

Trichophyton Antigens Associated with IgE Antibodies and Delayed Type Hypersensitivity

SEQUENCE HOMOLOGY TO TWO FAMILIES OF SERINE PROTEINASES*

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The dermatophyte fungus *Trichophyton* exhibits unique immunologic properties by its ability to cause both immediate and delayed type hypersensitivity. An 83-kDa *Trichophyton tonsurans* allergen (Tri t 4) was previously shown to elicit distinct T lymphocyte cytokine profiles *in vitro*. The homologous protein, Tri r 4, was cloned from a *Trichophyton rubrum* cDNA library, and the recombinant protein was expressed in *Pichia pastoris*. This 726-amino acid protein contained an arrangement of catalytic triad residues characteristic of the prolyl oligopeptidase family of serine proteinases (Ser-Asp-His). In addition, a novel *Trichophyton* allergen, encoding 412 amino acids, was identified by its human IgE antibody-binding activity. Sequence similarity searches showed that this allergen, designated Tri r 2, contained all of the conserved residues characteristic of the class D subtilase subfamily (41–58% overall sequence identity). Forty-two percent of subjects with immediate hypersensitivity skin test reactions to a *Trichophyton* extract exhibited IgE antibody binding to a recombinant glutathione *S*-transferase fusion protein containing the carboxyl-terminal 289 amino acids of Tri r 2. Furthermore, this antigen was capable of inducing delayed type hypersensitivity skin test reactions. Our results define two distinct antigens derived from the dermatophyte *Trichophyton* that serve as targets for diverse immune responses in humans.

Dermatophyte fungi of the genus *Trichophyton* colonize keratinized tissues in humans including nails, hair shafts, and the stratum corneum of the skin. *Trichophyton tonsurans*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum* are common causes worldwide of tinea capitis, athlete's foot, and onychomycosis (infection of the nail beds) (1). An estimated 30–70% of adults are asymptomatic carriers of these pathogens, and the incidence of symptomatic disease increases with age (2). The immune response to antigens derived from *Trichophyton* is unique in that both immediate hypersensitivity (IH)¹

and delayed type hypersensitivity (DTH) skin test reactions are induced. Studies suggest that the nature of the underlying immune response to *Trichophyton* antigens is related to the severity of dermatophytosis; IH skin tests are associated with chronic recurrent infections characterized by low-grade inflammatory lesions and the presence of IgE antibodies (Ab) (4–7). In contrast, DTH reactions are associated with highly inflamed lesions that resolve spontaneously and a resistance to re-infection (4, 8–13). The implication of these findings is that cell-mediated immune responses to *Trichophyton* are more effective at eradicating infection and may confer protection. Chronic dermatophytosis has been associated with allergic disease in the respiratory tract in individuals with immediate hypersensitivity (14–17). Furthermore, exposure to *Trichophyton* proteins may result in bronchial sensitization and symptomatic asthma that can be controlled with systemic antifungal therapy (7, 18, 19).

Experimental mouse models support a role for distinct T lymphocyte helper subsets in fungal infections (20). Furthermore, there is mounting evidence that a dichotomy in the immune response to a variety of pathogens, including *Trichophyton*, exists in humans and that these responses are regulated by distinct CD4⁺ T cell subsets (20–28). Characterization of antigens derived from *Trichophyton* provides a model system for studying both IgE antibody- and cell-mediated immune responses in humans; elucidation of the amino acid sequences of these antigens is relevant to structural analyses of intrinsic antigenic properties governing diverse immune responses and to the identification of antigenic determinants associated with immediate and delayed type hypersensitivity. Furthermore, elucidation of the biologic function of these unique antigens may define a role in fungal pathogenicity.

We previously demonstrated that an 83-kDa *T. tonsurans* antigen (Tri t 4) elicited IH and DTH skin test reactions in different individuals (27). IH skin tests were associated with IgG, IgE, and IgG4 Ab specific for Tri t 4, whereas DTH reactions were associated with only low levels of IgG Ab. In addition, short-term T cell lines specific for Tri t 4 had distinct cytokine profiles characteristic of a Th1 and Th2/ThO phenotype that correlated with skin test reactivity *in vivo* (28). Here we describe the molecular cloning and expression of the Tri t 4 homologue, Tri r 4, produced by *T. rubrum* and define its limited sequence identity to the prolyl oligopeptidase family of serine proteinases. In addition, we characterize a novel *T. rubrum* allergen (Tri r 2) that has a high degree of sequence identity to the subtilase enzyme family; this protein exhibits

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF082515 (Tri r 2) and AF082514 (Tri r 4).

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¹ The abbreviations used are: IH, immediate hypersensitivity; DTH,

delayed type hypersensitivity; Ab, antibody or antibodies; n-Tri t 4, natural Tri t 4; r-Tri r 4, recombinant Tri r 4; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

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1      AATTTTCGGCAGGAGCTTGTATCACGCATATCAAGAGAGAAGAAGAAGAAGCTATTAACCTTAGCTCCATTACACA
77    ATGGCAGCAGCAAAATGGTTGATTGCCTCCTTGGCATTTCGCTCAGGGCTTGCATTACCCAGAGGACTTCATCTCTGCTCCACGA
    M A A A K W L I A S L A F A S S G L A F T P E D F I S A P R
167   AGAGGAGAGGCCATCCAGACCCCAAGGGTGAGCTTGGCGTCTTCCATGTCTCCAAGTACAACCTTCGACAAGAAGGACGCCCATCCGCG
    R G E A I P D P K G E L A V F H V S K Y N F D K K D R P S G
257   TGGAACTCTTGAACCTCAAGAAGCGTGACATCAACGTTCTTACCACCGACAGTGATGTTTCTGAGATCACCTGGCTCGGAGACGGCACC
    W N L L N L K N G D I N V L T T D S D V S E I T W L G D G T
347   AAGGTCGTCTACATTAACGGAAGTACTCCGTCAGGGGGGTGTTGGTATTGGATTTCAGATGCCAAGAAGCTTCGGCAATGCCTACAAG
    K V V Y I N G T D S V K G G V G I W I S D A K N F G N A Y K
437   GCGCGTTCCGTCACCGGAGCGTTCTCTGGCCTCAAGCTTGCCAAAGTCGGAGACAAGATCAACTTCGTTGGTTACGGTCAATCCACAAC
    A G S V N G A F S G L K L A K S G D K I N F V G Y G Q S T T
527   AAGGGTGACCTCTACAATGAGGCCCGCTAAGGAGGCTGTCAGCTCTGCTCGCATCTACGACAGTCTTTTTCGTTCCGCACTGGGATACC
    K G D L Y N E A A A K E A V S S A R I Y D S L F V R H W D T
617   TATGTTGGGACTCAGTTCAATGCCGTCTTCTCTGCTACTCTCACCAAGAGCGGAGACAAGTACAGCTTCGATGGCAAGCTCAAGAAGCTC
    Y V G T Q F N A V F S G T L T K S G D K Y S F D G K L K N L
707   GTTCAACCCGTCAGTACGCTGAGTCTCTTACCCACCATTCGGCGGCTCTGGTACTACGATCTCTCTCTGATGGCAAGACCGTCGCG
    V Q P V K Y A E S P Y P P F G G S G D Y D L S S D G K T V A
797   TTCATGAGCAAGGCCAGAGCTTCCAAGGCCAAGCTCACACCTCTTACATCTTCTCGTTCCACACGATGGTTCGCGCTCGTGTAG
    F M S K A P E L P K A N L T T S Y I F L V P H D G S R V A E
887   CCCATTAACAAGCGCAATGGTCTCGCACTCCACAGGGCATTGAGGGTGCTTCTCCAGCCAGTCTTCTCTCCAGATGGCAAGAGAAT
    P I N K R N G P R T P Q G I E G A S S S P V F S P D G K R I
977   GCCTACCTCCAGATGGCAGCTAAGAAGTACGAGTCTGACCGCGTGTCTCATCCATTCGTTGAGGTTGGCACTAACAAGCAGTTTCAGCGC
    A Y L Q M A A K N Y E S P Y P P F G G S G D Y D L S S D G K T V A
1067  ATTGCCAGCAACTGGGACCGTTCTCCCGAGGCTGTCAAGTGGTCTTCTGATGGCGCACCCCTCTACGTCACTGCTGAGGACCAAGCTTACC
    I A S N W D R S P E A V K W S S D G R T L Y V T A E D H A T
1157  GGAAGGCTCTTACCCTCCCGCTGATGCTCGCGACAACCAAGCTGCTGTGTCAAGCACGACGGTTCGCTCAGCTCTTTTACTTC
    G K L F T L P A D A R D N H K P A V V K H D G S V S S F Y F
1247  ATCGGCAGCTCCAAATCTGCTCTCATCAGCGGTAACCTCTCTGTTGCCAAGCTCTCTACAGGTTGCTACTCCGACCGCCCTAACCGC
    I G S V K S V L I S G N S L W S N A L Y Q V A T P D R P N R
1337  AAGCTCTTCTATGCCAACGAGCATGATCCTGAGCTCAAGGCTTGGTCCCAAGATATTGAGCCTCTCTGGGTGATGGTGTCTCGCACC
    K L F Y A N E H D P E L K G L G P N D I E P L W V D G A R T
1427  AAGATCCACTCTTGGATCGTCAAGCCAAACCGGTTTCGATAAGAACAAGGCTACCCACTTGCTTTCCTCATCCAGGTGGTCTCTCAGGGC
    K I H S W I V K P T G F D K N K V Y P L A F L I H G G P O G
1517  TCCTGGGGTGACAAGTGGAGCACCGCTGGAACCCACGCTCTGGGGTGACAGGGATACGTTGCTGCTCTCTCAACCCAACTGGTAGC
    S W G D N W S T R W N P R V W A D Q G Y V V V A P N P T G S
1607  ACTGGTTTCGGCCAGAAGCTGACCGACACATCACCAAGACTGGGGTGGTCTCCATACAAGGATCTTGTCAAGATCTGGGAACACGTC
    T G F G Q K L T D D I T N D W G G A P Y K D L V K I W E H V
1697  CACGACCACATCAAGTACATTGACACTGACAACGGTATTGCTGCCGGTCCCTCTCGGTGGTTTCATGGTCAACTGGATCCAGGGACAA
    H D H I K Y I D T D N G I A A G A S F G G F M V N W I Q G Q
1787  GACCTCGGACGCAAGTCAAGGCCCTCGTCTCCCATGACGGTACCTTGGTGGATCTCCAAGATCGGAAGTACGAGCTCTTCTTCATT
    D L G R K F K A L V S H D G T F V G S S K I G T D E L F F I
1877  GAACACGACTTCAACGGAACCTTCTTCGAGGCTCGCCAGAACTATGACCGCTGGGATTGCTCTAAGCCAGAGCTCGTCGCTAAGTGGTCC
    E H D F N G T F F E A R Q N Y D R W D C S K P E L V A K W S
1967  ACCCTCAGCTCGTCCCAACGACTTCGACTTCCGCTCTCCGTTGCTGAGGGTGTGCGTCTCTTCAACGCTCTCCAGGAGAAGGGT
    T P Q L V V H N D F D F R L S V A E G V G L F N V L Q E K G
2057  GTTCCAGCCGTTTCTTAACTTCCCGATGAGACCCATTGGGTACCAAGCCAGAGAAGTCTCTCGTTTGGCACCAGCAGGTCTCTCGGA
    V P S R F L N F P D E T H W V T K P E N S L V W H Q Q L G
2147  TGGGTCAACAAGTGGTCTGGCATCAACAAGCAACCTAAGTCCATCAAGCTTTCGGATTGCCCATTCAGGTGCTGACCATGAGGCT
    W V N K W S G I N K S N P K S I K L S D C P I E V V D H E A
2237  CACTCTTACTTCTGACTACTAGACTCTTTATGCTTAACGCGACCATTTGATGTCATGCTTTATAATAGCAATGCATATATTGTAATAT
    H S Y F D Y ***

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FIG. 1. Nucleotide and deduced amino acid sequences of recombinant Tri r 4. The NH₂-terminal amino acid residues (positions 1–19) contain the conserved features of a signal peptide with a predicted cleavage site between Ala¹⁹ and Phe²⁰. Underlined regions represent amino acid sequences previously obtained for the NH₂ terminus and for six enzymatically generated peptides of natural Tri t 4. Catalytic triad residues (●) and four potential sites of N-linked glycosylation (X) are indicated. The stop codon TAG is shown (*).

human IgG and IgE Ab binding properties and the ability to induce DTH skin test reactions.

EXPERIMENTAL PROCEDURES

cDNA Cloning—Cultures of *T. rubrum*, *T. mentagrophytes*, and *T. tonsurans* were established in 25 ml of Sabouraud dextrose broth, and culture filtrates were screened using an assay for Protein IV as described previously (27). The *T. rubrum* cultures produced the highest concentration of Protein IV and were selected for construction of a cDNA library. Natural Protein IV was previously isolated from *T. tonsurans*, and this protein is now correctly termed Tri t 4 in keeping with allergen nomenclature. Thus, the homologous protein produced by *T. rubrum* is Tri r 4. Six grams of *T. rubrum* cells harvested on day 7 were washed in phosphate-buffered saline and ground with a mortar and pestle pre-cooled at –70 °C. Messenger RNA was isolated from 6 g

of culture material using a FastTrack kit (Invitrogen, Carlsbad, CA). A *T. rubrum* cDNA library was prepared from 10 µg of mRNA in the UniZAP-XR phagemid expression vector (Stratagene, La Jolla, CA). cDNA clones were identified by screening the library with either a 1:5000 dilution of serum obtained from a mouse immunized with natural Tri t 4 (n-Tri t 4) or a 1:2 dilution of an IgE serum pool from four individuals with high titer IgE antibodies and IH skin test reactions to a *Trichophyton* extract (29). Selected cDNA clones were screened against individual sera from 10 subjects with IH skin test reactions to *Trichophyton* and five individuals with DTH or negative skin test reactions. DNA sequencing was carried out by automated sequencing (ABI Prism 377, Applied Biosystems, Inc., Foster City, CA). Sequences obtained were compared with the National Biomedical Research Foundation, Swiss-Prot, and GenBank™ Data Banks using FASTA. Sequence alignments were performed using the GCG program. The pres-

	532-----546	615-----630	649-----661
	ooo *o oo	ooo o* oo	o * o
Tri r 4	NGIAAGASFGGFMVN---	LVVHNDFFRLSVAEG---	FPDETHWVTKPEN
ACPH	HVALMGSGHGGFISC---	LLMLGQEDRRVPFKQG---	YPKSTHALSEVEV
DAP2	KISLFGWSYGGYLT---	LLMHGTGDDNVHFQNS---	FPDSHDSIRYHNA
DPP6	RVAVFGKDYGGYLT---	LIHPTADEKIHQHT---	YPDESIFYTSSSL
DPP4	RIAIWGSYGGYVTS---	LLHGTADDNVHFQNS---	YTDEDHGIASSTA
DAP1	KIAIWGSYGGFTSL---	FIVHGTDDNVHIQNT---	FPDSHDSIRYHNA

FIG. 2. Comparison between conserved regions and catalytic triad residues of recombinant Tri r 4 and serine proteinases of the prolyl oligopeptidase family. Residues are numbered according to Tri r 4. Asterisks denote catalytic residues, and identical residues are depicted in boldface type. Conservative substitutions of hydrophobic residues present in at least four family members are indicated (○). ACPH, human acylaminoacyl peptidase; DAP2, *Saccharomyces* dipeptidyl aminopeptidase B; DPP6, human dipeptidyl peptidase IV-like protein; DPP4, human dipeptidyl peptidase IV; DAP1, *Saccharomyces* dipeptidyl aminopeptidase C.

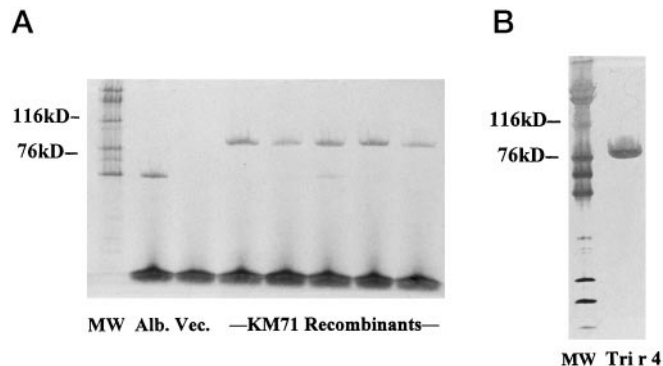


FIG. 3. SDS-PAGE analysis of recombinant Tri r 4 expressed in *P. pastoris*. A, analysis of culture supernatants 24 h after induction of protein expression revealed an 85-kDa band in all putative recombinants tested (Coomassie Blue staining), whereas no protein was visible in a control recombinant containing vector (Vec.) alone (third lane). Expression levels were comparable to an albumin (Alb.) control (second lane). B, recombinant Tri r 4 was purified from culture supernatants by affinity purification using immobilized nickel chelate. The recombinant antigen migrated as a single band on silver-stained SDS-polyacrylamide gel.

ence of signal peptides was confirmed using the prediction algorithm developed by Nielsen *et al.* (30).

Expression of Recombinant Tri r 4 in *Pichia pastoris*—Recombinant Tri r 4 (r-Tri r 4) was expressed in *P. pastoris* as a hexahistidine-tagged protein using the pPICZαA expression vector (Invitrogen). Plasmid DNA (50 ng) encoding Tri r 4 was used as a template to generate a 2178-bp DNA fragment by polymerase chain reaction. The following primers for polymerase chain reaction were synthesized: 5'-CCGGAAT-TCTTTACCCAGAGGACTTC-3' (sense), containing an *EcoRI* restriction site; and 5'-GCTCTAGAGCGTCGAAGTAAGAGTGAGC-3' (anti-sense), containing an *XbaI* restriction site. The 2178-bp polymerase chain reaction-amplified DNA fragment was ligated into *EcoRI*-*XbaI*-digested pPICZαA. *Escherichia coli* strain TOP10F⁺ was transformed, and plasmid DNA was purified from Zeocin-resistant transformants selected on low salt LB medium containing 25 μg/ml Zeocin. Yeast strain KM71 was transformed by electroporation (Bio-Rad GenePulser; 1500 V, 25 microfarads, 200 S) with 5 μg of DNA linearized by digestion with *PmeI*. Transformants were selected on yeast extract peptone dextrose agar containing 100 μg/ml Zeocin after incubation at 30 °C for 3 days. A single colony was used to inoculate 10 ml of buffered glycerol complex medium, and cultures were grown at 30 °C in a shaking incubator (300 rpm) until the culture reached $A_{600\text{ nm}} = 2.5$. The process was repeated using 10 ml of culture to inoculate 1 liter of medium. After reaching $A_{600\text{ nm}} = 2.5$, cells were harvested by centrifugation (3000 × *g* for 5 min) and resuspended in 100 ml of buffered methanol complex medium containing 0.5% methanol. Expression of r-Tri r 4 was induced at 30 °C in the presence of methanol for 4 days. The recombinant protein was purified from culture supernatants using immobilized nickel chelate (Probond resin, Invitrogen). Purity was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) with silver staining, and protein yields were measured by the Bradford assay. Proteins expressed in pPICZαA contain an NH₂-terminal α factor signal sequence that targets expressed proteins to the secretory pathway and into the culture medium. Proteins also contain carboxyl-terminal hexahistidine and Myc epitope tags. NH₂-terminal amino acid sequence analysis of r-Tri r 4 by Edman degradation confirmed cleavage of the signal se-

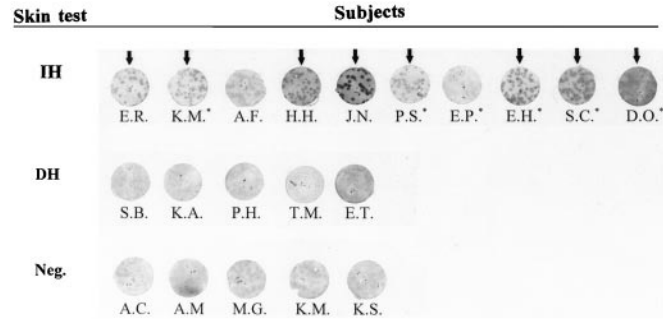


FIG. 4. Human IgE antibody binding to *T. rubrum* cDNA clone tr6B. Five clones encoding a single protein were obtained after screening a *T. rubrum* cDNA library with pooled IgE antibodies. Individual sera from 10 subjects with IH skin test reactions and from five subjects with DTH or negative (Neg.) skin test reactions were used to screen each clone by plaque immunoassay. Results are shown for clone tr6B. Similar binding patterns were observed for all five clones. Arrows denote subjects with IgE Ab binding, and individuals with bronchial reactivity to *Trichophyton* are indicated (*).

quence and the presence of the first 22 NH₂-terminal residues corresponding to those of n-Tri r 4.

Expression of Tri r 2 in *E. coli*—Plasmid DNA containing *T. rubrum* clone 9A (~1500 bp) was used as a template to generate an 867-bp DNA fragment encoding the carboxyl-terminal 289 amino acids corresponding to the putative mature form of Tri r 2. Primers for polymerase chain reaction incorporated *EcoRI* and *XhoI* restriction sites to allow subcloning into the pGEX-4T-3 expression vector and were as follows: 5'-CCGGAATTCGGGCACTAACCTCACC-3' (sense), containing an *EcoRI* restriction site; and 5'-CCGCTCGAGTTGCGCGTGC-3' (anti-sense), containing an *XhoI* restriction site. The 867-bp polymerase chain reaction-amplified DNA fragment was ligated into *EcoRI*-*XhoI*-digested pGEX-4T-3. Expression of the 29-kDa putative mature form of Tri r 2 as a fusion protein with glutathione *S*-transferase (GST) was induced in *E. coli* strain BL21 with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C. The recombinant protein (GST-Tri r 2) was purified from cell lysates using glutathione-Sepharose (yield of ~2–5 mg/liter of culture). Amino acid sequencing of the fusion protein by Edman degradation confirmed the presence of the first 5 NH₂-terminal residues of the GST moiety. Purified GST-Tri r 2 was dialyzed against phosphate-buffered saline, and purity was analyzed by silver-stained SDS-PAGE for the purpose of skin testing. The recombinant protein was also purified by electroelution from a 12% acrylamide gel for the purpose of radiolabeling (31).

Assays of Enzymatic Activity—Recombinant GST-Tri r 2 and Tri r 4 proteins were tested for proteolytic activity using a variety of substrates. Proteinase K (Sigma) was used as a positive control for all assays. Negative controls included reactions containing no putative enzyme. Glutathione *S*-transferase served as an additional negative control for assays of GST-Tri r 2 enzymatic activity. All assays were performed in duplicate. Substrates tested included azoalbumin, azocasein, azocollagen, and keratin according to methods previously described (32, 33). Briefly, 10 μg of the sample to be tested was incubated with 0.1 mg of azoalbumin, 2% (w/v) azocasein, 5 mg of azocollagen, or 5 mg of keratin azure in reaction volumes of 1, 0.2, 0.5, and 0.3 ml, respectively. Reactions were terminated after incubation for 24 h at 37 °C (azocasein, azocollagen, and keratin) or after 30 min at 20 °C

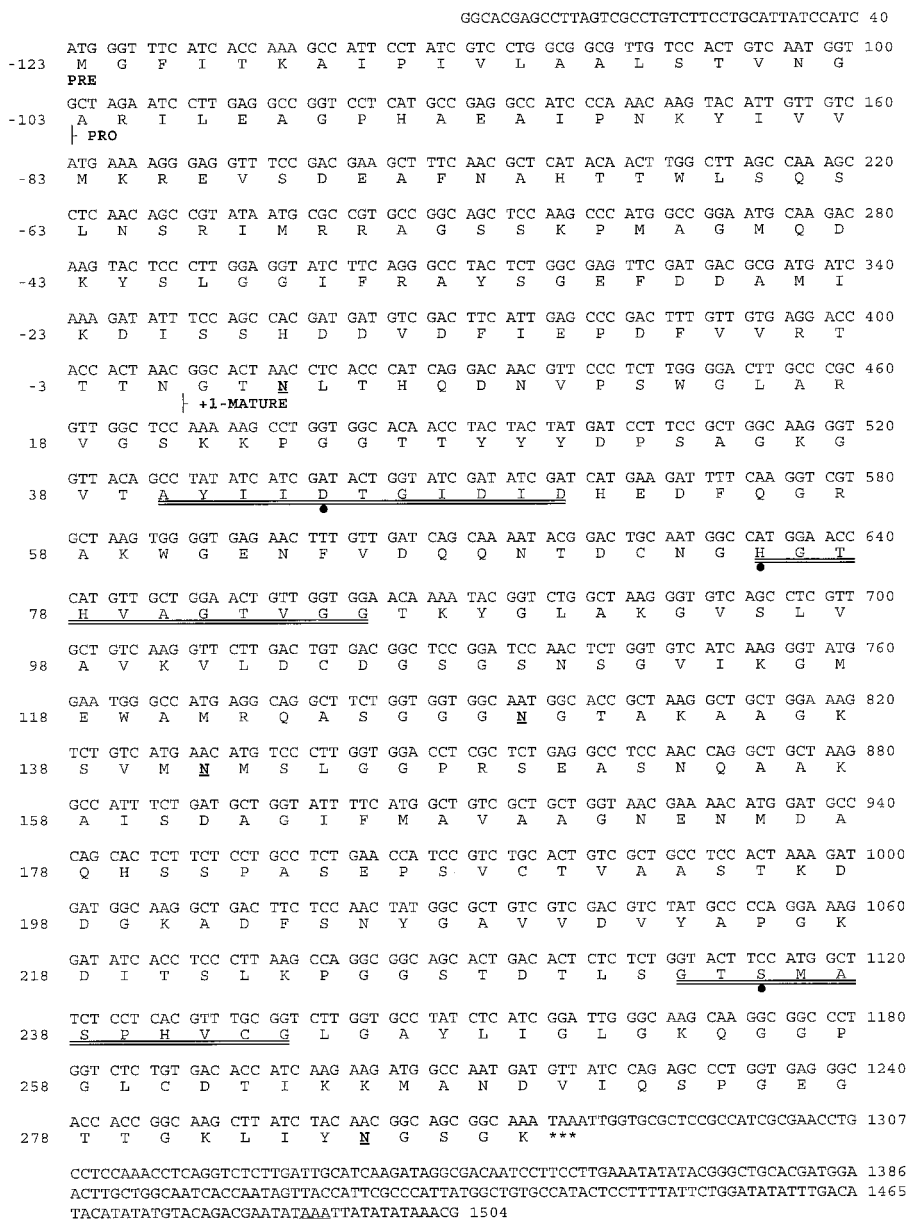


FIG. 5. Nucleotide and deduced amino acid sequences of *T. rubrum* cDNA clone 9A encoding Tri r 2. The NH₂-terminal amino acid residues (positions 1–20) contain the conserved features of a signal peptide. Double-underlined regions represent conserved amino acid motifs flanking catalytic triad residues (●) characteristic of the subtilase family of serine proteinases. The putative signal peptide (PRE), the propeptide region (PRO), and the mature form (MATURE) are shown. Four potential sites of N-linked glycosylation are indicated (boldface and underlined). The stop codon TAA (*) is shown, and a polyadenylation signal sequence (ATAAA) in the 3'-non-coding region is underlined.

(azobalumin). Reaction products were measured spectrophotometrically. One unit of activity was defined as an increase in absorbance of 0.01. Recombinant Tri r 2 was also tested using the anilide substrate succinyl-(Ala)₃-p-nitroanilide (32). Briefly, reactions contained a final concentration of 10 µg/ml Tri r 2 and substrate concentrations of 0.1–2.0 mmol/liter; assays of 1-ml volume were incubated at 20 °C for 1 h, and the absorbance of liberated nitroaniline was measured spectrophotometrically.

Immunoassays for IgG and IgE Antibodies to Tri r 2 Fusion Protein—IgE and IgG Ab to GST-Tri r 2 were measured using an antigen binding radioimmunoassay according to methods previously described (27). Serum samples diluted 1:2 and 1:10 (IgE Ab assay) or 1:12.5 and 1:50 (IgG Ab assay) were incubated with ¹²⁵I-labeled GST-Tri r 2 (~120,000 cpm added) for 4 h at room temperature. IgE myeloma serum (patient P. S.) diluted 1:300 was used as carrier in the IgE binding assay. Immune complexes were precipitated overnight at 4 °C with 50 µl of sheep anti-human IgE or 50 µl of sheep anti-human IgG (Binding Site, Inc., San Diego, CA), and precipitates were counted in a γ-counter. Quantitation of IgG Ab was carried out using a control curve constructed with pooled sera from patients K. M., J. C., and H. W., assigned to contain 2000 units/ml IgG antibodies. IgE Ab measurements are expressed as counts bound per min. Specificity of antibody binding to Tri r 2 was assessed by comparing values obtained for sera pre-absorbed with GST (5 mg of GST/ml of cyanogen bromide-activated Sepharose) and non-absorbed sera.

Human Subjects and Skin Testing—Sera were obtained from 73 subjects previously skin-tested with 0.03 ml of Hollister-Stier *Trichophyton* mixture containing *T. tonsurans*, *T. rubrum*, and *T. mentagrophytes* species (1:200, w/v). Intradermal skin testing was done with 0.03 ml of purified GST-Tri r 2 at 1 and 10 µg/ml diluted in 0.05% human serum albumin in phenol/saline solution. Prick testing was carried out prior to intradermal testing using a 10-fold higher concentration of protein. Test sites were examined at 20 min after injection and at 24 and 48 h. Positive delayed reactions were defined as erythema of ≥5-mm diameter at 24 h. Subjects were skin-tested with purified GST as a negative control. Skin testing of human subjects using GST-Tri r 2 was approved by the University of Virginia Human Investigation Committee.

RESULTS

Molecular Cloning of Tri r 4—Screening a *T. rubrum* cDNA library with a human serum pool obtained from five individuals with high titer IgE Ab to n-Tri t 4 failed to identify positive plaques. However, screening the library with polyclonal serum obtained from a mouse immunized with n-Tri t 4 resulted in identification of three clones containing insert sizes of ~1100 bp (clone tr2), 1600 bp (clone tr1), and 2300 bp (clone tr3). Nucleotide sequence analysis confirmed that all clones encoded

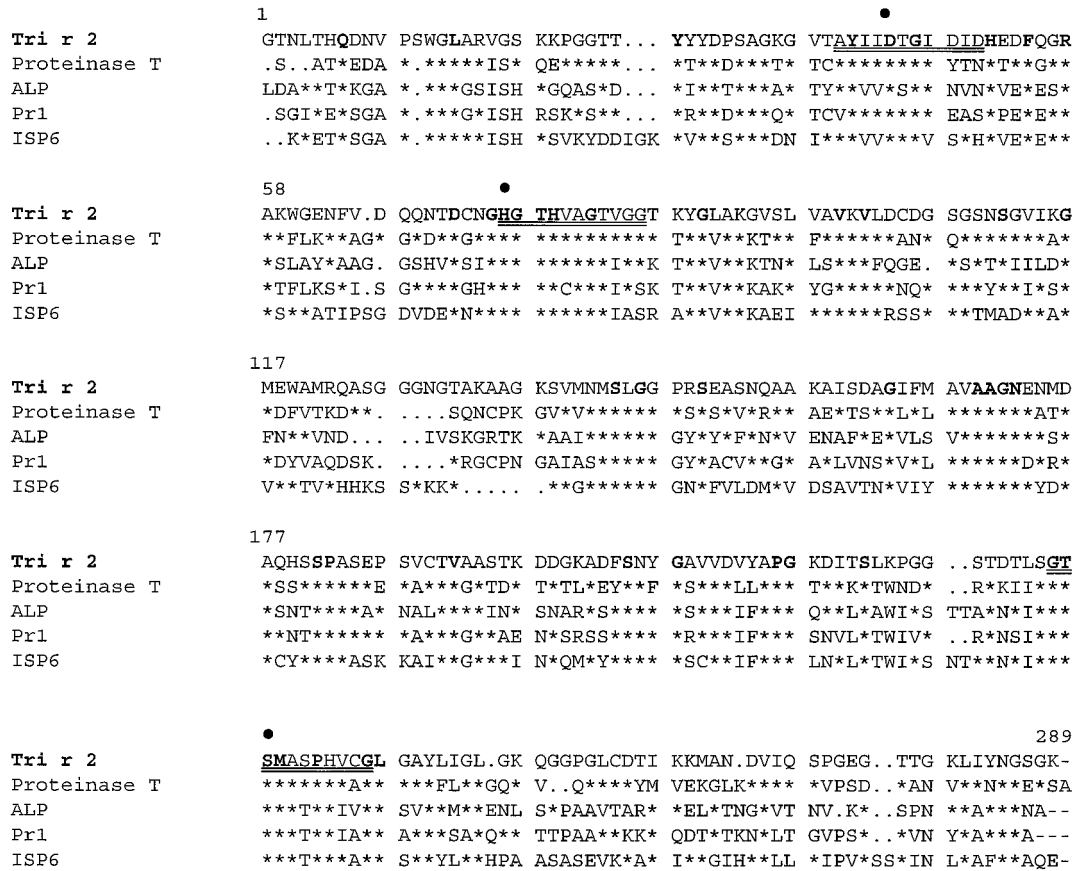


Fig. 6. Sequence alignment of the Tri r 2 putative mature form with catalytic domains of other fungal subtilases. Tri r 2 showed high sequence identity to proteinase T produced by *T. album* (GenBank™ P20015), an alkaline proteinase (ALP) derived from *A. fumigatus* (GenBank™ P28296), proteinase Pr1 from *M. anisopliae* (GenBank™ P29138), and proteinase ISP6 from *S. pombe* (GenBank™ P40903). Alignments obtained using the GCG program are shown. Conserved residues of the class D subfamily of subtilase enzymes (**boldface**) and catalytic residues (●) are indicated. Asterisks denote identical residues.

the same protein. Clone *tr3* contained an open reading frame of 2178 nucleotides encoding a 726-amino acid protein (Fig. 1). An estimated molecular mass of 78,193 Da ($pK = 2.2$) without the signal peptide sequence was consistent with a non-glycosylated form of n-Tri t 4. Four potential sites of N-linked glycosylation were identified, and the presence of a signal sequence with a predicted site of cleavage between Ala¹⁹ and Phe²⁰ was determined. Amino acid sequences of the amino terminus and six enzymatically generated internal peptides of n-Tri t 4 (comprising 108 residues) aligned with the deduced amino acid sequence of clone *tr3*. This confirmed that *T. rubrum* clone *tr3* encoded a protein with high amino acid sequence homology to n-Tri t 4, and we have designated this recombinant protein Tri r 4.

Amino acid sequence similarity searches identified homology between r-Tri r 4 and the prolyl oligopeptidase (S9) family of serine proteinases. These enzymes contain the distinctive Ser-Asp-His arrangement of catalytic triad residues in the carboxyl-terminal portion of the molecule. A short region spanning ~250 residues was identified within r-Tri r 4 that contained sequence similarity to prolyl oligopeptidases derived from other eukaryotic sources (20–25% identity, 231–282-amino acid overlap). A Gly-X-Ser-X-Gly motif comprising the nucleophile serine at position 539 was present within this region. The highest sequence similarity was between r-Tri r 4 and human acylaminoacyl peptidase (25.6% identity and 57% similarity in a 242-amino acid overlap) (Fig. 2). Additional homologues included *Saccharomyces* dipeptidyl aminopeptidases B and C and dipeptidyl peptidase IV (DPP4) and dipeptidyl peptidase IV-like proteins (DPP6) derived from several mammalian species.

Recombinant Tri r 4 was expressed in *P. pastoris* using the pPICZαA vector system. SDS-PAGE analysis at 24 h after induction of protein expression revealed the presence of an 85-kDa band in all recombinants selected consistent with a glycosylated form of r-Tri r 4 containing carboxyl-terminal Myc epitope and hexahistidine tags (Fig. 3A). The recombinant protein was purified by affinity purification from culture supernatants harvested on day 4, resulting in a yield of ~200 mg/liter of culture. The pure protein migrated as a single 85-kDa band on SDS-PAGE (Fig. 3B).

Identification of a Novel *T. rubrum* Allergen as a Member of the Subtilase Family of Serine Proteinases—Since screening the *T. rubrum* cDNA library with an initial human serum pool (Pool 1) failed to identify positive plaques, a second pool was established (Pool 2) in order to screen for additional putative *T. rubrum* allergens. Sera were obtained from four individuals with high IgE antibody titers and IH skin test reactions to a *Trichophyton* extract. All subjects had chronic dermatophytosis, whereas three had asthma and positive bronchial provocation to *Trichophyton*. Five positive clones were identified with insert sizes of ~900 bp (clone *tr6D*), 1000 bp (clone *tr6C*), 1200 bp (clone *tr6B*), 1300 bp (clone *tr6A*), and 1500 bp (clone *tr9A*). Nucleotide sequence analysis confirmed that all five clones encoded the same protein and that this protein was unrelated to Tri r 4. A representative clone (*tr6B*) was screened with sera obtained from individuals with different skin test reactivity to *Trichophyton*. Eight of 10 subjects with IH skin test reactions displayed IgE antibody binding to this clone, whereas sera from DTH and negative skin test subjects yielded no positive responders (Fig. 4). Clone 9A contained an open reading frame

encoding 412 amino acids with an estimated molecular mass of 42,632 Da and a pK value of 1.9 (Fig. 5). The first 20 NH₂-terminal residues contained the conserved features of a signal peptide, and four potential sites of N-linked glycosylation were identified.

Sequence similarity searches showed a significant homology between the deduced amino acid sequence of clone 9A and serine proteinases of the subtilase family (S8) derived from other fungal species (Fig. 6). Conserved amino acid motifs were identified flanking aspartic acid, histidine, and serine residues, which form the catalytic triad characteristic of this enzyme family. Over 70 subtilases are currently known, belonging to four subfamilies. The deduced amino acid sequence of clone 9A contained all of the conserved residues characteristic of the class D subfamily, which consists of enzymes found only in yeast, fungi, and Gram-negative bacteria (Fig. 6). The highest degree of amino acid sequence identity was between Tri r 2 and proteinase T produced by the thermophilic fungus *Tritirachium album* (58.2% identity in a 304-amino acid overlap). Other enzymes with striking homologies included proteinases derived from the pathogenic fungus *Aspergillus fumigatus* (41.3% identity), the insect-colonizing fungus *Metarhizium anisopliae* (42.3%), and the yeast *Schizosaccharomyces pombe* (41%) (Fig. 6). Sequence alignments identified the presence of a putative pro-region in Tri r 2 (residues 21–123) with a predicted cleavage site between asparagine and glycine residues (positions 123 and 124, respectively) generating a putative mature product (positions 124–412) with an estimated molecular mass of 29,171 Da (pK = 1.9) (Fig. 5).

Demonstration of the Immune Response to Tri r 2—The putative mature form of Tri r 2 was produced in *E. coli* using the pGEX-4T-3 vector. The resulting GST fusion protein purified from bacterial lysates by glutathione affinity chromatography migrated as a single 57-kDa band on SDS-PAGE, consistent with the presence of a GST moiety fused to the 29-kDa putative mature form of Tri r 2. Specific IgE Ab were measured in 73 sera: the prevalence of IgE Ab was significantly higher among subjects with IH skin test reactions (43%) compared with those with DTH or negative skin test reactions (12%) ($p < 0.01$) (Fig. 7A). It has been established that GST exhibits IgE Ab binding properties (34); however, absorption of sera with GST did not reduce the prevalence of IgE Ab binding to Tri r 2. The prevalence of IgG Ab was relatively high in all skin test groups. However, mean levels of IgG Ab were significantly higher in subjects with immediate reactions compared with those with delayed or negative skin test reactions ($p < 0.01$) (Fig. 7B). Recombinant ¹²⁵I-GST-Tri r 2 showed strong reactivity with IgG Ab (up to 46,000 cpm bound) and IgE Ab (up to 10,500 cpm bound), demonstrating that the putative mature form of Tri r 2 retained B cell epitopes. Intradermal skin testing was used to evaluate the reactivity of recombinant Tri r 2 *in vivo*. Five of nine individuals with delayed reactions to the *Trichophyton* mixture showed a positive delayed type hypersensitivity reaction maximal at 24 h; four of these subjects are shown in Table I.

Activity against Protein Substrates—Given the amino acid sequence homology to known proteinases, the enzymatic activity of recombinant Tri r 2 and Tri r 4 was tested using a variety of general proteolytic substrates including albumin, casein, collagen, and keratin. No proteolytic activity was observed for GST-Tri r 2 for any of the substrates tested when compared with a GST control. Furthermore, this protein exhibited no activity against the anilide substrate succinyl-(Ala)₃-p-nitroanilide. However, r-Tri r 4 exhibited weak activity against keratin (447 units/mg) compared with proteinase K (7490 units/mg) and showed no activity against the other substrates tested.

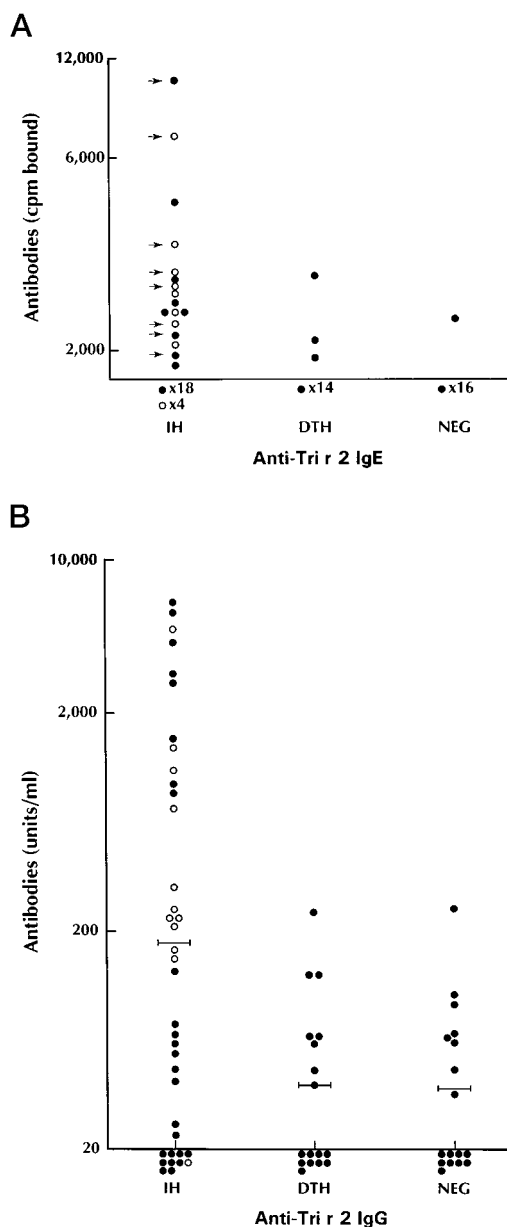


FIG. 7. **IgE and IgG antibody binding to recombinant Tri r 2.** Sera from 73 individuals with different skin test reactivities to the *Trichophyton* mixture were analyzed for IgE and IgG Ab binding to ¹²⁵I-labeled Tri r 2 fusion protein. A, the prevalence of IgE Ab binding in IH subjects and in those subjects with DTH or negative (NEG) skin test reactions was 43 and 12%, respectively. Arrows denote individuals with IgE Ab binding as determined by plaque immunoassay. B, mean IgG Ab levels were significantly higher in subjects with immediate reactions ($p < 0.01$). Bars represent geometric means. Individuals with bronchial reactivity to *Trichophyton* are indicated (○).

DISCUSSION

We have reported the amino acid sequences and expression of two distinct proteins derived from the dermatophyte fungus *Trichophyton*. The 83-kDa mannose-rich natural glycoprotein (n-Tri t 4) was previously shown to elicit IH and DTH skin test reactions in different individuals (27). To our knowledge, this is the first reported sequence of a fungal antigen associated with distinct skin test reactions. The homologous recombinant protein produced by *T. rubrum* (r-Tri r 4) is a 726-amino acid protein with limited amino acid sequence homology to the prolyl oligopeptidase (S9) family of serine proteinases. Despite the relatively low amino acid sequence similarity (~20%), several characteristics provide convincing evidence that r-Tri r 4

TABLE I
Skin test responses and serum antibodies to a GST fusion protein containing the putative mature form of Tri r 2

Patient	Skin test to <i>Trichophyton</i> mixture ^a	Tri r 2		
		Skin test ^a	IgG ^b	IgE ^b
			units/ml	cpm bound
R. P.	DTH (10 × 11)	DTH (10 × 10)	<20	<1700
E. K.	DTH (10 × 11)	DTH (5 × 5)	<20	<1700
G. W.	DTH (12 × 12)	NEG	<20	<1700
C. D. ^c	IH/DTH (8 × 10)	DTH (5 × 5)	240	<1700
T. P. ^c	IH/DTH (15 × 15)	DTH (15 × 15)	60	2120

^a Values in parentheses represent diameter of induration (mm) on intradermal testing at 24 h. NEG, no response.

^b These were measured by antigen binding radioimmunoassay.

^c These individuals experienced a wheal and flare preceding the delayed reaction.

belongs to this family of proteins: the distinctive arrangement of catalytic triad residues and their localization in the carboxyl-terminal region of the molecule, the presence of conserved amino acids flanking putative catalytic residues, its high molecular mass consistent with other members of this family, and a large variable NH₂-terminal portion. In contrast to subtilases, S9 peptidases do not exist as proenzymes and are synthesized in an active form (35, 36). These enzymes, which may be either cytosolic or membrane-bound, exhibit restricted specificities that may limit degradation of other cell proteins (37). Some family members have been reported to be involved in a variety of nonenzymatic physiologic processes; for example, the membrane glycoprotein dipeptidyl peptidase IV (CD26) plays a role in cell-matrix adhesion and transmembrane signaling (38–40). Recombinant Tri r 4 exhibited a low level of proteolytic activity against keratin. Dermatophyte fungi are adapted to infect keratinized tissues by virtue of their ability to utilize keratin as a nutrient source. Whether the natural Tri r 4 and Tri t 4 proteins are functionally keratinolytic *in vivo* remains to be established. If this proves to be the case, the enzymatic activity of these proteins could facilitate colonization and may contribute to pathogenicity.

P. pastoris was selected for expression of r-Tri r 4 since a eukaryotic system is more appropriate for expression of fungal antigens, and high yields of foreign proteins, including some allergens, were previously reported (41, 42). Yields of recombinant protein were very high (~200 mg/liter of culture). SDS-PAGE analysis suggested that r-Tri r 4 was glycosylated to a degree comparable to the natural antigen (~5% carbohydrate by weight). Despite this, preliminary studies suggest that r-Tri r 4 exhibits partial loss of B cell epitopes as determined by decreased binding to IgG antibodies compared with natural antigen (data not shown). We hypothesize that partial loss of conformational epitopes on r-Tri r 4 may result from incorrect folding owing to its large size, a factor that may also contribute to its low enzymatic activity. Preliminary results have also shown that recombinant Tri r 4 failed to elicit DTH skin test responses in three individuals with DTH responses to natural Tri t 4. These findings are surprising since only linear antigenic determinants are required for initiation of T cell responses *in vitro*. Since recombinant Tri r 4 is derived from a *T. rubrum* cDNA library, and natural Tri t 4 was purified from a *T. tonsurans* extract, this raises the possibility that antigenic properties differ between homologous proteins derived from the two fungal species. Alternatively, it could be hypothesized that conformational epitopes or post-translational modifications of linear antigenic determinants required for DTH responses fail to occur in the recombinant protein. Similar findings have been demonstrated for a ribosomal protein derived from *Brucella melitensis* (43). This antigen typically induces DTH responses in *Brucella*-sensitized guinea pigs. However, recombinant antigen expressed in *E. coli* produced no skin response. It was concluded that post-translational acylation of protein is re-

quired for DTH activity. Recombinant Tri r 4 will serve as a valuable tool for distinguishing the relevance of conformational epitopes or post-translational modifications in the induction of DTH responses in humans.

The second antigen defined is an allergen with high amino acid sequence similarity to serine proteinases of the class D subtilase subfamily. Eight of 10 subjects with IH skin test reactions to *Trichophyton* displayed IgE antibody binding to this allergen, five of whom had bronchial reactivity to *Trichophyton*. True subtilisins derived from bacteria are among the best characterized of the subtilase enzyme family. Subtilisin Carlsberg (Alcalase), a class A subtilase produced by *Bacillus licheniformis*, is one of several subtilases used in detergent formulations. Soon after the initiation of large-scale production of enzyme-containing detergents, allergic respiratory reactions to the enzyme components were noted among factory workers (44, 45). Thus, Tri r 2 is a member of the same enzyme family as an antigen previously related to asthma.

Bacterial expression of the putative mature form of Tri r 2 in the absence of a fusion partner resulted in rapid degradation during purification. One possible explanation is that the predicted site of cleavage of the pro-region is incorrect and that the presence of additional NH₂-terminal flanking residues is required for stabilization of the carboxyl-terminal domain containing active-site residues characteristic of the subtilase family. Alternatively, the presence of the entire pro-region may be required to serve as a template for correct folding of this domain, as has been demonstrated for other subtilase enzymes (46); however, attempts to express Tri r 2 with the putative pro-region were unsuccessful. Production of the putative mature form of Tri r 2 as a GST fusion protein facilitated stabilization of this domain. Members of the class D subtilase subfamily have been shown to exhibit cuticle-degrading and elastase activities. The class D subtilase ALP (alkaline proteinase), produced by the pathogenic fungus *A. fumigatus*, exhibits elastase activity and has been proposed to contribute to fungal persistence in allergic individuals (47). However, no enzymatic activity of recombinant Tri r 2 was demonstrated. It is possible that Tri r 2 is not an enzyme; however, given the high degree of homology to subtilase enzymes, especially in the putative active site, it appears more likely that the lack of activity reflects features intrinsic to the recombinant protein. These may include suboptimal processing of the recombinant protein owing to the absence of the putative pro-region, lack of post-translational modification, or the presence of the NH₂-terminal GST moiety. Alternatively, inappropriate substrates may have been selected for study.

Tri r 2 expressed as a GST fusion protein was shown to exhibit IgE and IgG Ab binding characteristics in addition to mediating DTH skin test reactions. These findings suggest that expression of the carboxyl-terminal 289 amino acids containing the putative mature form of the protein was sufficient for

immunologic function. This is important since the absence of the pro-region or amino-terminal flanking residues could possibly influence immunologic properties. Tri r 2 is a novel antigen in that it is the first recombinant protein demonstrated to induce both IgE Ab- and cell-mediated responses in humans. Furthermore, the high prevalence of IgE antibodies suggests that this protein is an important allergen among patients with chronic dermatophyte infection.

Dermatophytosis is an important clinical problem both because of its chronicity and because current antifungal therapy is only curative in a small proportion of cases. Identification of the antigenic determinants associated with protective cell-mediated immune responses would make it possible to design peptide or recombinant protein vaccines to modify the natural course of the disease. The 29-kDa antigen Tri r 2 is a good candidate for the application of overlapping peptide methodology to define immunodominant epitopes in individuals with either IgE antibody or DTH reactions. Thus, definition of these proteins will make it possible to investigate T cell recognition associated with different responses. Characterization of *Trichophyton* antigens provides unique molecular tools not only for the development of immunotherapeutic strategies related to management of chronic dermatophytosis and the associated allergic disease, but also for the analysis of immunologic mechanisms governing diverse immune responses in humans.

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