgE binding directed against the fusion partner GST – might lead to misinterpretations with regard to the IgE reactivity of the recombinant allergen.

In conclusion, for production of recombinant Der p 2, the mature allergen is preferred over expression as a fusion protein. Furthermore, the expression plasmid pSAY1 is a suitable tool to express other allergens in the mature form as well.

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