Allergens, IgE, mediators, inflammatory mechanisms

Cloning and expression in yeast *Pichia pastoris* of a biologically active form of Cyn d 1, the major allergen of Bermuda grass pollen

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Background: *Pollen of grasses, such as Bermuda grass* (Cynodon dactylon), *represent a major cause of type I allergy.*
Objective: *In this report we attempted to clone and express a biologically active form of recombinant Cyn d 1, the major allergen of Bermuda grass pollen, in the yeast* Pichia pastoris.
Methods: *Clones encoding Cyn d 1 were isolated by screening a Bermuda grass pollen complementary DNA library with specific monoclonal antibodies and by polymerase chain reaction amplification. Recombinant Cyn d 1 was expressed in* Escherichia coli *and yeast. The expressed proteins were analyzed by Western blotting to assess binding to Cyn d 1–specific monoclonal antibodies and IgE from sera of patients allergic to Bermuda grass pollen.*
Results: *Two isoforms of Cyn d 1 were cloned. Recombinant Cyn d 1 expressed in bacteria bound two monoclonal antibodies raised against Cyn d 1 but was not recognized by IgE from sera of patients allergic to Bermuda grass pollen. Cyn d 1 expressed in yeast bound both the monoclonal antibodies and human IgE.*
Conclusion: *An IgE-reactive Cyn d 1 was expressed in yeast but not in bacteria, suggesting that posttranslational modifications (e.g., glycosylation), which occur in eukaryotic cells such as yeast, are necessary for the production of a biologically active allergen. (J Allergy Clin Immunol 1996;98:331-43.)*

Key words: *Cyn d 1, Bermuda grass, pollen allergens, cDNA cloning, expression, yeast, IgE binding*

Grass pollen is a major cause of seasonal hay fever and allergic asthma in spring and summer. Pollen from a number of grasses has been implicated in this reaction. In cool temperate climates, grasses such as ryegrass, Kentucky bluegrass, and timothy grass (subfamily, Pooideae) are clinically significant. However, in warm temperate and subtropical environments pollen of Bermuda grass (subfamily, Chloridoideae) becomes an important source of allergens.

Abbreviations used

IPTG:	Isopropyl beta thiogalactopyranoside
mAb:	Monoclonal antibody
ORF:	Open reading frame
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
SDS-PAGE:	Sodium dodecylsulfate–polyacrylamide gel electrophoresis

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Individuals sensitive to the allergens from one grass are often sensitive to those of a number of other grasses.[1] Immunologic cross-reactivity among the allergens from these grasses is demonstrated by RAST inhibition experiments in which pollen extracts of one grass are able to inhibit the binding of IgE to extracts from other grasses. This is particularly true for pollen of grasses within the subfamily Pooideae.[2-4] In contrast, little inhibition is observed when pollen extracts of these grasses are used to inhibit the binding of IgE to Bermuda grass extracts, suggesting that the IgE-binding epitopes of Bermuda grass allergens are distinct from those of the Pooid grasses.[3, 4]

The major allergens that elicit an allergic reaction to pollens from the Pooid grasses are those of Groups 1 and 5 (previously Group IX). In ryegrass, Lol p 1 and Lol p 5 (previously described as *Lol p* Ib[5] or *Lol p* IX[6]) together can inhibit virtually all the IgE binding to crude pollen extracts.[7] There is allergenic cross-reactivity among Group 1 and Group 5 allergens from different grasses.[8, 9] For the Group 1 allergens, this can be explained by the presence of an IgE epitope that is shared by Group 1 allergens from at least five grass genera of the subfamily Pooideae.[10] This epitope has been localized within a 28 amino acid peptide of Lol p 1, which is highly homologous with peptides from four other grass Group 1 allergens.[10] Other shared epitopes may also exist on both Group 1 and Group 5 allergens from different grasses.

It is not known whether the major allergen of Bermuda grass, Cyn d 1, shares IgE epitopes with Group 1 allergens from other grasses, but monoclonal antibodies (mAbs) raised against Cyn d 1 have been shown to recognize Group 1 allergens from other grasses.[11, 12] The NH_2-terminal amino acid sequence of Cyn d 1 is similar to those of Lol p 1 and Poa p 1,[12] and it shares a number of physicochemical characteristics with other Group 1 allergens.

Cyn d 1 is a glycoprotein[11] to which more than 87% of individuals sensitive to Bermuda grass pollen are allergic.[13, 14] A number of isoforms of Cyn d 1 differ in both isoelectric point and molecular mass. The major isoforms are 31 and 32 kd,[11, 12] but 23 and 29 kd isoforms have also been identified.[15]

In this article we report the isolation and expression of a biologically active form of the gene encoding Cyn d 1 by using both complementary DNA cloning and polymerase chain reaction (PCR) amplification. Comparison of its deduced amino acid sequence with that of Lol p 1[16] shows that there is considerable homology among the proteins. Although recombinant Cyn d 1 expressed in *Escherichia coli* is not recognized by IgE from the sera of patients allergic to Bermuda grass, the recombinant protein expressed in yeast does bind IgE, suggesting that posttranslational modification is important for IgE binding to Cyn d 1. It is possible that the non-IgE-reactive, bacterially expressed, Cyn d 1 may be useful as a therapeutic tool and that the IgE-reactive, yeast-expressed Cyn d 1 may be useful as a diagnostic tool.

METHODS

Plant material

Bermuda grass pollen was obtained from Greer Laboratories Inc. (Lenoir, N.C.) as a dry, nondefatted pollen and stored at −20° C until required.

Monoclonal antibodies

Monoclonal antibodies 1D1, 3A2, and 4D2 were produced as described by Smith et al.[12]

Construction of cDNA libraries

RNA was isolated from Bermuda grass pollen by using a modification of the guanidinium thiocyanate method.[17] After the pollen was ground in guanidinium thiocyanate buffer (5 mol/L guanidinium thiocyanate in 25 mmol/L sodium citrate [pH 7.0], 0.1 mol/L β-mercaptoethanol, 0.5% sarcosyl), the solution was repeatedly extracted with phenol-chloroform-isoamyl alcohol (25:24:1) until the interface was clear after centrifugation. The aqueous phase was then treated as previously described.[17]

Messenger RNA was isolated by using an mRNA purification kit (Pharmacia LKB, Uppsala, Sweden). Complementary DNA was synthesized by using a You-Prime cDNA synthesis kit (Pharmacia LKB) according to the manufacturer's instructions, and the linkered cDNA was inserted into the expression vector λgt11 (Promega Corp., Madison, Wis.).

Screening of cDNA library

Lambda-gt11 cDNA libraries or purified λgt11 clones were incubated for 15 minutes at 37° C with *E. coli* Y1090 cells and then plated onto Luria broth plates containing 0.1 μg/μl ampicillin. After 3 hours of growth at 42° C, the plates were overlaid with 10 mmol/L isopropyl-β-thiogalactopyranoside (IPTG)–impregnated nitrocellulose filters (Hybond-C extra; Amersham International plc, Buckinghamshire, U.K.) in order to induce production of fusion protein. The plates were then incubated for 4 hours at 37° C. Replica filters were obtained by overlaying the plate with a second filter for 4 hours. Unreacted binding sites on the plaque lifts were blocked by incubation of the nitrocellulose filters in powdered milk (10% in phosphate-buffered saline (PBS: 150 mmol/L NaCl, 16 mmol/L Na_2HPO_4, 4 mmol/L NaH_2PO_4, pH 7.2). The lifts were then incubated in mAb (hybridoma supernatant) for 1.5 hours at room temperature, washed twice in PBS containing 0.1% Tween-20 and twice in PBS alone, then incubated in

TABLE I. Oligonucleotides

Name	Nucleotide sequence	Specificity	Strand	Position
AL	5′p-AATGATCGATGCT *Cla* I	NA	NA	NA
AP	5′GGGTCTAGAGGTACCGTCCG *Xba* I *Kpn* I	NA	NA	NA
AT	5′GGGTCTAGAGGTACCGTCCGATCGATCATT *Xba* I *Kpn* I *Cla* I	NA	NA	NA
CD-4	5′GGGGATCCGAGGCCGTCCTTGAA *Bam* HI	Clone 18B	Noncoding	52 < 68
CD-5	5′GATGTGCTCGTAGTTCTT	Clone 18B	Noncoding	148 < 165
CD-13	5′TTTCTAGAGCCATCGGCGACAAGCCAGGGCCC *Xba* I	Clone 14a1	Coding	107-130
CD-15	5′GCGTACTTCACGAGCAGCGCCAGGTAATT	Clones 2B & 3B	Noncoding	178 < 206
CD-16	5′TTGAATTCGACACGGCGGAACTGCAGCAT *Eco* RI	Clones 2B & 3B	Noncoding	97 < 117
CD15′N	5′GGGAATTCGCCATCGGCGACAAGCCAG *Eco* RI	Clone 14a1	Coding	107-125
CD15′	5′GGGAATTCGTGGCTGCGATGGTGGCC *Eco* RI	Clone 14a1	Coding	77-94
Cd13′B18	5′CCCTGCAGATGGAGGATCATCGTCTC *Pst* I	Clone 18B	Noncoding	604 < 621

AL, AP, and AT are synthetic link oligonucleotides that do not correspond to the sequence of Cyn d 1. Position numbers correspond to the numbering of the nucleotide sequence of the clones shown in Fig. 2. Restriction endonuclease sites added for cloning purposes are underlined.
NA, Not applicable.

peroxidase-labeled anti-mouse Ig (Silenus, Vic, Australia) (1:500 in PBS–bovine serum albumin) for 1 hour at room temperature. This was followed by washing and color development with the enzyme substrate as described by Singh and Knox.[18]

Plaques that were antibody-positive were designated 1B to 35B. They were cut out of the plate, purified, and retested for antibody binding.

DNA was isolated from plaque-purified phage by using a liquid lysate method.[19] Inserts recovered from *Eco*RI digestion were ligated into pGEM3Z (Promega) or pBluescript vectors (Stratagene, La Jolla, Calif.) for nucleotide sequencing.

PCR amplification of clones encoding Cyn d 1

Single- or double-stranded cDNA was synthesized by using either a cDNA Synthesis System Plus kit (BRL Life Technologies, Gaithersburg, Md.) or a Promega cDNA synthesis kit. Amplification of the 5′ region of the Cyn d 1 transcript was done with the anchored PCR method.[20, 21] Briefly, anchored PCR involves the amplification of cDNA, with a poly(dG) tail added to its 3′ termini, primed by an oligonucleotide consisting of a poly(dC) tail attached to a sequence with convenient restriction sites (termed the anchor) and a single synthetic oligonucleotide (or a degenerate pool of oligonucleotides) corresponding to a known tract of amino acids in the protein of interest. For example, in our study double-stranded cDNA was synthesized from total RNA by using oligo dT as a primer, blunt-ended with T4 polymerase, and blunt end–ligated to self-annealed AT and AL primers. Oligonucleotides AP (anchor) and CD-5 were then used to prime double-stranded cDNA in the first amplification. Oligonucleotides AP and CD-4 (which is nested internally compared with CD-5) were then used as primers in a reaction, which used 5% of the first amplification as a template (Table I). The use of internally nested oligonucleotides in the second amplification reaction provided convenient restriction endonuclease sites for subcloning.

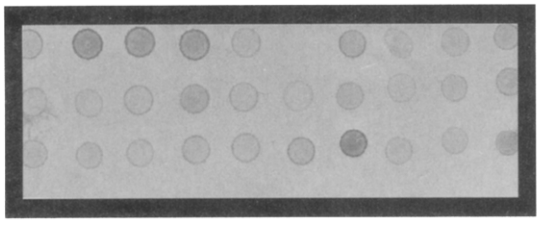
PCR was also used to amplify different isoforms and the full coding region of Cyn d 1. DNAs corresponding to the NH_2-terminal region of clone 2B or 3B were amplified with oligonucleotides CD-13 and CD-15 in the primary amplification. CD-13 and CD-16 (which is nested internally to CD-15) were then used to prime the secondary reaction.

The full coding region of Cyn d 1 was amplified from first-strand cDNA (primed with oligo dT) by using oligonucleotides CD15′N and CD13′. The sequences of all oligonucleotides are shown in Table I.

PCR products were recovered by sequential chloroform and phenol and chloroform extractions, followed by precipitation at −20° C with 0.5 volumes of 7.5 mol/L ammonium acetate and 1.5 volumes of isopropanol. After precipitation, the DNA was washed with 70% ethanol, cut with the appropriate restriction enzymes and electrophoresed on a 3% low-melting-point agarose gel (Seaplaque; FMC Corp., Bioproducts Div., Rockland, Maine) or a 1% agarose gel (Promega). The appropriate-sized DNA bands were cut out, and the DNA was ligated into digested vector

1B	2B	3B	4B	5B	6B	7B	8B	13B	15B
16B	18B	19B	20B	21B	22B	23B	24B	25B	26B
27B	28B	29B	31B	32B	33B	34B	35B	36B	37B
λ	λ								

A.



B.

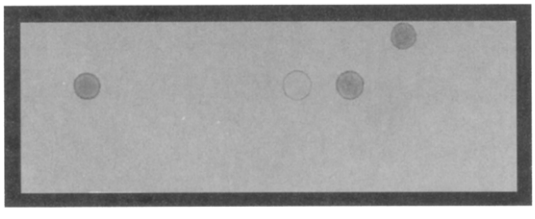


FIG. 1. Specificity of mAbs for clones expressing regions of Cyn d 1. Recombinant fragments of Cyn d 1 (clones 1B to 35B) were expressed in λgt11, and proteins were transferred to nitrocellulose. Proteins were then screened for binding of mAbs: **A,** 3A2 and **B,** 4D2. The grid above shows the name of the clone corresponding to each spot. Dots denoted λ are proteins expressed by nonrecombinant λgt11.

for nucleotide sequencing. The vectors used were M13mp19 (New England Biolabs, Beverly, Mass.), pUC (New England Biolabs), or pBluescript (Stratagene).

Nucleotide sequencing

DNA sequences were determined by the dideoxy chain termination method[22] with T7 DNA polymerase (T7 DNA sequencing kit; Pharmacia LKB) or Sequenase (United States Biochemical Corp., Cleveland, Ohio).

Expression of Cyn d 1 in *E. coli*

Clone CD1 was subcloned into the expression vector p*Trc* 99A. Soluble bacterial proteins were isolated from cultures after recombinant protein production was induced with 1 mmol/L IPTG according to the method of Amman et al.[23] The proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Singh et al.,[5] the proteins were transferred to nitrocellulose, and the Western blot was probed with mAbs and IgE as described in screening of cDNA libraries above and by Ong et al.[24]

Expression of Cyn d 1 in the yeast *Pichia pastoris*

Clone CD1 was inserted into the multiple cloning sites of the yeast expression vector pHIL-S1 (Invitrogen Corp., San Diego, Calif.). The constructs were sequenced to confirm that the open reading frame (ORF) of the cloned insert was in the same reading frame as the vector's secretion signal sequence. This is needed for effective secretion of the recombinant CD1 protein. Constructs were then linearized with the restriction enzyme *Bgl*II and used to transform yeast spheroplasts.[25, 26] Since the transformants will not be producing alcohol oxidase (the product of the *AOX*1 gene) because the *AOX*1 gene has been disrupted with the cloned insert, they cannot efficiently metabolize methanol as a carbon source and therefore grow poorly on media supplemented with methanol as the sole carbon source.[27, 28] The transformant cells were then grown to saturation for 2 days at 30° C in buffered minimal glycerol-complex medium, and the secretion of the recombinant CD1 protein was induced with methanol.[29] After 2 days of expression, the cells were pelleted, and the supernatant was analyzed for the product with SDS-PAGE and Western blotting; 30 μg of total protein was loaded per well and resolved on 10% polyacrylamide gel. Western blots were probed with Cyn d 1–specific mAb 3A2 and IgE from a panel of six sera from patients allergic to Bermuda grass pollen, followed by horseradish peroxidase–labeled anti-mouse Ig (for detection of mAb binding) and iodine 125–labeled anti-human IgE (for detection of IgE binding), as described previously.[24]

RESULTS

Isolation of cDNA clones encoding Cyn d 1

Monoclonal antibodies directed against Cyn d 1 were used to isolate cDNA clones encoding Cyn d 1. The preparation and specificity of these mAbs has been described elsewhere.[12] Thirty-five positive clones were isolated from a Bermuda grass pollen cDNA expression library with mAb 3A2. These were retested to confirm binding to mAb 3A2, and eventually 28 positive clones were plaque-purified (Fig. 1). Proteins expressed by these cDNA clones were also tested for binding to mAb 4D2 (Fig. 1). Fusion proteins of only four clones bound to mAb 4D2. Three of these clones (18B, 22B, and 23B) and four clones that expressed fusion proteins recognized only by mAb 3A2 (2B, 3B, 21B, and 33B) were chosen for further study. IgE binding to fusion proteins expressed by the 29 clones was not detected.

Sequence of cDNA clones isolated with mAb probes

The nucleotide sequences of the seven selected clones were determined (Fig. 2). They ranged in size from 424 to 834 nucleotides. The cDNA clones

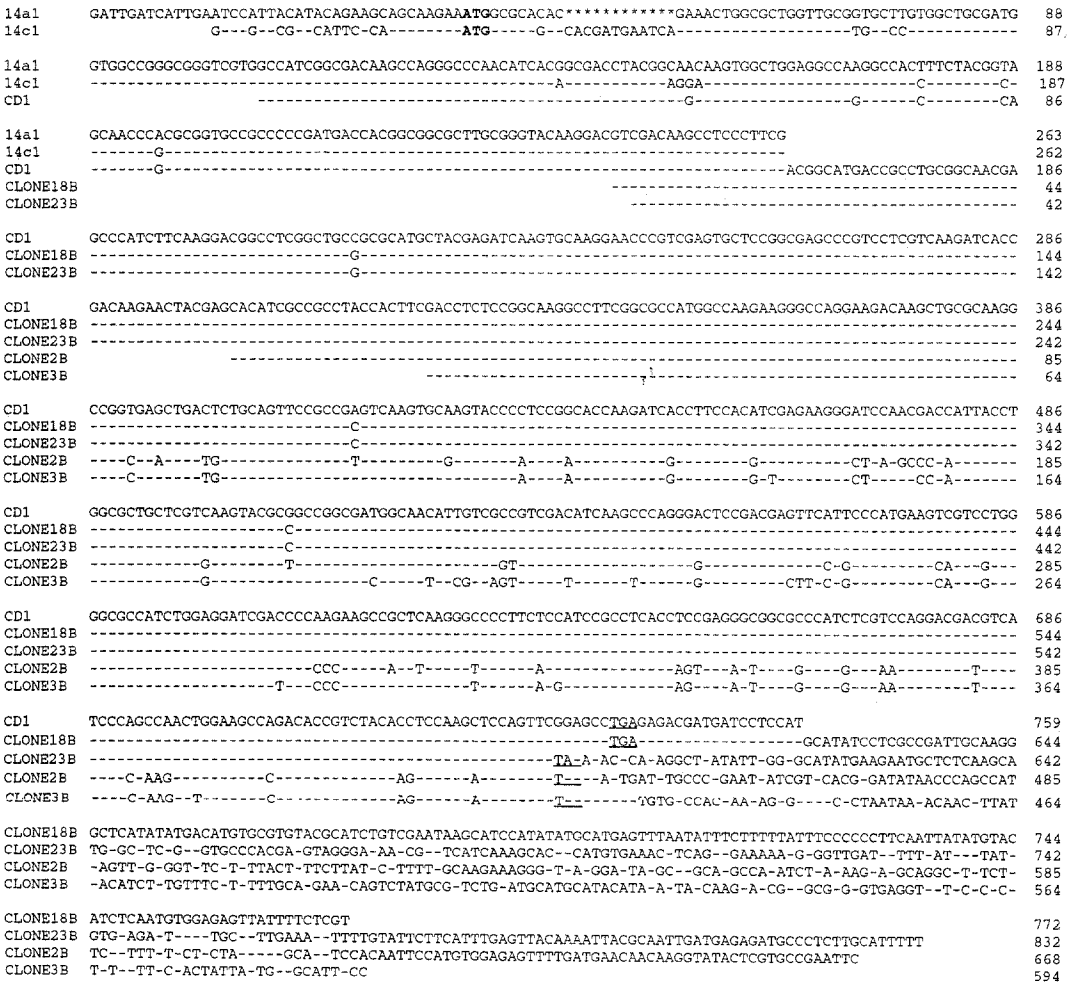


FIG. 2. Comparison of DNA sequences of cDNA and PCR-derived clones encoding Cyn d 1. Nucleotides identical to those of clone 14a1 or CD1 are indicated by *a dash.* The proposed start point of translation is indicated by *bold letters. Underlined nucleotides* indicate stop codons. Numbers to the right of the nucleic acid sequence refer to the numbering for each clone. *Asterisks* indicate gaps that have been inserted for maximum homology.

that were sequenced can be separated into two groups on the basis of sequence homology: those with sequence most similar to clone 18B (clones 21B, 22B, 23B, and 33B) and those most similar to clone 2B (clone 3B). There is 85% identity between the nucleotide sequences of clones 18B and 2B (Fig. 2).

The deduced amino acid sequences of the isolated clones are given in Fig. 3. The longest ORF (200 amino acids) was encoded by clone 18B. None of the clones contained the known NH_2-terminal amino acid sequence for Cyn d 1, indicating that they did not encode the complete Cyn d 1 protein. However, the clones do show homology with the known sequence of Lol p 1,[16, 30] suggesting that they encode a Group 1 allergen. Within the overlapping portions of clones 18B, 21B, 22B, 23B, and 33B, no amino acid sequence differences are encoded by the five clones (Fig. 3). Clones 22B and 23B encode proteins that are two and three amino acids shorter, respectively, than 18B. These clones also have different 3′ untranslated sequences compared with 18B and each other. Clone 33B, although identical to clone 18B within the ORF (67 amino acids), has a different 3′ untranslated sequence (data not shown).

Within the clone 2B family, there is more variation in the amino acid sequence. There are five amino acid differences between the proteins encoded by clone 2B (145 amino acids) and 3B (138 amino acids) (96.3% identity, 1.5% similarity) (Fig. 3). The proteins encoded by these clones are shorter than 18B by two amino acids at their C-terminal. There is 84.1% amino acid identity

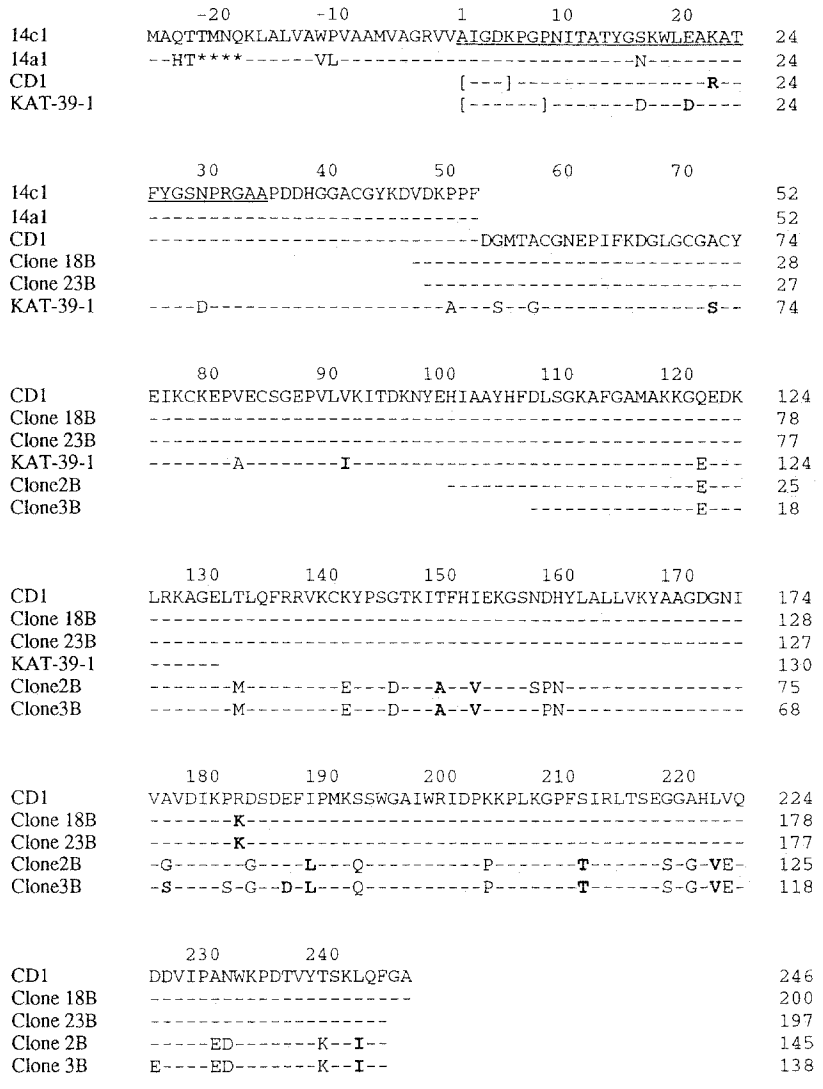


FIG. 3. Comparison of deduced amino acid sequences of PCR-generated clones encoding Cyn d 1 (CD1, 14a1, 14c1, and KAT-39-1) with that of cDNA clones isolated by using an mAb probe (2B, 3B, 18B, and 23B). Residues identical to 14c1 or CD1 are indicated by *a dash*. Amino acids similar to 14c1 or CD1 are indicated by *bold letters*. Amino acids said to be similar are: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; F, Y, and W. Numbers to the right of the sequence refer to the numbering for each clone. Numbers above the sequence refer to the numbering for clones 14c1 or CD1. Amino acids that have previously been identified by amino acid sequencing are *underlined*. Residues in brackets are those encoded by the oligonucleotide used to prime the PCR.

(4.1% similarity) between the proteins encoded by clone 18B and clone 2B and 80.1% identity (7% similarity) between those encoded by clones 18B and 3B (Fig. 3).

PCR amplification and nucleotide sequencing of clones encoding Cyn d 1

The 5′ region of Cyn d 1 was cloned by anchored PCR with oligonucleotides based on the noncoding strand of clone 18B. The resultant two partial clones overlap by 19 nucleotides with the 5′ end of clone 18B. The nucleotide and deduced amino acid sequences of these PCR-derived clones, 14a1 and 14c1 are shown in Figs. 2 and 3. The deduced amino acid sequences of these clones include a region with homology to all the isoforms of Cyn d 1 for which an NH_2-terminal amino acid sequence has been determined.[5, 11, 12, 15] The putative trans-

lation initiation codon of 14a1 corresponds to nucleotides 41-43 (Fig. 2). Surrounding this start point are nucleotides with 78% identity with the consensus sequence for initiation of translation in plants (AACAATGGC).[31] There is an in-frame stop codon at nucleotides 11-13 (Fig. 2), which further supports the identification of nucleotides 41-43 as the translation initiation codon of 14a1. Clone 14a1 encodes a leader sequence of 22 amino acids before the start of the mature protein[5, 11] (Fig. 3). The leader sequence encoded by 14c1 has three amino acid differences compared with 14a1 and is longer by four amino acids. The expected start point of translation for 14c1 is the methionine encoded by nucleotides 28-30 (Figs. 2 and 3).

Oligonucleotides corresponding to the 5′ end of the Cyn d 1 clone were synthesized by using sequences conserved between clone 14a1 and 14c1. These were used with a 3′ oligonucleotide (based on the noncoding strand of clone 18B) to amplify a clone encoding the coding region of Cyn d 1 (for nucleotide sequence see Fig. 2). Clone CD1 encodes only the mature Cyn d 1 protein (Figs. 2 and 3). The ORF of CD1 is 738 nucleotides and encodes 246 amino acids. In the region that overlaps with clone 18B, there is one nucleotide difference, which results in a conservative amino acid substitution of lysine (position 137, clone 18B) for arginine (position 201, clone CD1) (Fig. 3). There are two amino acid substitutions (one conservative) between 14a1 and CD1 in the region in which they overlap and one conservative amino acid substitution between 14c1 and CD1 (Fig. 3). The protein encoded by clone CD1 has one potential N-glycosylation site (Asn-X-Ser/Thr) at asparagine 9.

A more complete clone corresponding to clones 2B or 3B, designated KAT-39-1, was isolated after PCR amplification with oligonucleotides corresponding to the NH_2-terminal region of Cyn d 1 (CD-13) and to regions that are common for both 2B and 3B (CD-15, CD-16). The nucleotide and deduced amino acid sequences of KAT-39-1 were determined (Figs. 2 and 3) and compared with other clones encoding Cyn d 1. The nucleotide sequences of KAT-39-1 and clone 3B are identical in the region in which they overlap (Fig. 2), suggesting that KAT-39-1 is derived from the same transcript as clone 3B. There are eight nucleotide differences between KAT-39-1 and clone 2B (Fig. 2), but their deduced amino acid sequences are identical in the region in which they overlap (Fig. 3). There are 11 amino acid differences between the proteins encoded by CD1 and KAT-39-1 in the region in which they overlap (Fig. 3). If the nucleotide sequences of KAT-39-1 and clone 3B are combined, the Cyn d 1 isoform that they encode (Cyn d 1.3) has 244 amino acids, two fewer than that encoded by clone CD1 (Cyn d 1.CD1). There is 86.1% identity between Cyn d 1.3 and Cyn d 1.CD1.

The deduced amino acid sequence of CD1 (Cyn d 1.CD1) and a combined KAT-39-1/clone 3B (Cyn d 1.3) sequence have been compared with the amino acid sequences of Lol p 1[16] and Sor h 1[32] (Fig. 4). There is 70.7% homology (62.2% identity, 8.5% similarity) between Cyn d 1.CD1 and Lol p 1 and 72.3% homology (63.8% identity, 8.5% similarity) between Cyn d 1.CD1 and Sor h 1. There is 74.1% homology (66.3% identity, 7.8% similarity) between Cyn d 1.3 and Lol p 1 and 73.8% homology (66% identity, 7.8% similarity) between Cyn d 1.3 and Sor h 1. No significant homology with proteins of known function was identified when the sequence of Cyn d 1 was compared with those from protein databases.

Expression of Cyn d 1 in *E. coli*

The coding region of Cyn d 1 (expressed by clone CD1) was expressed in the vector p*Trc* 99A. A bacterial protein extract from this clone was separated by SDS-PAGE and a Western blot of the gel screened with mAbs 3A2 and 4D2 and IgE from the sera of patients allergic to Bermuda grass. The mAbs bound to a protein of approximately 30 kd (Fig. 5). We could not detect binding of IgE (from the sera of patients allergic to Bermuda grass pollen) to this protein.

To confirm that denaturation on the SDS polyacrylamide gel was not affecting IgE binding to this recombinant protein, we tested dot blots of the bacterial extract for IgE binding. No binding was detected (results not shown).

To confirm mAb and IgE specificity for natural Cyn d 1, Western blots of protein extracts from Bermuda grass pollen separated by SDS-PAGE were probed with mAbs 3A2 and 4D2 and serum from a patient allergic to Bermuda grass. Both mAbs and IgE bound to proteins of 31 to 32 kd (Fig. 5).

Expression of Cyn d 1 in *Pichia pastoris*

Recombinant Cyn d 1 protein (encoded by clone CD1) was secreted into the media by the yeast expression vector pHIL-S1 on induction with methanol. Presence of recombinant Cyn d 1 in the media was detected by SDS-PAGE, followed by Western blot probed with Cyn d 1–specific mAb

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                    10         20         30         40         50
Cyn d I.CD1   AIGDKPGPNITATYGSKWLEARATFYGSNPRGAAPDDHGGACGYRNVDKP   50
Cyn d I.3     -X-------------D-----K------D--------------------A   50
Lol p Ia      IAKVP-------E--D---D-KS-W--K*-T--G-K-N------KD---A   49
Sor h Ib      PPKVAK-K--------D---RK--W--K*-T--G---N------KD-N-A   49

                    60         70         80         90        100
Cyn d I.CD1   PFDGMTACGNEPIFKDGLGCGACYEIKCKEPVECSGEPVLVKITDKNYEH   100
Cyn d I.3     ---S--G--------------S---------A---------I---------   100
Lol p I       --N---G-NGT------R---S-F----TK-ES----A-T-T---D-E-P   99
Sor h I       --NS-G----L----------S-F----DK-A-----A-V-H---M---Q   99

                   110        120        130        140        150
Cyn d I.CD1   IAAYHFDLSGKAFGAMAKKGQEDKLRKAGELTLQFRRVKCKYPSGTKITF   150
Cyn d I.3     --------------------E----------M---------E---D---A-   150
Lol p I       --P-------H---S-----E-Q---S----E-----------D---P--   149
Sor h I       --------A-H----------E--A---IIDMK---------**-E-V--   147

                   160        170        180        190        200
Cyn d I.CD1   HIEKGSNDHYLALLVKYAAGDGNIVAVDIRPKDSDEFIPMKSSWGAIWRI   200
Cyn d I.3     -V-----PN---------------S----S-G--D-L---Q---------   200
Lol p I       -V--A--PN---I----VD---DV-----KE-GK-KW-EL-E----V---   199
Sor h I       -V-----PN--------VD---D--G---KE-GG-AYQ-L-H-------K   197

                   210        220        230        240
Cyn d I.CD1   DPKKPLKGPFSIRLTSEGGAHLVQDDVIPANWKPDTVYTSKLQFGA       246
Cyn d I.3     --P-------T------S-G-VE-E----ED-------K--I--         244
Lol p I       -TPDK-T---TV-Y-T---TKSEVE----EG--A--S-SA-           240
Sor h I       -SD--I-F-VTVQI-T---TKTAY-----EG--A--T--A-           238
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FIG. 4. Comparison of deduced amino acid sequences of Cyn d 1 with other Group 1 allergens. Cyn d 1.CD1 is the deduced amino acid sequence of clone CD1. Cyn d 1.3 is derived from the deduced amino acid sequences of clones KAT-39-1 and 3B. Residues identical to Cyn d 1.CD1 are indicated by *a dash.* Amino acids similar to Cyn d 1.CD1 are indicated by *bold letters.* Amino acids said to be similar are: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; F, Y, and W. *Underlined* amino acids are those that are different from that found in Cyn d 1.CD1 but the same as that in Cyn d 1.3. *Asterisks* indicate gaps that have been inserted for maximum homology. Numbers above the sequence refer to the numbering for Cyn d 1.CD1; numbers to the right of the sequence refer to the numbering for each protein. [a]Sequence of Lol p 1 is from Griffith et al.[16] [b]Sequence of Sor h 1 is from Avjioglu.[32]

3A2 and serum IgE from six patients allergic to Bermuda grass pollen, from which one is shown as a representative sample in Fig. 5. No antibody-binding protein was detected with the nonrecombinant constructs, which were used as negative controls (Fig. 5). Both mAb 3A2 and human IgE antibody bound to a 39.8 kd protein, but only mAb 3A2 recognized a 30.2 kd protein (Fig. 5). This lower band is probably a breakdown product of recombinant Cyn d 1, which still possesses the mAb 3A2 epitope but not the IgE epitope.

DISCUSSION

Through cDNA cloning and PCR amplification we have isolated clones encoding the major Bermuda grass pollen allergen Cyn d 1. The ORF of the clone encoding the complete Cyn d 1 polypeptide (CD1) encodes a protein of 246 amino acids with one possible N-glycosylation site. The predicted mass of the mature protein is 26.8 kd. This differs from that observed for major isoforms of the natural protein (31 and 32 kd), but the difference could be accounted for by glycosylation. Binding of concanavalin A to Cyn d 1 indicates that it is an N-linked glycoprotein.[11, 12]

Two signal peptides have been identified for Cyn d 1. One of these proteins is four amino acids longer than the other and otherwise differs by substitution of three amino acids. Because these clones resulted from a single PCR reaction, they may represent PCR-generated errors, but if they represent real differences, they may result in a difference in the targeting of the Cyn d 1 proteins. Unlike Lol p 1,[16, 29] the signal peptides of Cyn d 1 do not follow the "(−3, −1) rule" for cleavage of the signal peptide.[33] The rule states that residues at positions −1 and −3 in the signal peptide are small and neutral and that aromatic, charged, and large

polar residues are absent from these positions. In Cyn d 1, arginine is in the −3 position. The signal peptides do, however, have the domain structure usually seen in these peptides, (e.g., relatively hydrophilic NH_2-terminus with one or two basic amino acids; an apolar, largely hydrophobic core; and a relatively hydrophilic COOH-terminus, ending with an amino acid with a small side chain [valine in the case of both Cyn d 1 signal peptides]).[34]

The NH_2-terminal regions encoded by 14a1, 14c1, and KAT-39-1 show identity with those published for Cyn d 1. The NH_2-terminal sequence encoded by clone 14c1 has complete identity with the 26 and 31 kd isoforms of Cyn d 1 reported by Matthiesen et al.[15] The deduced NH_2-terminus expressed by clone KAT-39-1 shows strong homology with a 32 kd isoform of Cyn d 1 (Cyn d 1a) reported by Smith et al.[12] It is possible that the two isoforms cloned here represent isoforms of Cyn d 1 that differ in molecular mass.

It is somewhat unusual that of the clones isolated, all appear to have different 3′ untranslated regions, and none of them have polyadenylation signals or poly(A) at the 3′ end. Thus it is not possible to determine whether the different clones are transcribed from the same or different genes. On the other hand, it may be possible that some of the 3′ untranslated sequence is not intrinsic to the Cyn d 1 gene and might instead represent a sequence ligated to it during cDNA synthesis and library construction. Indeed, the isoallergens identified here may be the result of transcription from a number of genes or different alleles of the same gene. For example, there are three gene copies of the maize Group 1 homolog, Zea m 1,[35] and because of the close relationship between maize and Bermuda grass, multiple genes encoding Cyn d 1 could be expected. It has not been determined whether all the isoforms are expressed in a single plant. Rafnar et al.[36] and Griffith et al.[37] identified four families of Amb a 1 allergens. One particular plant was found to express seven unique Amb a 1 mRNA transcripts.

Griffith et al.[37] also examined the distribution of Amb a 1 isoforms in plants from different geographic locations (in the United States). There was no pattern in the distribution of isoforms. The Bermuda grass pollen used to extract RNA for the cDNA library and PCR was obtained from the United States. It is possible that some different isoforms of Cyn d 1 may be expressed in the pollen of Bermuda grass collected from other areas of the world. With Lol p 1, however, the same poly-

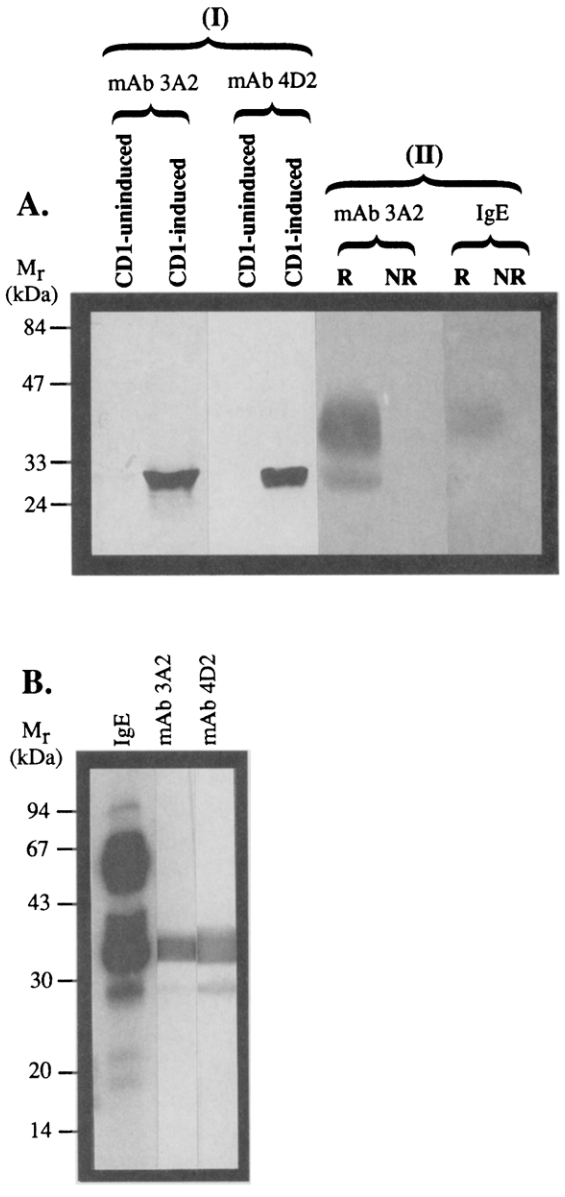

FIG. 5. Specificity of mAbs and IgE for recombinant and natural Cyn d 1. **A,** cDNA encoding the complete coding region of Cyn d 1 (CD1) was expressed in *E. coli (I)* and yeast *(II).* Bacterial proteins from cultures that had not been induced with IPTG *(CD1-uninduced)* and those that had been induced *(CD1-induced)* were then separated by SDS-PAGE and transferred to nitrocellulose. Yeast proteins secreted into the media from recombinant cells *(R)* and non-recombinant cells *(NR)* were also separated by SDS-PAGE and transferred to nitrocellulose. Western blots were then probed with mAbs 3A2 and/or 4D2 and IgE from six individual sera of patients allergic to Bermuda grass pollen, from which one is shown as a representative sample. **B,** Total Bermuda grass pollen proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blots were then probed with IgE, mAb 3A2, or mAb 4D2, followed by ^{125}I-labeled anti-human IgE (for detection of IgE binding) and horseradish peroxidase–labeled anti-mouse Ig (for detection of mAb binding). M_r, Molecular mass.

TABLE II. Comparison of *C*-terminal amino acid sequences of Group 1 allergens

Grass	Allergen	Amino acid sequence	IgE binding
Lolium perenne	Lol p 1*†	(YTTEGGTKSEVEDVIPEGWKADTSYSAK)	+
Lolium perenne	Lol p 1†	----------F----------	+‖
Festuca elatior	Fes e 1†	----------A------	+‖
Festuca elatior	Fes e 1†	----------	+‖
Poa pratensis	Poa p 1†	--------A-A---------V----E	+‖
Agrostis alba	Agr a 1†	--------A-A----------E	+‖
Anthoxanthum odoratum	Ant o 1†	------K-V-A---------V----E	+‖
Cynodon dactylon	Cyn d 1‡	L-S-S-GHV-Q------D--P--V-KS-IQF	−
Cynodon dactylon	Cyn d 1§	L-S---AHLVQD----AN--P--V-TS-LQFGA	−

Amino acids that do not differ from Lol p 1 are indicated by a hyphen. *Underlined* amino acids indicate that the amino acid is similar to that of Lol p 1. Amino acids said to be similar are: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; F, Y, and W.

*Deduced from cDNA clone 13R.[16]

†Determined by protein microsequencing.

‡Deduced from cDNA clone 3B.

§Deduced from cDNA clone 18B.

‖Although the IgE epitope has been localized to this region by inhibition between mAbs and IgE, these peptides have not been shown to bind IgE. A peptide with the sequence shown by the amino acids of Lol p 1 in brackets has been shown to bind IgE from the sera of individuals allergic to rye-grass.[43]

morphisms in Lol p 1 were detected in RNA from pollen collected in both the United States[30] and Australia.[16]

The primary amino acid sequences of three Group 1 allergens (Lol p 1, Cyn d 1, and Sor h 1) have been determined.[16, 30, 32] There is a strong degree of homology among the three proteins, as would be expected from the NH_2-terminal sequences that have been determined for Group 1 allergens.[11, 15, 38-40] Homology between Lol p 1 and Cyn d 1 isoforms varies: there is 62.2% identity (70.7% homology) with the isoform encoded by clone CD1 and 66.3% identity (74.1% homology) with the isoform encoded by clones KAT-39-1 and 3B (Cyn d 1.3). This would be expected to result in similar IgE responsiveness to both proteins by patients allergic to grass pollen. However, a number of studies have shown that allergens of Bermuda grass have little allergenic cross-reactivity with those of other grasses,[2-4, 41] and separate diagnosis and treatment of patients allergic to its pollen are required.[1] Three IgE epitopes have been identified on Cyn d 1 through RAST inhibition with anti-Cyn d 1 mAbs.[42] Two mAbs (when diluted 1:50) inhibited the binding of IgE to Cyn d 1 by more than 30%. These mAbs bind specifically to Cyn d 1 but not to any other Group 1 allergens tested, and it has been suggested that the IgE epitopes were thus specific to Cyn d 1.[42] These results are consistent with the lack of cross-reactivity between Cyn d 1 and other Group 1 allergens.

It also appears that Cyn d 1 does not share the conserved C-terminal IgE epitope found in Group 1 allergens from five other grasses: *Lolium perenne, Festuca elatior, Poa pratensis, Anthoxanthum odoratum,* and *Agrostis alba*.[8, 10] This epitope has been localized to a C-terminal tryptic peptide isolated from Group 1 allergens.[10] A 25 amino acid synthetic peptide based on the sequence of the Lol p 1 peptide has also been reported to bind IgE.[43] Esch and Klapper[10] suggested that allergenic cross-reactivity between the above closely related grasses could be ascribed to this conserved IgE epitope. All the Cyn d 1 clones that span this region encode a sequence homologous but not identical to this peptide (Table II). However, the fusion proteins expressed by these clones do not bind IgE, suggesting that Cyn d 1 does not share the C-terminal IgE epitope. In earlier work, we showed that IgE affinity-purified from the C-terminal half of recombinant Lol p 1 does not bind to Cyn d 1.[12]

The amino acid substitutions between Cyn d 1 and the other Group 1 allergens may explain the observed absence of IgE binding to the C-terminal of Cyn d 1. Comparison of the deduced amino acid sequence of Cyn d 1 isoforms identified here with the corresponding 28 amino acid C-terminal Lol p 1 peptide[10] reveals 12 amino acid substitutions between the 3B protein (Cyn d 1.3) and Lol p 1 and 14 between the clone CD1 protein and Lol p 1 (Table II). However, a number of these substitutions occur at positions that differ among the five grasses for which the common epitope has been identified. Four positions have nonconservative substitutions in both Cyn d 1 isoforms (com-

pared with Lol p 1) but not in the other grasses. These extra amino acid substitutions may result in loss of IgE binding to this region.

The lack of IgE reactivity in the C-terminal of Cyn d 1 cannot account for the lack of IgE reactivity of the complete Cyn d 1 protein encoded by clone CD1. Natural Cyn d 1 shows high IgE reactivity on Western blots (as indicated in this article and other studies[13, 14]), and glycosylation is not necessary for IgE binding to natural Cyn d 1, because enzymatic deglycosylation of natural Cyn d 1 did not abolish IgE recognition (data not shown). However, recombinant Cyn d 1 expressed as a bacterial fusion or a nonfusion protein does not bind IgE. On the other hand, the IgE-binding capacity is restored when Cyn d 1 is expressed in the yeast *Pichia pastoris.* Unlike bacteria, yeast has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modifications. Indeed, the recombinant Cyn d 1 expressed in yeast is approximately 8 kd greater in molecular mass than the natural allergen (~32 kd), which suggests that the yeast protein may possess N-linked glycosylation of the high mannose type. However, the length of the oligosaccharide chains added posttranslationally to proteins in *Pichia* species (average, 8 to 14 mannose residues per side chain) are much shorter than those in the yeast *Saccharomyces cerevisiae* (50 to 150 mannose residues).[44, 45] Although it may be debated that the observed IgE binding to the *Pichia*-expressed Cyn d 1 may be due to nonspecific binding to the sugars, this does not appear to be the case because no IgE binding was detected with the negative control natural yeast proteins, of which many are known to be also glycosylated, and because the deglycosylated natural Cyn d 1 recognized IgE (data not shown). Moreover, sera of patients allergic to grass pollen that displayed no IgE binding with natural Cyn d 1 did not demonstrate any binding to *Pichia*-produced Cyn d 1 and vice versa (data not shown). Another attractive feature of *Pichia* species in this study was that high expression levels (~1.5 gm/L of media) of Cyn d 1 were obtained. *Pichia pastoris* is known to secrete only very low levels of natural proteins, which together with the very low amount of protein in minimal *Pichia pastoris* growth medium,[28] means that the secreted recombinant Cyn d 1 comprises the vast majority of the total protein in the medium. This may serve as the first step in purification of the protein.

Taken together, these results suggest that the IgE-binding epitopes of Cyn d 1 are dependent on the conformation of the molecule, where posttranslational modification of the protein may play a critical role, rather than on its linear sequence. In contrast, there is little difference in binding of IgE by natural and recombinant Lol p 1,[7] indicating that most epitopes present on natural Lol p 1 are also present on the recombinant molecule and that the major IgE-binding epitopes of Lol p 1 are not dependent on conformation. Thus Cyn d 1 and Lol p 1 may have distinct IgE-binding epitopes, in spite of their high sequence identity (64% amino acid identity). This would explain the limited cross-inhibition of IgE binding by Bermuda grass and ryegrass pollen extracts.[3, 4] Similar findings have been reported for the ragweed allergens Amb a 1 and Amb a 2. Both the natural allergens bind IgE from patients allergic to ragweed to a similar extent, but although they show 65% amino acid identity, recombinant Amb a 2 does not bind IgE, whereas recombinant Amb a 1 does bind IgE.[46]

In conclusion, we have isolated clones encoding two isoforms of Cyn d 1, which differ in the length of the mature protein. We have successfully expressed one of these isoforms (CD1) in yeast, and unlike the bacterially expressed protein, the yeast recombinant protein demonstrated IgE-binding capacity. Lack of IgE reactivity with C-terminal fragments of recombinant Cyn d 1, expressed in bacteria, suggests that Cyn d 1 does not share the C-terminal IgE epitope found in Lol p 1 and four other Group 1 allergens.[8, 10] It is possible that Cyn d 1 has unique IgE epitopes compared with Group 1 allergens from the Pooid grasses and that IgE epitopes of Cyn d 1 are dependent on the conformation of the protein. It is proposed that hyposensitization treatment with recombinant allergens can be used for alleviation of the allergic response. The non-IgE-binding form of Cyn d 1, as expressed in *E. coli,* could be one candidate for evaluation as a therapeutic agent. Moreover, because IgE-reactive recombinant Cyn d 1 can be readily produced in large amounts in yeast, its potential as a diagnostic tool in grass pollen allergy can now be seriously evaluated.

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