

IgE-binding proliferative responses and skin test reactivity to Cop c 1, the first recombinant allergen from the basidiomycete *Coprinus comatus*

Karl A. Brander, PhD,^a Peter Borbély, MD,^a Reto Crameri, PhD,^b Werner J. Pichler, MD,^a and Arthur Helbling, MD^a Bern and Davos, Switzerland

Background: Basidiomycetes spores are ubiquitously distributed, found throughout the year in outdoor and indoor air, and represent relevant sources of aeroallergens associated with allergy and asthma.

Objective: Cloning and characterization of *Coprinus comatus* (shaggy cap mushroom) allergens is essential to elucidate their molecular characteristics and to improve the diagnosis of allergy. **Methods:** A complementary DNA (cDNA) library of *C. comatus* displayed on phage surface was screened with sera of basidiomycete-sensitized individuals. Subcloning and high-level expression of one of the enriched cDNAs allowed the isolation of a [His]₆-tagged recombinant protein formally termed rCop c 1. The allergenic properties of rCop c 1 were investigated in vitro by ELISA, inhibition experiments, immunoblots, and proliferation assays and in vivo by skin tests.

Results: The rCop c 1-encoding cDNA spans 435 bp and contains an open reading frame of 246 bp, predicting a protein of 8.96 kd without significant sequence homology to known proteins. Immunoblots with [His]₆-rCop c 1 fusion protein show a background free IgE-binding band of the expected size that can be completely inhibited by crude *C. comatus* extracts in ELISA. rCop c 1 induced specific proliferative responses in PBMCs of *C. comatus*-sensitized individuals. The incidence of sensitization to rCop c 1 among 92 sera of basidiomycete-sensitized individuals tested in ELISA was 25%, indicating that Cop c 1 is an intermediate allergen. However, prick tests showed that less than 2 pmol of the rCop c 1 protein was able to induce strong specific skin reactions in sensitized individuals.

Conclusions: rCop c 1, the first cloned allergen from the genus *Coprinus*, fulfills all the criteria required to be classified as a clinically relevant allergen. The data demonstrate at the molecular level the presence of sensitizing molecules among Basidiomycetes, the most important source contributing to the total spore load in the outdoor air. (J Allergy Clin Immunol 1999;104:630-6.)

Key words: Basidiomycetes, mushrooms, *Coprinus comatus*, complementary DNA cloning, filamentous phage, recombinant allergens, airborne allergens

Fungal spores are ubiquitous air contaminants generally accepted as a relevant source of aeroallergens associated with allergy and asthma.¹ Although allergenic reactions have been demonstrated beyond doubt for extensively studied fungal species such as *Alternaria*,² *Aspergillus*,³ and *Cladosporium*,⁴ a detailed study of the clinical reactivity related to other species of airborne fungi is much more difficult because of the absence of reliable antigens.^{5,6} Among these, Basidiomycetes (fleshy mushrooms) represent the morphologically most complex fungi and there are at least 14,000 different species reported, including mushrooms and puffballs, which are prominent sources for airborne spores. Basidiospores are mainly found in the outdoor air where they outnumber the pollen load¹ and contribute to the total spore load of the air in the range of 5% to 30%. Their presence is virtually perennial, with marked peaks during spring and fall.²⁻⁴ The basidiospore load in indoor air is not well documented, but some studies show that the spores are easily transported from the outside, contributing to a continuous exposure.⁷ Because of their size, 3 to 20 μ m in diameter, basidiospores can partially overcome the mucociliary clearance and become deposited in the nonciliated epithelial surface of the lower respiratory tract, where they can release allergenic components and start the cascade leading to sensitization of predisposed individuals.^{5,8} In more than 3800 tested subjects referred to our outpatient clinic, the incidence of sensitization to 3 basidiomycetes was found to be in the range of 3.5% (unpublished data). One of the tested basidiomycetes was *Coprinus comatus* (shaggy cap) belonging to the genus *Coprinus* (inky cap), which is commonly present in Europe with more than 200 different species.⁸ *C. comatus* is an allergologically relevant representative of this genus, as shown by the high sensitization rate of 58% among basidiomycete-sensitized individuals.⁹ However, reliable extracts of basidiomycetes are not available and therefore these estimates might be inaccurate. *C. comatus* was found to show an extensive cross-reactivity with other basidiomycetes⁹ and was therefore chosen for further investigation.

The goal of our project was to clone and characterize *C. comatus* allergens to be used for diagnostic purposes and to study at the molecular level cross-reactivity among basidiomycetes allergens. Here we present sequence analysis and immunologic characterization of a first complementary DNA (cDNA) obtained by screening of a *C. comatus*

From the Institute of Immunology and Allergology, Inselspital, Bern,^a and the Swiss Institute of Allergy and Asthma Research, Davos, Switzerland.^b

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Reprint requests: Arthur Helbling, MD, Institute of Immunology and Allergology, University Hospital, CH-3010 Bern, Switzerland.

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Abbreviations used

aa:	Amino acid
AU:	Arbitrary unit
bp:	Base pair
cDNA:	Complementary DNA
SPT:	Skin prick test

cDNA library displayed on the surface of filamentous phage.¹⁰ The allergen, formally termed Cop c 1 according to the recommendations of the International Allergen Nomenclature Committee,¹¹ represents the first molecularly characterized allergen of the genus *Coprinus*. With the exception of Psi c 2 of *Psilocybe cubensis*,¹² Cop c 1 is the only Basidiomycete allergen so far described.

MATERIAL AND METHODS

Synthesis and screening of a *C comatus* pJuFo library

Total RNA of young cap tissue of *C comatus* was isolated with RNA-easy (AxonLab) according to the manufacturer's instructions. Messenger RNA was isolated from total RNA with oligodeoxythymidine spin columns (Qiagen). For the generation of a unidirectional λ -ZapII cDNA library, 5 μ g of messenger RNA was converted to cDNA with use of unidirectional cDNA synthesis kits (Stratagene). The linked and ligated cDNA was packed in vitro with Gigapack II Gold packaging extracts (Stratagene), resulting in an library with a primary size of 1.5×10^6 plaque-forming units. The method used for the construction of the pJuFo surface display library was as described.¹³ λ -ZapII cDNA library 100 times the primary size of *C comatus* were excised in vivo. A total of 4.5 μ g cDNA inserts, obtained by *Xba*I/*Kpn*I digestion of the excised phagemid, were gel purified, electroeluted, and ligated to 1.5 μ g of *Xba*I/*Kpn*I-restricted pJuFo plasmid. The primary library size, as determined by ampicillin-resistant colonies, was 7.5×10^7 colony-forming units. Just before the screening process this library was freshly amplified¹³ and subjected to 5 rounds of panning against serum IgE from *C comatus*-sensitized individuals as ligand, immobilized on solid phase with antihuman IgE mAb TN-142 as the capturing antibody.¹³ The serum used for screening was obtained by pooling serum of 4 *C comatus*-sensitized subjects.⁸

Cloning and production of recombinant Cop c 1

A first discrimination of the selectively enriched clones was performed with *Xba*I/*Kpn*I digestion of miniprep DNA¹⁴ of randomly picked clones. The Cop c 1 insert was 450 base pairs (bp) in length and accounted for 23% of all clones analyzed. Cop c 1 cDNA (accession number AJ132235) was sequenced (Microsynth), analyzed with DNAsis software (Hitachi Software, San Bruno, Calif), and compared with databases with BLAST and FASTA. Analysis of the predicted protein sequence was performed with various tools provided on the Swiss-Prot home page (University of Geneva). For production of recombinant protein, the cDNA insert encoding Cop c 1 was isolated from phagemid DNA by *Bam*HI-*Kpn*I restriction and ligated into a modified pQE 30 high-level expression vector (Qiagen) restricted with *Bgl*II-*Kpn*I. The ligation mixture was transformed into *Escherichia coli* XL-1 blue cells, transformants grown in liquid culture to verify the nucleotide sequence and used

to produce hexahistidine-tagged recombinant protein. After a single-step purification over Ni⁺⁺-chelate affinity columns (Qiagen), protein concentrations were determined by the Bradford-based protein assay (Bio-Rad) and dialyzed stepwise against 4, 2, and 1 mol/L urea at 4°C with a final dialysis step against PBS for 6 hours at room temperature. Molecular size and purity of the recombinant protein were analyzed by polyacrylamide gradient gels (10%-20%), and samples were stored at -20°C.

Recombinant Cop c 1-specific IgE-binding assays

The presence of rCop c 1-specific IgE in 92 sera of individuals sensitized to *C comatus* was determined by ELISA and immunoblotting. Twelve additional sera from nonatopic individuals or from atopic patients lacking sensitization to *C comatus* served as negative controls. A standard direct solid-phase ELISA was used to detect anti-Cop c 1 IgE, as described.^{15,16} Results were recorded after 1 hour of incubation at 37°C by determining the absorbency (OD at 405 nm), and readings were expressed as arbitrary units (AU).

Immunoinhibition ELISA was performed as described.¹⁷ Briefly, sera were preincubated with 0.001 to 100 μ g/mL of the test allergen or with 30 to 4000 μ g/mL of total *C comatus* extract for 2 hours at 37°C. The sera were subsequently added to the plates coated with rCop c 1. The percentage of inhibition was calculated from the decreasing OD by setting the AU value without competing antigen in the fluid phase as 0% inhibition. Immunoblot analysis was carried out as recently described.⁹ For Western blot analysis, 30 ng of rCop c 1 and 20 μ g of total protein extract of *C comatus* were separated on a 10% to 20% gradient SDS-PAGE gel (Bio-Rad). For controls, 20 μ g of total protein of uninduced XL1-blue culture and 1 lane with molecular weight markers were included.

Lymphocyte transformation test

PBMCs were isolated from *C comatus*-sensitized individuals shown to possess rCop c 1-specific IgE in ELISA, as well as from atopic control subjects. Aliquots of 1×10^5 cells were cultured in triplicate in U-bottom 96-well plates in the presence of different concentrations (0.01-100 mg/mL) of either dialyzed rCop c 1 or total *C comatus* protein extract for 6 days. Cultures were pulsed with tritiated thymidine (0.5 μ Ci) and incubated for a further 14 hours. Thereafter, cells were harvested, and incorporated radioactivity was determined on a liquid free β -counter (INB 384, Inotech). Stimulation index values were calculated as counts per minute in culture with antigen relative to counts per minute in culture without antigen. Average stimulation index values were calculated as the mean of 2 repetitive assays, each in triplicate, and displayed against the antigen concentration used.

Skin prick tests with rCop c 1

Dialyzed rCop c 1 was serially diluted in a solution containing 50% glycerol, 85 mmol/L sodium chloride, and 30 mmol/L sodium bicarbonate. The lowest concentration prepared was 1 fmol/ μ L corresponding to 11 pg/ μ L and the highest was 43.5 pmol/ μ L (479 ng/ μ L). All solutions were sterile filtrated with Acrodisc PF 0.2 μ m (Gelman Science) and stored at -20°C until used. Skin prick tests (SPTs) were performed as previously described,⁸ starting with histamine solution (0.01%) and diluting solution as positive and negative controls, respectively, and the lowest concentration of the allergen preparation. Results were recorded after 15 minutes and skin tests were continued with the next higher allergen concentration. A reaction was regarded as positive if the wheal was >3 mm.¹⁸ Ethical approval was obtained from the local committee and study subjects consented before testing.

RESULTS

Library synthesis, screening, and sequence analysis of Cop c 1

C. comatus spores are difficult to harvest because ripe caps disintegrate into an inky, spore-containing slurry. We therefore decided to use young cap tissue because allergens of basidiomycetes are not only confined to spores but are also present in stalk, mycelium, and gills.¹⁹ We first constructed a unidirectional λ -ZapII library that resulted in a primary size of 1.5×10^6 plaque-forming units. In a second step, cDNAs isolated from the in vivo excised λ -ZapII library were used to construct a pJuFo phage surface display library that yielded a primary size of 7.5×10^7 colony-forming units. A pool consisting of 4 sera from *C. comatus*-sensitized individuals was used to selectively enrich phage displaying IgE-binding molecules. After 5 rounds of panning, restriction analysis of 96 randomly picked colonies showed a variety of 7 different inserts. The insert encoding Cop c 1 was the most frequent (23%) and was estimated to span 450 bp in length. The insert was DNA sequenced and was compared with known sequences deposited in the databases. The cDNA, 435 bp in length, consists of an open reading frame of 246 bp and a 3' untranslated region of 186 bp (Fig 1, A). The open reading frame encodes a protein of 81 aa, whereas the recombinant Cop c 1 protein was expressed as N-terminal fusion containing a [His]₆-tail and 16 aa derived from the multicloning site of the excised SK- plasmid (Fig 1, B). The derived rCop c 1 fusion protein spans 103 aa, predicting a gene product with a calculated mass of 11.4 kd and a theoretical pI of 9.5. Homology searches with this DNA and deduced aa sequence of Cop c 1 did not allow us to clearly assign the gene product to any class of known protein.

Further analysis with scans on PROSITE at the Swiss Institute of Bioinformatics²⁰ revealed 2 leucine zipper motifs (Fig 1, C, upper sequence, boxed). The N-terminal leucine zipper motif²¹ is not a perfect match because the 2 first leucines are separated by 7 instead of 6 aa. The C-terminal motif matches perfectly the consensus leucine zipper motif (L-6 aa -L -6 aa -L- 6 aa -L). Two different repeats as indicated by overhead lines and underlining arrows, respectively (lower sequence) were identified. Repeats marked with overhead lines have the L-4-L - consensus (L- 4 aa -L) and are present 6 times. Repeats marked by arrows, the P-5-L - consensus (P- 5 aa -L), are present 7 times.

Production and purification of recombinant Cop c 1

The Cop c 1 cDNA insert was subcloned in frame into pQE30 and transformed into XL1-Blue cells, and protein expression was induced with isopropyl thiogalactose. Although *E. coli* strain XL1-Blue is not an ideal host for protein expression, a yield of 80 mg of rCop c 1 per liter of bacterial culture was obtained after purification over Ni⁺⁺-nitrilotriacetic acid columns. For refolding, the protein was dialyzed in stepwise lowered urea concentra-

tions and in a final step against PBS. Purity was assessed with an overloaded lane (20 μ g of rCop c 1) on denaturing-reducing SDS-PAGE and subsequent Coomassie staining. The preparation was actually free of *E. coli* contaminants and a single band of about 11 kd corresponding to the expected molecular weight of the fusion protein was detected (data not shown).

IgE immunoblotting

The IgE-binding capacity of rCop c 1 was analyzed by Western blotting with use of a serum pool from *C. comatus*-sensitized individuals. Thirty nanograms of rCop c 1 exhibits a clear, background-free band with the expected size without any traces of IgE-binding contaminants (Fig 2). Total protein extract of *C. comatus* has been included as positive control and a whole pattern of IgE-reactive proteins can be observed. The assay is free of "systemic" background, as demonstrated by complete lack of signals on the marker lane included as negative control.

IgE-ELISA with rCop c 1 and inhibition studies

The reliability of the ELISA used to detect Cop c 1-specific IgE was demonstrated by the fairly good correlation between serum dilutions and OD readings (data not shown). Subsequently, we investigated the prevalence of rCop c 1-sensitized subjects among 92 individuals with a positive SPT to basidiomycetes. Twelve additional atopic and nonatopic control sera were used as negative controls. Although none of the negative control sera reacted with rCop c 1, 23 sera (25%) of basidiomycete-sensitized subjects were found to contain significant levels of rCop c 1-specific IgE. Forty-four of the 92 sera (48%) were from individuals with a positive SPT to *C. comatus* but did not contain rCop c 1-specific IgE. The remaining 25 sera (27%) of basidiomycete-positive individuals had a negative SPT for *C. comatus* and were also negative on rCop c 1 ELISA. If the 23 rCop c 1-positive sera were compared with the corresponding SPT results with *C. comatus*, 18 of the 23 sera correlated perfectly, whereas 5 did not. Except for 1 of these 5 noncorrelating sera all AUs were very low but still positive.

Specific IgE binding to rCop c 1 was inhibited in 2 ways: first by autoinhibition (Fig 3, A) and second by inhibition with *C. comatus* total protein extract (Fig 3, B). Complete inhibition was observed at a concentration of 100 μ g/mL when rCop c 1 was inhibited by itself (Fig 3, A). An unrelated recombinant *C. comatus* protein served as negative control and did not interfere with the specific IgE. Solid-phase bound rCop c 1 protein was almost completely inhibited by *C. comatus* total protein extract at concentrations of 3 to 4 mg/mL (Fig 3, B).

Proliferative response to rCop c 1

Because allergen-specific IgE production depends on T-cell help, PBMCs from 3 individuals sensitized to *C. comatus* as well as from 3 atopic and nonatopic control subjects without sensitization to *C. comatus* were stimulated in vitro with different concentrations of rCop c 1

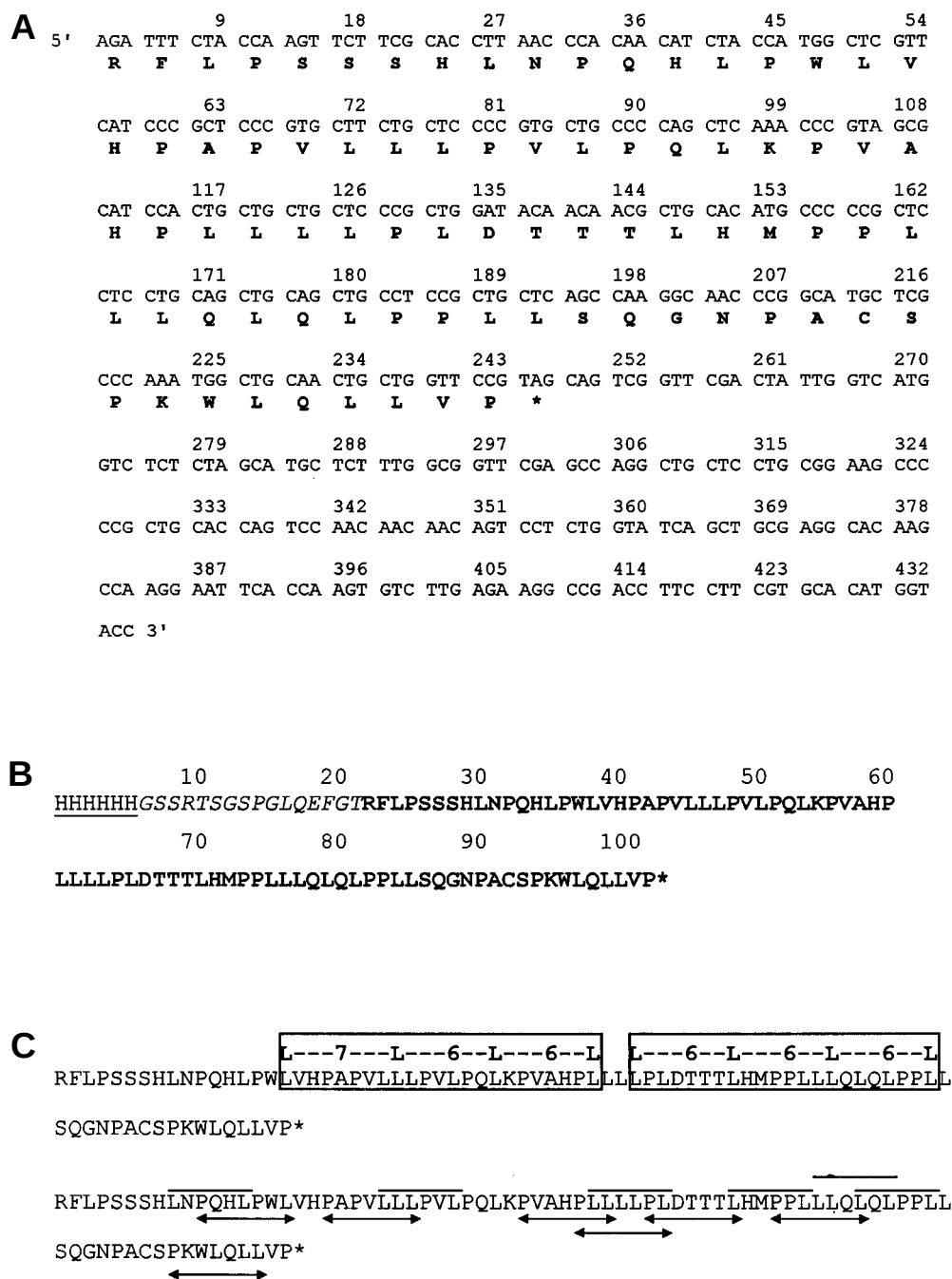


FIG 1 A, cDNA sequence of Cop c 1 and deduced amino acid sequence (accession No. AJ132235). **B**, Deduced amino acid (aa) sequence of recombinant Cop c 1 protein. *Underlining*, N-terminal His-tag; *italics*, aa that derive from multicloning site of λ -ZapII phagemid SK-; *bold*, sequence encoded by Cop c 1. **C**, The same deduced aa sequence is shown twice. *Upper sequence*, Two leucine zipper motifs termed N- and C-terminal; *lower sequence*, 2 different repeats, 6 times L-4-L and 7 times P-5-L.

and total protein extract of *C comatus*. In control individuals no response to the recombinant protein could be observed, whereas in *C comatus*-sensitized individuals a clear proliferative response could be detected. Comparable results were obtained when the total protein extract of *C comatus* was assayed, except that a single control sub-

ject with grass pollen allergy showed a weak proliferative response to high extract concentrations (Fig 4, B).

SPT reactivity of rCop c 1

To demonstrate the *in vivo* relevance of rCop c 1, SPTs were performed with atopic control and *C coma-*

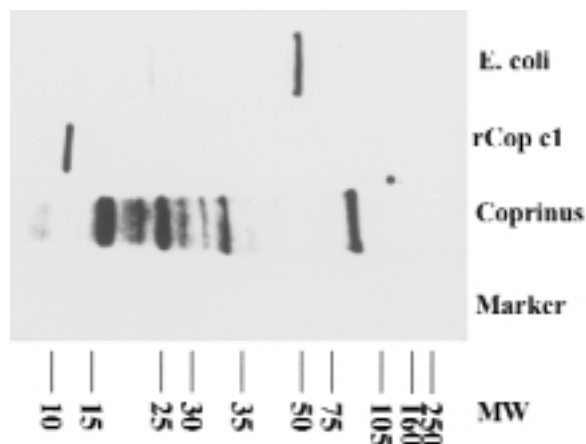
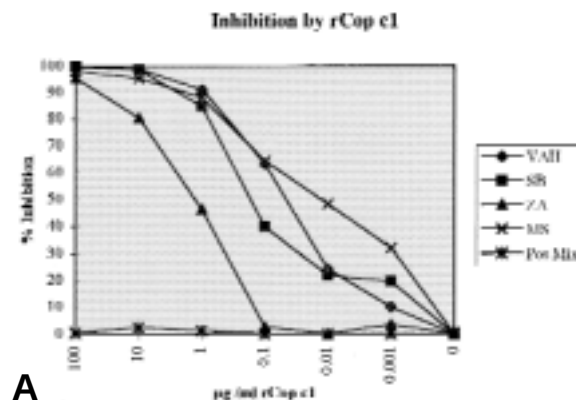


FIG 2. rCop c 1 is recognized by serum IgE in immunoblot. Separated proteins were 20 μ g of total *E coli* extract, 30 ng of Cop c 1, and 20 μ g of *C comatus* protein extract. Marker, Molecular weight marker as control; MW, molecular weight in kilodaltons. Mix of sera of 7 basidiomycete-sensitized subjects diluted 1:10 served as probe. Bound IgE was detected with TN-142 and goat antimouse horseradish peroxidase. Visualization was with enhanced chemoluminescence and 30 seconds of exposure.

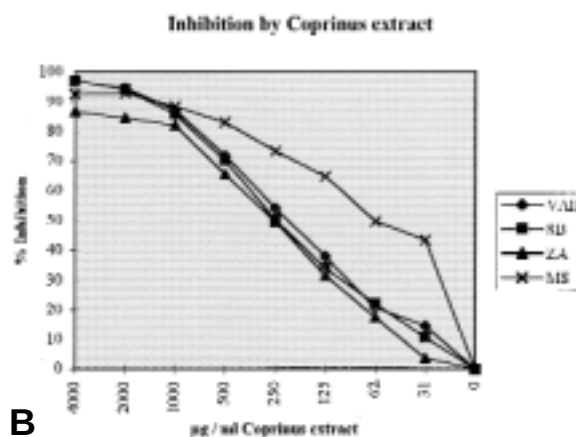
tus-sensitized subjects. The recombinant protein rCop c 1 was applied in SPTs at different concentrations to the forearm of control persons and *C comatus*-sensitized individuals. All 5 tested persons reacted positively to histamine challenges and were negative with diluting solution. A positive wheal-and-flare reaction was restricted to individuals with rCop c 1-specific serum IgE and was observed with concentrations as low as 100 fmol/ μ L rCop c 1 (Fig 5) corresponding to a total challenge with 15 ng of rCop c 1 protein. A gradual increase of the wheal was observed with increasing concentrations of rCop c 1.

DISCUSSION

Screening of a *C comatus* cDNA library displayed on phage surface¹⁰ with serum IgE of *C comatus*-sensitized individuals yielded in a variety of clones harboring different-sized cDNA inserts. One clone carrying a 450-bp cDNA insert was chosen for further analysis because of its high frequency of 23% among 96 cDNA clones analyzed. Sequence analysis of the DNA and derived aa sequence did not identify the function of the gene product, although some weak homologies to zein, a 10-kD storage protein of *Zea mays*²² could be found. Analysis of the protein sequence with Prosite²⁰ revealed 2 closely adjacent leucine zipper motifs (Prosite pattern No. PDOC00029). However, the motif toward the N-terminus is imperfect because of the spacing of 7 instead of 6 aa between the first 2 leucines. Despite the presence of a perfect leucine zipper motif toward the C-terminus of the protein, no further or extended homologies to regulatory proteins, which are prominent members of the class of proteins containing leucine zipper motifs,²³ could be found. In addition, 2 different repeats, coined P-5-L (7



A



B

FIG 3. Competitive inhibition of IgE binding to solid-phase coated rCop c 1 in ELISA. **A**, Sera of 4 *C comatus*-sensitized patients were inhibited with different amounts of rCop c 1. Pos Mix, Pool of 4 sera of *C comatus*-sensitized subjects inhibited with unrelated recombinant protein (accession No. AJ006788) of *C comatus* as negative control. **B**, Same 4 sera as in **A** but inhibited with total protein extract of *C comatus*.

times) and L-4-L (6 times), were found to be present in Cop c 1. However, these structural features do not allow us to assign a distinct biologic function to the gene product except the ability to bind human serum IgE, which could be clearly demonstrated (see below). The DNA sequence analysis makes us assume that Cop c 1 is not full length because neither a methionine nor a 5' untranslated leader sequence could be identified. Despite the application of various techniques and several attempts, we were not able to isolate the suspected incomplete 5' region of the cDNA.

The production of recombinant N-terminally His-tagged fusion protein (rCop c 1) and purification over Ni⁺⁺-nitrilotriacetic acid columns resulted in high yields (80 mg/L culture) of pure rCop c 1 protein further used in different in vitro and in vivo assays. Purity assessed by SDS-PAGE and immunoblotting showed a single background-free IgE-binding band of the expected size (Fig

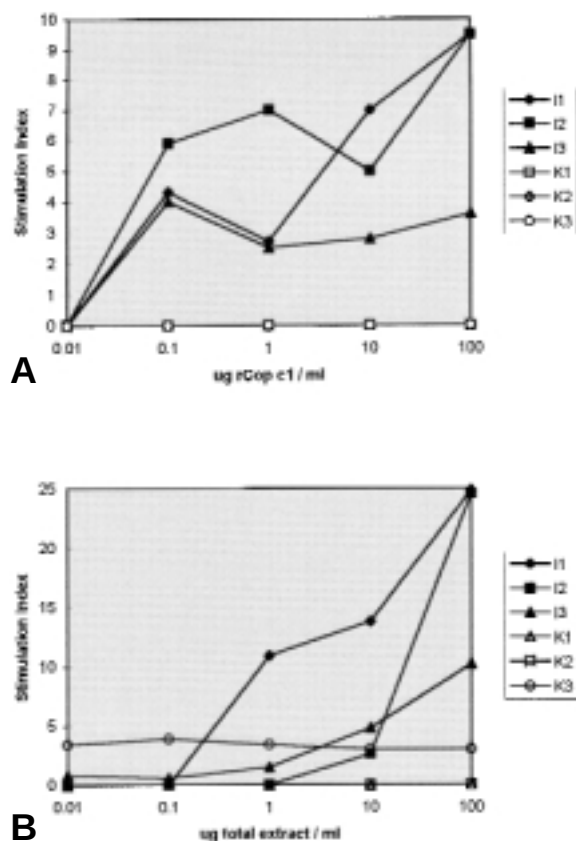


FIG 4. Proliferative response of PBMCs to rCop c 1 and total protein extract. Representative dose responses to rCop c 1 (A) and total protein extract of *C. comatus* (B). *C. comatus*-sensitized subjects (I1-I3, solid symbols) and atopic control subjects (K1-K3, open symbols) are identical in both figures. No response was observed in control subjects after stimulation with rCop c 1 as antigen. Because no response was observed with rCop c 1 in control subjects, all 3 lines are overlapping in A. The same is observed in B with 2 control subjects.

2). The pure protein was recognized by serum IgE from 25% of the *C. comatus*-sensitized individuals in ELISA. From these 23 sensitized individuals showing Cop c 1-specific IgE, 18 reacted strongly whereas 5 patients had low IgE values. The limited skin test data available do not allow us to define a cutoff value with regard to the level of specific IgE level needed to elicit a positive skin reaction. As expected, none of the individuals and control subjects without detectable IgE antibodies to Cop c 1 reacted in SPTs against the recombinant protein preparations. However, proliferative responses to PBMCs of sensitized individuals to Cop c 1 stimulation show the presence of Cop c 1-specific T cells and the lack of responses in *C. comatus*-sensitized individuals without IgE against Cop c 1 indicate that the recombinant antigen did not induce nonspecific effects (Figs 4 and 5). Taken together, these results clearly show that Cop c 1 represents an allergenic protein able to stimulate specific T-cell proliferation and to elicit type 1 skin reactions in sensitized individuals with high specificity and sensitivity. Cop c 1, together with a panel of additional *C. comatus*

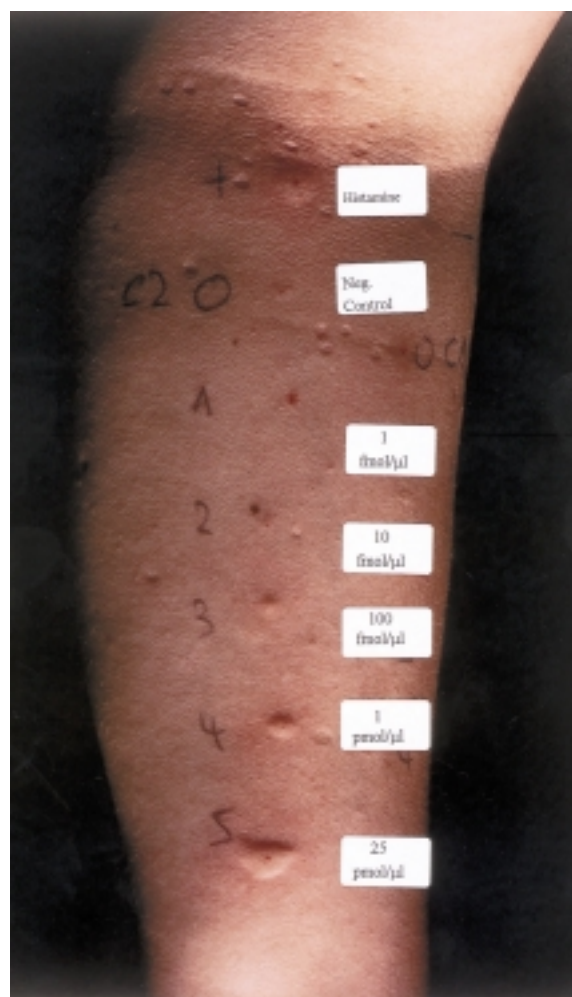


FIG 5. Skin test reactivity to rCop c 1. For SPTs indicated concentrations of rCop c 1 were applied and results were recorded after 15 minutes. Histamine dihydrochloride 0.01% and sodium chloride solution 0.9% were used as positive and negative controls, respectively.

allergens, already isolated from a cDNA library and currently under investigation, will serve as a starting point to develop sensitive and standardized reagents²⁴ with a high potential to substantially increase the sensitivity and specificity of the diagnosis of *C. comatus*-mediated allergic reactions.

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