Molecular cloning and immunological characterisation of Cyn d 7, a novel calcium-binding allergen from Bermuda grass pollen

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Received 31 October 1996; revised version received 18 December 1996

Abstract A cDNA coding for a newly identified Bermuda grass pollen allergen, Cyn d 7, with significant sequence similarity to Ca^{2+}-binding proteins, was isolated from a cDNA expression library using serum IgE from an allergic individual. The deduced amino acid sequence of Cyn d 7 contained two typical Ca^{2+}-binding sites (EF hand domains). Depletion of Ca^{2+} with EGTA led to a loss of IgE-binding capacity of rCyn d 7. A synthetic peptide based on domain II showed high IgE reactivity. Cyn d 7 therefore represents a grass pollen allergen that belongs to a novel class of Ca^{2+}-binding proteins.

Key words: *Cynodon dactylon*; cDNA cloning; Calcium-binding protein; IgE; Cross-reactivity; Amino acid sequence

1. Introduction

Up to 20% of the human population in developed countries suffers from IgE-mediated atopic diseases such as allergic rhinitis, conjunctivitis and bronchial asthma [1]. The major outdoor cause of allergy is airborne grass pollen [2]. In warm temperate and sub-tropical climates, such as northern and central Australia, Taiwan, southwestern United States and South Africa, Bermuda grass (*Cynodon dactylon*), is an important source of allergens. In Australia, Bermuda grass flowers in summer and autumn extending the hayfever season from early spring, in which other grasses (i.e. rye-grass and canary grass) are considered to be the dominant source of pollen.

Bermuda grass belongs to the sub-family Chloridoideae which is distantly related to other sub-families that contain clinically significant grasses. As a result, differences in the immunological reactivity of the allergens of Bermuda grass compared to other grasses have been noted in a number of studies [3–5]. Moreover, individuals allergic to Bermuda grass require separate diagnosis and treatment [6]. Therefore, molecular and immunological characterisation of Bermuda and other grass pollen allergens are of paramount importance in order to gain a better insight into the molecular basis of allergenicity and provide material for possible use in the future treatment of grass pollen allergy.

We have cloned and characterized the genes encoding the major allergens of two clinically important grasses; Lol p 1 from rye-grass [7] and Cyn d 1 from Bermuda [8]. Although comparison of the deduced amino acid sequences of Cyn d 1 and Lol p 1 indicates a significant degree of identity (64.2%), their immunological reactivities are different. Some individuals have IgE that recognises Lol p 1 but does not recognise Cyn d 1 [8].

Although the major allergens from most of the clinically significant grass pollens have been cloned and their immunological properties characterised, no information is available regarding the biological function of these proteins. However, one example of a well described pollen allergen from the birch tree is profilin, an actin-binding protein [9–11]. Since profilin is suggested to be involved in the acrosomal reaction of *Thyone* sperm [12,13] and also predominantly expressed in the pollen of different unrelated plant species, it is likely that profilins play important roles in plant sexual reproduction and in particular in pollen germination [10]. It is well established that pollen germination and tube growth are dependent on the presence of Ca^{2+} ions and the intracellular levels and the distribution of Ca^{2+} is tightly controlled by Ca^{2+}-binding proteins such as calmodulin [14,15] and such Ca^{2+}-binding proteins have been recently identified as allergens in birch tree pollen [16]. Therefore, the occurrence of similar protein allergens in pollens of distantly related plants (i.e. birch tree and Bermuda grass) deserves further study.

In this paper, we report the cDNA sequence and immunological characterisation of a novel Bermuda grass pollen allergen, designated Cyn d 7, as a Ca^{2+}-binding protein. This is the first cloned grass pollen allergen with a known biological function. Furthermore, a synthetic peptide based on the sequence of Ca^{2+}-binding domain II showed high IgE reactivity, highlighting the importance of the Ca^{2+} domain in allergenicity and its possible role in human autoimmunity in allergy to pollens of distantly related plants. Ca^{2+}-binding proteins serve as receptors for calcium signals in plant and animal cells. Such a signalling function may be important in determining the allergenicity of this protein. On the other hand, cloning of this allergen will make a significant contribution to defining allergens and providing material and information for B cell epitope and T cell studies. It is anticipated that its usefulness could extend into developing reagents to improve diagnosis and immunotherapy of grass pollen allergy.

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Abbreviations: aa, amino acid(s); Ab, antibody(ies); Bet v 3, major allergen(s) of birch pollen; Bet v 4, major allergen(s) of birch pollen; bp, base pair(s); BSA, bovine serum albumin; cDNA, DNA complementary to RNA; Cyn d 1, major allergen(s) of Bermuda grass pollen; Cyn d 7, major allergen(s) of Bermuda grass pollen; Ig, immunoglobulin(s); IUIS, International Union of Immunological Societies; Lol p 1, major allergen of rye-grass pollen; nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); rCyn d 7, recombinant Cyn d 7; rBet v 4, recombinant Bet v 4; SDS, sodium dodecyl sulfate.

The nucleotide and amino acid sequences of Cyn d 7 have been submitted to the EMBL and Genbank databases under the accession numbers X91256 and U35683, respectively.

PII S0014-5793(96)01520-7

2. Materials and methods

2.1. Isolation and sequencing of cDNA clones

Total RNA was extracted from mature anthers of Bermuda grass (*Cynodon dactylon* (L.) Pers.) using the method of Chomczynski and Sacchi [17]. Polyadenylated RNA was isolated using an oligo(dT) affinity column (Pharmacia, Sweden) and cDNA library constructed in λgt11 expression vector using a Time-Saver cDNA synthesis kit (Pharmacia, Sweden). Immunological screening was performed by probing duplicate filters with sera from Bermuda grass pollen allergic individuals. The positive clones were identified using rabbit anti-human IgE antibodies (DAKO, USA) followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Promega, USA). The cDNA inserts recovered from positive clones were ligated into plasmid vector, pBluescript KS(+) (Stratagene, USA). DNA sequencing was performed on double-stranded DNA by the dideoxy chain-termination method using a T7 DNA sequencing kit (Pharmacia, Sweden). Specific oligonucleotide primers were used to obtain the complete sequence along both strands. Sequence analysis, including aa deduction, was performed using the computer program DNA Strider (Commissariat a l'Energie Atomique, France). The nt and aa sequences of Cyn d 7 were searched for comparison with other sequences in the Angis database which incorporates databases from the following major sources: GenBank, EMBL and NBRF nucleic acid libraries; NBRF PIR protein and Swiss-Prot libraries. The nt and deduced aa sequences of the Cyn d 7 cDNA have been submitted to the EMBL and GeneBank databases under the accession numbers X91256 and U35683, respectively.

2.2. Expression and immunological analysis

Immunological screening was accomplished as described by Suphioglu and Singh [18]. Briefly, λgt11 Cyn d 7 clones were plated and fusion protein plaque lifts were taken on Hybond-C extra nitrocellulose membranes (Amersham, UK). Filter sectors were either incubated in serum only (diluted 1:4 in S buffer: PBS, 0.5% BSA), serum in the presence of $CaCl_2$ (S buffer containing 10 mM $CaCl_2$) or serum in the presence of EGTA (S buffer containing 10 mM EGTA, pH 7.5). IgE-binding was detected using ^{125}I-anti-human IgE (Kallestad, USA) as described by Suphioglu et al. [19].

2.3. Peptide synthesis and immunological analysis

Two 12-mer peptides, based on the deduced aa sequences of domains I and II of Cyn d 7 shown in Fig. 1, were synthesised on an automated Advanced ChemTech Multiple Peptide Synthesiser model MPS 350 (Advanced ChemTech, USA) employing Fmoc chemistry. The peptides were assembled sequentially on C-terminal Fmoc-protected aa WANG resins (25 μM; Auspep; Australia). Briefly, the aa was deprotected by piperidine (25% in DMF; Auspep; Australia), washed seven times with DMF (Auspep; Australia), coupled twice with the next aa residue for 30 min each in presence of HOBt and DIC (Auspep; Australia), washed three times with DMF, and the same steps repeated sequentially until the assembly of all aa were completed. After final deprotection, the resin was washed three times with absolute ethanol and dried overnight at 35°C under vacuum. The dried resins were incubated in cleaving solution (95% TFA, 4% phenol, 1% ETT; Auspep; Australia) for 6 h at room temperature (~18°C) and the cleaved peptides precipitated in ice-cold DEE (Auspep; Australia) for 30 min. Precipitated peptides were isolated on qualitative filter paper no. 1 (Whatman, UK) and eluted with 30% acetonitrile in water and then freeze-dried. The lyophilised peptides were resuspended in distilled water to give a 1.6 mM stock solution and analysed by FPLC (Pharmacia, Sweden) and mass spectrometry to check mass, sequence and purity. The peptides were immobilised onto NC filters (BA 0.45 μm, Schleicher and Schuell, Germany) by using a modified method of Sithigorngul et al. [20]. 2 μl drops of the peptide stock solutions were spotted on the NC filters about 1 cm apart. After the strips were dried and baked at 80°C for 1 h, they were fixed by exposure, in a tightly sealed plastic box, to vapor from 0.2% glutaraldehyde (Serva Feinbiochemica GmbH and Co., Germany) in PBS at 37°C overnight followed by fixation in fresh 2% glutaraldehyde in PBS at room temperature for 1 h. After washing thoroughly with distilled water, the NC filters were blocked, washed, screened for IgE-binding and detected with ^{125}I-labelled anti-human IgE, as described in Section 2.2 above.

2.4. Inhibition studies

Expression plasmid containing the Bet v 4 cDNA was constructed in the vector pMW175 [21] which is based on the original pET vectors of Studier et al. [22]. For expression of the pMW175/Bet v 4 plasmid, competent *E. coli* strain BL21(DE3) was transformed and selected on plates containing 100 mg/l ampicillin [22]. A single transformant colony was picked and grown to an OD_{600} of 1.0. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 1.0 mM, and incubation continued for 6 h at 37°C. After expression, cells were harvested by centrifugation, and pellets resuspended in 50 mM Tris-HCl, pH 7.5 containing 220 mM NaCl. Cells were then disrupted by freezing in liquid nitrogen followed by thawing at 37°C. This step was repeated twice. rBet v 4 was recovered in the supernatant after centrifugation at 30 000×*g* for 25 min at 4°C. rBet v 4 was purified from crude *E. coli* lysates by chromatofocusing on a PBE-94 (Pharmacia, Sweden) exchanger column and reverse-phase HPLC, as previously described [23]. Briefly, bacterial lysates were prepared in 25 mM imidazole-HCl buffer, pH 7.4. After treatment with Biocryl BPA-1000 (Tosohaas, Stuttgart, Germany) and centrifugation, the clear supernatant was loaded onto a PBE-94 exchanger column equilibrated with imidazole buffer, at room temperature. Bound proteins were eluted with aqueous 12.5% (v/v) Polybuffer 74/HCl (Pharmacia), pH 4.0. Fractions containing Bet v 4, as determined by dot-blot IgE immunoassay, were pooled and subjected to HPLC employing a linear gradient of 2-propanol created within 60 min at room temperature (C8 Hypersil-WP 300 column, 10 mm, 8×250 mm; solvent A: 0.1% trifluoroacetic acid in water; solvent B: 90% 2-propanol, 0.1% trifluoroacetic acid; gradient of 0–60% solvent B; flow rate 1.0 ml/min). As Bet v 4 contains no tryptophan, tyrosine, or cysteine residues, UV absorbance was monitored at 214 nm. Protein concentration was determined by the micro-Kjeldahl method using glycine as standard [24].

Protein plaque lifts of Cyn d 7 were prepared as described in Section 2.2. Filter sectors were incubated in serum only (diluted 1:4 in S buffer: PBS, 0.5% BSA), serum pre-incubated for 1 h with 100 μg peptide 1, 100 μg peptide 2 or 100 μg rBet v 4. IgE-binding was detected as described in Section 2.2 above.

3. Results and discussion

3.1. Molecular cloning and characterisation of Cyn d 7

A mature Bermuda grass anther λgt11 cDNA expression library was screened with pooled sera of grass pollen allergic patients. Four clones that bound strongly to IgE antibodies (Ab) were identified and plaque-purified. The purified clones gave no signal when screened with non-allergenic human sera. The ability to bind to specific IgE Ab in sera of grass pollen sensitive individuals identified these clones as encoding allergens. Consequently, all four clones were transformed into plasmid vectors and their nucleic acid sequences determined. The sequences of three clones could not be identified with any protein of known function in the databases. The deduced aa sequence of the other cDNA, clone E3, when compared with sequences in EMBL and Swiss-Prot databases revealed a significant sequence similarity with calcium-binding proteins including the birch pollen allergen Bet v 4 (Fig. 1). Moreover, there was a strict conservation of aa residues representing the two calcium-binding domains (Fig. 1). The deduced aa sequence identities between Cyn d 7 and other calcium-binding proteins ranged from 32.9% (with *A. thaliana* touch-induced calmodulin-related protein 3, calmodulin from the fungus *F. oxysporum* and *S. dubia* mRNA for caltractin) to 66% (Bet v 4) (Table 1). However, in all cases the highest sequence similarities were observed with the calcium-binding domains (e.g. 83.3% between domains I and II of Cyn d 7 and Bet v 4). The sequence of clone E3 contains an ORF of 246 bp encoding a mature protein of 82 aa (~9 kDa). Clone E3 was consequently named Cyn d 7 in accordance with IUIS nomenclature [25].

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                                    --Domain I--
Cyn d 7     KTMADT------GDMEHIFKRFDTNGDGKISLAELTDALRTLG-STSAD      42
Bet v 4     DHPQDK------AERERIFKRFDANGDGKISAAELGEALKTLG-SITPD      45
B34669      ------LTDDQITEYKESFRLFDKNGDGSITKKELGTVMRSLGQNPTKA      45
ATHACR      KTMADKLTDDQITEYRESFRLFDKNGDGSITKKELRTVMFSLGKNRTKA      136
CATR_T      ------LTEEQKQDIREAFDLFDTDGSGTIDAKELKVAMRALGFEPKKE      43
CELC24      ------VGEIREDDLKGIFREFDLNGDGYIQREELRAVMQKMGQSPTED      57
MCZP        RKMKD---TDNEEEVREAFKVFDKDGNGYITVEELTHVLTSLGERLSQE      121
CATR_C      ------LTEEQKQEIREAFDLFDTDGSGTIDAKELKVAMRALGFEPKKE      64
CALM_C      RKMKD---TDSEAEIAEAFKVFDRNGDGKISAAELRHLLTSIGEKLSDA      120
JU0232      RKMKD---TDSEEEIREAFKVFDRDNDGFTSAAELRHVMTSIGEKLTDD      119
SDCALT      ------LTEEQKQEIREAFDLFDTDGSGTIDAKELKVAMRALGFEPKKE      63
                        En  nn  n# # #G I#  #n  nn  n
                        .    *  **  .  * *   **    .  .*
                        XXXXXXXXX++++++++++/////////

                      --Domain II-
Cyn d 7     EVQRMMAEIDTDGDGFIDFDEFISFCNANPGLMKDVAKVF               82
Bet v 4     EVKHMMAEIDTDGDGFISFQEFTDFGRANRGLLKDVAKIF               85
B34669      DLQDMMNEVDLDGDGTIDFPEFLNLMA------------                72
ATHACR      DLQDMMNEVDLDGDGTIDFPEFLYLMAKNQGHDQAPRHTK               176
CATR_T      EIKKMIADIDKDGSGTIDFEEFLQMMTAKMGERDSREEIM               83
CELC24      ELDAMFQAADKDCDGNIDFQEFLVIAKANPLSLSLKAVFE               97
MCZP        EVADMIREADTDGDGVINYEEFSRVISSK-----------               150
CATR_C      EIKKMISEIDKDGSGTIDFEEFLTMMTAKMGERDSREEIL               104
CALM_C      DVDQMIKEADTNNDGEIDIQEFTSLLAAK-----------               149
JU0232      EVDEMIREADQDGDGRIDYNEFV-----Q-----------               143
SDCALT      EIKKMIADIDKDGSGTIDFEEFLQMMTAKMGERDSREEIM               103
            En  nn  n# # #G I#  #n  nn  n
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            XXXXXXXXX++++++++++//////////
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Fig. 1. Comparison of Cyn d 7 with other proteins in the EMBL and Swiss-Prot databases exhibiting calcium-binding domains. Bet v 4: *Betula verrucosa* pollen allergen (Engel et al., unpublished; accession no. S54819); B34669: *Arabidopsis thaliana* touch-induced calmodulin-related protein 3 (accession no. B34669 [37]); ATHACR: *Arabidopsis thaliana* calmodulin-related protein (TCH3) gene (accession no. L34546 [38]); CATR_T: caltractin (centrin) from green algae *Tetraselmis striata* (accession no. P43646 [39]); CELC24: EF-hand calcium-binding protein from *Caenorhabditis elegans* cosmid (accession no. U40423 [40]); MCZP: calmodulin from the yeast *Schizosaccharomyces pombe* (accession no. A26614 [41]); CATR_C: caltractin (centrin) from green algae *Chlamydomonas reinhardtii* (accession no. P05434 [42]); CALM_C: calmodulin from the yeast *Candida albicans* (accession no. P23286 [43]); JU0232: Calmodulin from the fungus *Fusarium oxysporum* (Hoshino et al., unpublished; accession no. JU0232); SDCALT: *Scherffelia dubia* mRNA for caltractin (accession no. X69220 [39]). The two calcium-binding domains are indicated by + and the residues predicted to ligand calcium is shown by #; stars (★) indicate identical residues and dots (.) similar ones. Amino acids said to be similar are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W. Numbers to the right of the sequence refer to the amino acid residue numbers of each protein. Gaps represented by dashes (–) have been introduced to ensure maximum homology. The EF-hand model position numbers are from Kretsinger [44] with residues represented by X, + and / corresponding, respectively, to the E helix, calcium-binding loop, and F helix of the calcium-binding domains. The amino acids indicated by n are predicted to be on the hydrophobic face of the α-helices in calcium-binding proteins. The glutamic acid (E), the glycine (G) and the isoleucine (I) in the calcium-binding proteins are also conserved in Cyn d 7 except in domain I where glutamic acid (E) is replaced with a similar amino acid aspartic acid (D). The nt and deduced aa sequences of the Cyn d 7 cDNA have been submitted to the EMBL and GeneBank databases under the accession numbers X91256 and U35683, respectively.

Genes encoding major pollen allergens from rye-grass and Bermuda grass show pollen-specific expression [7,8]. Using RNA gel blot analysis, a partial clone of Cyn d 7 hybridised to a transcript of approx. 900 nucleotides in pollen RNA but no transcripts were detectable in RNA from other Bermuda grass tissues [26]. Moreover, the same clone of Cyn d 7 hybridised to transcripts in pollen of 13 other clinically significant grasses [26], suggesting that Cyn d 7 and related proteins play an important role in grass pollen development.

3.2. Immunological characterisation of Cyn d 7 protein

When recombinant fusion protein of Cyn d 7 was expressed in bacteria as plaque lifts and screened with sera from grass pollen allergic individuals, IgE binding was observed among >36% of the 30 sera tested (Fig. 2). When IgE, specific to a partial clone of Cyn d 7, was eluted off from the plaque lifts and used to screen a Western blot of SDS-PAGE resolved crude Bermuda grass pollen extract, a 12 kDa protein bound these Ab [26]. Similarly, after incubation of the Cyn d 7 protein plaque lifts in the same serum, IgE specific for the 12 kDa protein was depleted from the serum [26]. These results suggest that this 12 kDa protein represents natural Cyn d 7.

It has been reported that the binding of monoclonal antibodies raised against calmodulin requires Ca^{2+} [27] and that depletion of Ca^{2+} from recombinant birch pollen allergen Bet v 3 led to a loss of IgE-binding capacity [16]. We therefore tested whether the binding of allergenic patients' IgE is affected by addition and depletion of protein-bound Ca^{2+} using EGTA. Fig. 2 shows that while addition of Ca^{2+} slightly enhanced IgE reactivity, depletion of Ca^{2+} from rCyn d 7 led to an almost complete loss of the IgE-binding capacity.

In order to investigate whether the two Ca^{2+}-binding domains represent IgE epitopes, we produced synthetic peptides (12-mers) representing the two domains and tested their IgE-binding capacity. Although peptide 1 (based on aa sequence of domain I) did not bind IgE, peptide 2 (based on aa sequence of domain II) showed strong IgE binding, and this binding could be significantly inhibited with the addition of free peptide 2 (Fig. 3). However, IgE binding to peptide 2 was not Ca^{2+} dependent since neither the addition of Ca^{2+} nor

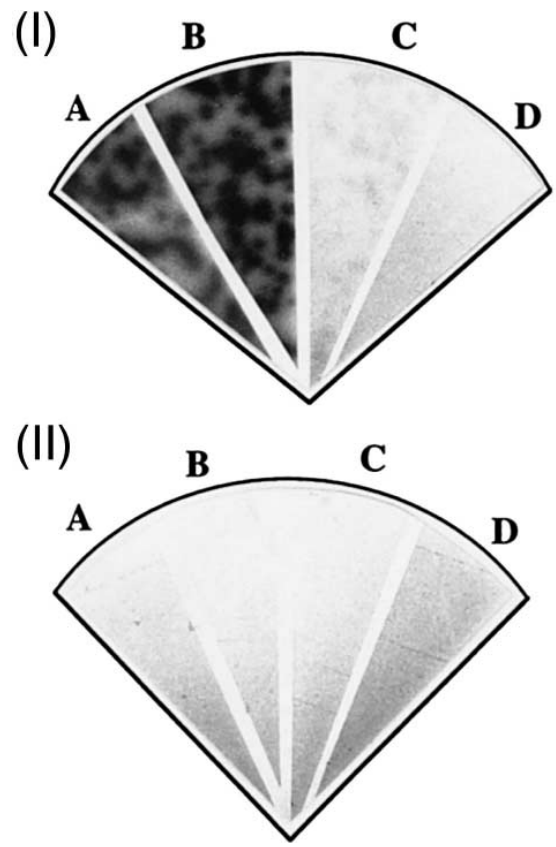

Fig. 2. Effect of protein-bound Ca^{2+} on the IgE-binding capacity of Cyn d 7. Plaque lift nitrocellulose sectors containing rCyn d 7 (A–C) or non-recombinant λgt11 control (D) were incubated with serum IgE from a Bermuda grass pollen allergic individual (I) or with serum IgE from a non-allergic individual (II). Filters were incubated either in serum only (A,D), or serum in the presence of $CaCl_2$ (B) or EGTA (C).

depletion of Ca^{2+} with EGTA generate a significantly different IgE-binding intensity to that shown in Fig. 3A (data not shown). This suggests that IgE binding may be directed to specific amino acid residue(s) that, unlike our observations for rCyn d 7, does not require Ca^{2+} for binding. In a similar study with Bet v 3 from birch pollen, Seiberler et al. [16], could not detect any IgE binding when inhibited with synthetic peptides based on all three calcium-binding domains of Bet v 3. As may be the case with our peptide 1, the lack of IgE binding to the three peptides of Bet v 3 may imply that the IgE-binding epitopes represented by these peptides may be conformational within the context of the natural folded protein. Indeed, while the SDS-PAGE resolved natural Cyn d 7 can be identified with affinity purified IgE antibodies specific to rCyn d 7, Seiberler et al. [16] reported that although rBet v 3, as plaque lifts, binds IgE, no IgE binding could be detected under the denaturing conditions of SDS-PAGE, indicating that the binding of allergenic patients' IgE might depend on the natural conformation of Bet v 3. However, it should also be stressed here that IgE-reactive, recombinant proteins, especially fusion proteins like Cyn d 7 and Bet v 3, are unlikely to mimic the natural conformation of these proteins. Despite this, natural Cyn d 7 can be identified with rCyn d 7-specific IgE Ab [26] indicating the presence of the recombinant epitopes within the context of the natural protein.

3.3. Immunological cross-reactivity between Cyn d 7 and Bet v 4 proteins

A major birch pollen allergen, Bet v 3, has been recently characterised as a Ca^{2+}-binding protein [16]. Unlike Cyn d 7, Bet v 3 has three calcium-binding domains and is a larger protein. On the other hand, a search of the EMBL and Swiss-Prot databases revealed a strong sequence similarity with another recently cloned Ca^{2+}-binding allergen from birch pollen, designated as Bet v 4. Bet v 4 is not only similar to Cyn d 7 in molecular mass but also shares two similar Ca^{2+}-binding domains (Fig. 1).

In order to assess whether Bet v 4 and Cyn d 7 were immunologically cross-reactive, we performed antibody depletion experiments (Fig. 4). Here, we have screened Cyn d 7 plaque lifts with serum from a Bermuda grass pollen allergic individual either in the absence or presence of purified rBet v 4, as the competing protein. Fig. 4 clearly shows that Bet v 4 has the capacity to compete with Cyn d 7 for the same IgE Ab and thus must be immunologically similar or cross-reactive. Plaque lifts screened under the same conditions using serum from a non-allergic individual indicated no binding.

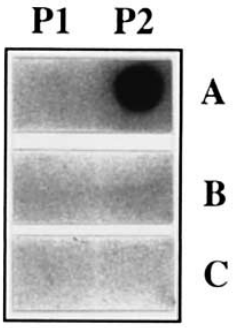

Fig. 3. IgE reactivity of synthetic peptides based on Ca^{2+}-binding domain I (P1) and domain II (P2) of Cyn d 7. (A) Peptides screened with serum of a Bermuda grass pollen allergic individual only. (B) Peptides screened with the same serum pre-incubated with 100 μg of P2. (C) Peptides screened with serum from a non-allergic individual.

In a further study, addition of peptide 2 (based on domain II) to allergic patient serum gave a significant reduction in IgE Ab binding to Cyn d 7 plaque lifts (Fig. 4). Moreover, when the aa sequences representing domain II of Cyn d 7 and Bet v 4 are compared there is a very high sequence identity (> 83%) (Table 1); the two different aa residues at positions 60 and 62 of Cyn d 7 sequence can thus be considered as not critical for IgE-binding. However, peptide 1 (based on domain I) has similar aa sequence identity (> 83%) (Table 1) but did not show any inhibitory effect on IgE-binding. These results clearly indicate that Ca^{2+}-binding domain II is also an allergenic epitope in Cyn d 7 and Bet v 4 and must exist as a linear epitope within the context of the folded natural proteins. On the other hand, the lack of IgE-binding and competitive effect of peptide 1 on IgE-binding reactivity of Cyn d 7 indicates that this domain does not represent an allergenic epitope or may be conformational in nature within the context of the folded natural protein.

3.4. Clinical and biological significance of Cyn d 7 protein

This is the first report of the molecular cloning and characterisation of a Ca^{2+}-binding allergen from grass pollen and the cloning of a grass pollen allergen with a known biological

Table 1
Percentage identity between the deduced aa sequence of Cyn d 7 and other calcium-binding proteins as shown in Fig. 1

Proteins	Source	Data-base name	% aa sequence identity with Cyn d 7	% aa sequence identity with domain I of Cyn d 7	% aa sequence identity with domain II of Cyn d 7
Bet v 4	*Betula verrucosa*	Bet v 4	65.9	83.3	83.3
Touch-induced calmodulin-related protein 3	*Arabidopsis thaliana*	B34669	32.9	48.3	75
Calmodulin-related protein (TCH3)	*Arabidopsis thaliana*	ATHACR	40.2	58.3	75
Caltractin (centrin)	*Tetraselmis striata*	CATR_T	35.4	50	66.7
EF-hand calcium-binding protein	*Caenorhabditis elegans*	CELC24	36.6	58.3	66.7
Calmodulin	*Schizosaccharomyces pombe*	MCZP	35.4	41.7	66.7
Caltractin (centrin)	*Chlamydomonas reinhardtii*	CATR_C	34.1	50	66.7
Calmodulin	*Candida albicans*	CALM_C	39	83.3	58.3
Calmodulin	*Fusarium oxysporum*	JU0232	32.9	58.3	66.7
Caltractin	*Scherffelia dubia*	SDCALT	32.9	50	66.7

References for the proteins are listed in Fig. 1.

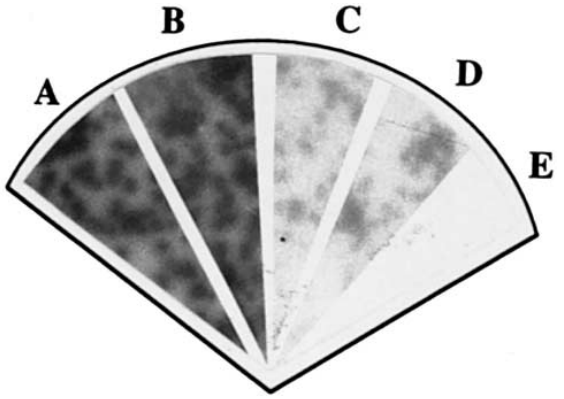

Fig. 4. Assessment of immunological cross-reactivity between rCyn d 7 and rBet v 4 from birch pollen. Protein plaque lifts of Cyn d 7 probed with serum from a Bermuda grass pollen allergic individual only (A); serum pre-incubated with 100 μg peptide 1 (based on domain I) (B); serum pre-incubated with 100 μg peptide 2 (based on domain II) (C); serum pre-incubated with 100 μg rBet v 4 (D); serum from a non-allergic individual (E).

function. Allergens of similar sequence structures and biological functions have been characterised in birch pollen (i.e. Bet v 3 and Bet v 4). However, although birch is botanically distantly related to Bermuda grass, evidence of the similarities between Cyn d 7 and Bet v 4 highlight the presence of strict conservation of certain genes through evolution.

There is very significant similarity between Cyn d 7 and other Ca^{2+}-binding calmodulins. Calmodulin is a highly conserved protein found in all eukaryotes with 98% similarity between vertebrates and plants [28]. The low level of similarity between Cyn d 7 and other plant calmodulins suggests that Cyn d 7 may not encode calmodulin itself. However, the high level of identity with the Ca^{2+}-binding domain of calmodulin suggests that Cyn d 7 may encode calmodulin-related Ca^{2+}-binding proteins. Indeed, this type of protein has been identified in mammals [28]. Calmodulin acts as a second messenger in many systems, binding Ca^{2+} and then interacting with enzymes to activate them. Calmodulin has been implicated in such important functions as mitosis [29], induced microtubule dissociation [30] and in pollen tube growth where it may be associated with the microfilament network and be involved in vesicle transport and cytoplasmic streaming [31]. Indeed, recent works have shown that tip-focused Ca^{2+} gradient is present in pollen tubes of *Agapanthus* [32], *Lilium*, *Tradescantia* and *Nicotiana* [33]. Therefore, since Ca^{2+}-binding proteins may have similar functions to those of calmodulin and transcripts similar to Cyn d 7 have been found in other grasses examined [26], we suggest that Cyn d 7 may play a pivotal role as a messenger in grass pollen development and especially in pollen tube growth with tip-focused Ca^{2+} gradients. Now that we have cDNA clones that encode Cyn d 7, we are interested in the localisation of Cyn d 7 in the germinating pollen; especially the tip of pollen tubes.

In terms of clinical importance, a number of low M_r allergens from Bermuda grass pollen have been previously identified but not fully characterised [34,35] and it is possible that the protein encoded by Cyn d 7 corresponds to one of these. Since allergens with significant similarity to calmodulin have also been recently identified in birch pollen [16], it may be possible that these Ca^{2+}-binding proteins are ubiquitous proteins like manganese superoxide dismutase from *Aspergillus fumigatus* which has been recently shown to have in vitro and in vivo humoral and cell-mediated autoimmune reactivity to human manganese superoxide dismutase in individuals allergic to *A. fumigatus* [36].

We have demonstrated in this study the occurrence of cross-reactivity and shared IgE-binding epitopes between Cyn d 7 and Bet v 4. An exciting feature of this study has been the finding that Ca^{2+}-binding domain II shows high IgE reactivity whereas domain I does not. It will be important in future work to immunodissect domain II further in terms of its Ca^{2+} binding and the apparent co-occurrence of IgE-binding by amino acid replacement studies. The availability of highly purified Cyn d 7, whether natural, recombinant or synthetic, will be useful in standardisation of allergen extracts for both diagnosis and treatment of allergies triggered by Ca^{2+}-binding proteins.

Acknowledgements: The authors wish to thank Dr. S. Krishnaswamy of Doncaster, Victoria for supply of sera, the Australian National Health and Medical Research Council, Asthma Foundation of Victoria, Australia and Grant P10019-MOB from the Fonds zur Förderung der Wissenschaftlichen Forschung, Vienna, Austria for financial support of this research.

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