

Mapping of an allergenically important determinant of grass group I allergens

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This report documents the mapping of the second major epitope, previously described as site D, of grass group I allergens to residues 23-35 of meadow fescue group I (STFYGKPTGAGPK). Mapping was accomplished by screening fractions from a meadow fescue group I tryptic digest for peptide(s) that inhibit the ability of monoclonal antibody 24.64 (specific for site D) to bind to immobilized group I allergen. One such peptide, representing residues 23-35 of meadow fescue group I, was identified. Additional studies with the use of synthetic analogs of this peptide demonstrate that it binds mAb 24.64 directly. Examination of extracts containing group I glycoproteins from seven other species of grass confirms antigenic cross-reactivity due to this peptide. We also report based on protein sequence analysis* that the amino terminal segment (which includes the site D epitope) of GpI allergens from seven different grass species is highly conserved and contains two hydroxyproline residues and an N-linked carbohydrate moiety. (*J Allergy Clin Immunol* 1997; 100:335-40.)

*PIR accession numbers are as follows: Fes e I = A58493, Ant o I.1 = B58493, Ant o I.2 = C58493, Dac g I = D58493, Poa p I = E58493, Agr a I.1 = F58493, Agr a I.2 = G58493.

Key words: Grass allergen, monoclonal antibody, epitope map, peptide

Group I (GpI) glycoproteins are isoallergens shared by many species of grass. They are major contributors to hay fever in individuals hypersensitive to grass¹ and have been extensively characterized. Complementary deoxyribonucleic acid (cDNA) clones encoding GpI from perennial ryegrass pollen have been isolated and the amino acid sequence deduced.^{2,3} GpI proteins that have been cloned at the cDNA level possess a considerable amount of both nucleotide and amino acid sequence homology,^{2,4} and GpI proteins from a variety of grasses display both antigenic⁵ and allergenic⁶ cross-reactivity. The existence of cross-reactive allergenic epitopes may provide an explanation for why individuals allergic to grass are frequently hypersensitive to more than one type of grass. Cross-reactivity rather than multiple sensitization may indeed be the principle mechanism by which individuals

Abbreviations used

ASN:	ASNPNYLAILK peptide
cDNA:	Complementary deoxyribonucleic acid
CGG-STF:	CGGSTFYGPKTGAGPK peptide
GpI:	Group I
HPLC:	High-performance liquid chromatography
JU:	June grass
KLH:	Keyhole limpet hemocyanin
mAb:	Monoclonal antibody
MF:	Meadow fescue
OR:	Orchard grass
PBS:	Phosphate-buffered saline
PR:	Perennial rye
PVDF:	Polyvinylidene difluoride
RT:	Redtop grass
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
STF:	STFYGKPTGAGPK peptide
SV:	Sweet vernal grass
TFA:	Trifluoroacetic acid
TI:	Timothy grass

display allergic reactivity to multiple grass species. The characterization of such cross-reactive epitopes may hold potential for the development of standardized diagnostic and therapeutic tools with which to approach immunotherapy for individuals allergic to grass.

Previous studies from this laboratory used monoclonal antibodies (mAbs) to study the antigenic and allergenic structure of GpI proteins.^{5,7,8} Monoclonal antibodies that are cross-reactive among GpIs of five species of grass were produced and characterized: MF (meadow fescue, *Festuca elatior*), PR (perennial rye, *Lolium perenne*), JU (june, *Poa pratensis*), RT (redtop, *Agrostis alba*), and SV (sweet vernal, *Anthoxanthum odoratum*). During experiments in which individual mAbs as well as pairs of mAbs were examined for their ability to inhibit human IgE from binding to purified GpI allergen, two particularly interesting mAbs were identified. MAbs 16.9 and 24.64, which define sites A and D, respectively, of the GpI molecule, were selected for further study. Together, these antibodies inhibit ~50% of the binding of IgE from pooled sera of patients allergic to grass to purified GpI allergen. Sites A and D are thus in some way meaningful during the immune and allergic response to grass GpI. Site A was previously mapped by this laboratory⁸ and confirmed by others⁹ to be the 29-residue peptide representing the carboxyl terminus of the GpI molecule. Amino acid sequence analysis of this

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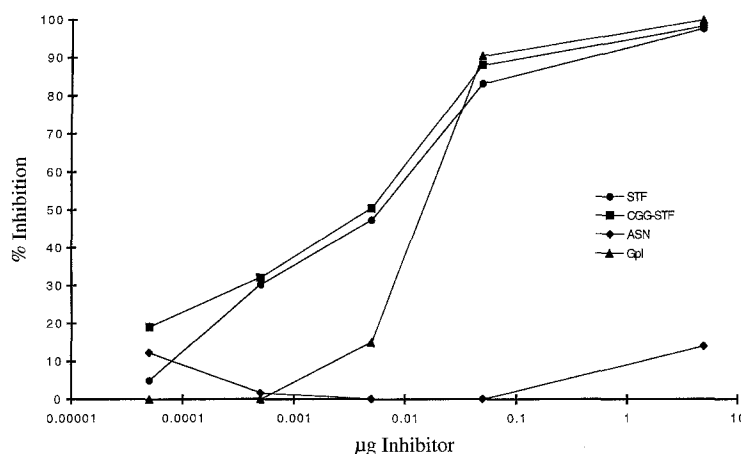


FIG. 1. Inhibition ELISA with immobilized GpI. Dilutions of STF, CGG-STF, ASN, and GpI were tested for their ability to inhibit the binding of mAb 24.64 to immobilized GpI. Percent inhibition is expressed as a percentage of a control with no inhibitor.

peptide epitope showed it to be highly homologous between GpI proteins of all five grasses investigated.⁸

In this report, we continue our studies of the B cell epitope structure of grass GpI allergens by characterizing the site D peptide recognized by mAb 24.64. In the course of this study, we determined the amino acid sequence of the first 35 residues of seven different GpI molecules. We have found that this entire region of the molecule is highly conserved between different species of grasses; it has at least two unusual features not reported when cDNA for GpI was cloned and its amino acid sequence was deduced.

METHODS

Antibodies

Monoclonal antibodies 24.64 (antisite D) and 16.9 (antisite A) used for GpI recognition have been described previously.⁷ Milligram quantities of the 24.64 mAb were produced by inducing ascites fluid in BALB/c mice. Mice were primed intraperitoneally with 0.5 ml of pristane (Aldrich, Milwaukee, Wis.). One week later, 7×10^6 mAb 24.64 hybridoma cells suspended in sterile phosphate-buffered saline (PBS) were injected intraperitoneally. Ascites fluid was harvested from the peritoneal cavity at appropriate intervals over the next several weeks. Antibodies from mAb 24.64 ascites fluid were precipitated with 45% ammonium sulfate, dialyzed against PBS (0.02 mol/L, pH 8.0, 0.15 mol/L NaCl), and coupled to Affigel 10 resin (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions to make a GpI-binding immunoaffinity column.

Antigens

GpI antigens were purified from MF whole pollen (Greer Laboratories, Lenoir, N.C.) by a series of chromatographic steps according to a procedure modified from Marsh.¹ Pollen was extracted overnight in 0.02 mol/L sodium-phosphate buffer at pH 7.2 and the extract was applied to a Sephadex G75 molecular sieving column (Pharmacia Inc., Piscataway, N.J.) equilibrated in the same buffer. The eluate, monitored at OD 280 nm, was screened by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE),¹⁰ and fractions containing proteins between 26,000 and 33,000 kDa were pooled for

further processing. The GpI-enriched sample was then applied to a DEAE Biogel column (Bio-Rad Laboratories) equilibrated in 0.015 mol/L Tris at pH 8.0 and eluted with a gradient of increasing NaCl (0 to 0.2 mol/L). Any remaining contaminants were removed by reducing (adding 0.1 mol/L dithioerythritol) and alkylating (adding 0.22 mol/L iodoacetic acid) the sample, passing it through the mAb 24.64 affinity column equilibrated in 0.02 mol/L sodium phosphate, pH 7.2, 0.15 mol/L NaCl, and eluting with 10% acetic acid in H₂O. Eluents appeared as a single band of ~30,000 kDa on SDS-PAGE gels and were recognized by mAb 24.64 ascites fluid in ELISA.

Whole grass extracts of PR, JU, RT, SV, TI (timothy grass, *Phleum pratense*), and OR (orchard grass, *Dactylis glomerata*) were reconstituted from lyophilized preparations of defatted pollen (Greer Laboratories). These were either used directly or partially purified to yield material enriched for GpI. The enrichment procedure consisted of a single fractionation on the Sephadex G75 column. OR and JU GpI-containing fractions were then run on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes for direct protein sequence analysis. Comparable G75 fractions from SV and RT pollen extracts were passed through the mAb 24.64 affinity column equilibrated in phosphate-NaCl, the column was washed with H₂O to remove all salts, and GpI glycoproteins were eluted with 10% acetic acid. After lyophilization, the purified GpI glycoproteins were subjected to protein sequence analysis. Amino acid sequencing was performed on a PROCISE 491 protein sequencer (Perkin Elmer/ABI, Foster City, Calif.) equipped with on-line phenylthiohydantoin.

ELISA

ELISAs were performed as modified from the method of Voller et al.¹¹ Antigens were immobilized on polystyrene microtiter plates (Immulon 2, Dynatech Laboratories, Chantilly, Va., or EIA/RIA, Costar, Cambridge, Mass.) by incubating overnight at 4° C in 0.015 mol/L Tris at pH 8.5. Plates were washed three times with dilution buffer: 0.015 mol/L Tris at pH 7.5, 0.1 mol/L NaCl, and 0.05% Tween 20. Primary antibody diluted in this buffer was added and incubated for 75 minutes at 37° C. After washing, alkaline phosphatase-labeled anti-mouse IgG (Southern Biotech, Birmingham, Ala.) diluted 1:4000 in dilution buffer was added and incubated for 45 minutes at 37° C. Bound antibody was detected by the addition of *p*-

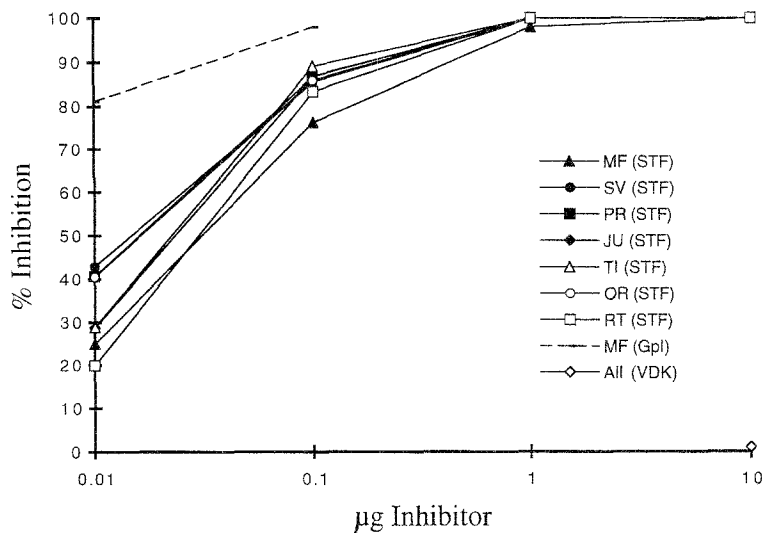


FIG. 2. Inhibition ELISA with immobilized grass extracts. Coated extracts are named next to the corresponding symbol, followed by inhibitors in parentheses. Grass extracts of MF, SV, PR, JU, TI, OR, and RT were coated onto a microtiter plate. STF was tested for its ability to inhibit mAb 24.64 binding to these seven GpI-containing extracts. As a negative control, an excess of peptide VDK was also tested against mAb 24.64 for binding to all seven extracts. VDK showed negligible inhibition for all seven extracts, and this is represented here by a point. As a positive control, the ability of GpI to compete with mAb 24.64 for binding to the extracts was also assessed. Only the results for Fes e 1 inhibition are shown. Results for all other extracts were very similar.

nitrophenyl substrate (Sigma Chemical Co., St. Louis, Mo.) dissolved in diethanolamine buffer. Absorbance was measured at 405 nm with a Bio-Tek (Winooski, Vt.) automated ELISA reader 1 hour after the addition of substrate. Incubation volumes were 100 µl for plate coating, 95 µl for primary antibody incubation, 90 µl for secondary antibody incubation, and 85 µl for developing solution. This stepwise decrease in volume was done to minimize the chance of antibody binding to the sides of the wells and being developed as a false-positive result.

Inhibition ELISAs were performed as described above, with an additional step in which primary antibody was incubated with inhibitor overnight at 4° C before the mixture was added to the wells. Parameters for inhibition ELISAs were established during preliminary experiments in which appropriate ratios of reactants were established. First, a "checkerboard" ELISA was performed in which dilutions of the Fes e 1 preparation ranging from 1:10² to 1:10⁵ were tested with dilutions of mAb 24.64 ascites fluid ranging from 1:10⁵ to 1:10⁹. Alkaline phosphatase-labeled anti-mouse IgG was used at a constant value of 1:4000. A 1:10⁴ dilution of GpI incubated with mAb 24.64 ascites diluted 1:10⁶ resulted in moderate development (absorbance at 405 nm = 0.6) after 1 hour. These conditions were then used for a preliminary inhibition assay in which GpI was used as the inhibitor. A control with no inhibitor was included for purposes of calculating percent inhibition. GpI was incubated with mAb 24.64 before being added to GpI-coated microtiter wells and the ELISA was performed as described above. During development, absorbance was measured at regular intervals. Percent inhibition was calculated by the equation: % inhibition = [(A₀ - A_i)/A₀] × 100, where A₀ represents absorbance with no competitor and A_i represents absorbance with competitor present. Under the conditions described, an amount of GpI equal to the GpI coated on the plate inhibited 75% of mAb 24.64 binding. A 10-fold increase in GpI inhibited 96% of the binding. These conditions were used for subsequent inhibition assays.

Enzyme digests and high-performance liquid chromatography separations

A quantity of 10 µg of TPCK (L-(tosylamido-2-phenyl)ethyl chloromethyl ketone)treated trypsin (Worthington, Freehold, N.J.) was added to approximately 1 mg of reduced and alkylated Fes e 1 in fresh 2% ammonium bicarbonate at pH 8.0, and incubated at 37° C for 5 hours. The digestion products were lyophilized, resolubilized in 0.1% trifluoroacetic acid (TFA), and separated by reverse-phase high-performance liquid chromatography (HPLC) by use of a 4.6 × 150 mm W-Porex C₁₈ column (Phenomenex, Torrance, Calif.) with 0.1% TFA and an increasing acetonitrile gradient. One-minute fractions were collected and screened for blocking activity by use of an inhibition ELISA. Those fractions that inhibited the ability of mAb 24.64 to bind to immobilized GpI were reprocessed through the same column with a modified gradient to enhance separation of peptides in the fractions. Individual peaks were collected and screened again for inhibitory activity.

Peptides

Peptides isolated by HPLC were subjected to amino acid sequence analysis either on glass fiber discs in the presence of polybrene or adsorbed to ProBlott PVDF membranes (Perkin Elmer/ABI, Foster City, Calif.) by the use of standard sequencer programs developed for the PROCISE protein sequencer. Synthetic peptides and homologs representing the defined peptide as well as control peptides were synthesized by the use of 9-fluorenylmethoxycarbonyl chemistry on a Rainin Symphony multiple peptide synthesizer (Rainin, Woburn, Mass.) and purified by the use of reverse-phase HPLC chromatography with a C₁₈ column in 0.1% TFA with an increasing acetonitrile gradient. Masses of synthetic peptides were verified with a MALDI-TOF mass spectrometer (Shimadzu/Kratos, Ramsey, N.J.). Peptides purified by HPLC were lyophilized, weighed, resolubilized in H₂O at 1 mg/ml, and stored at 20° C.

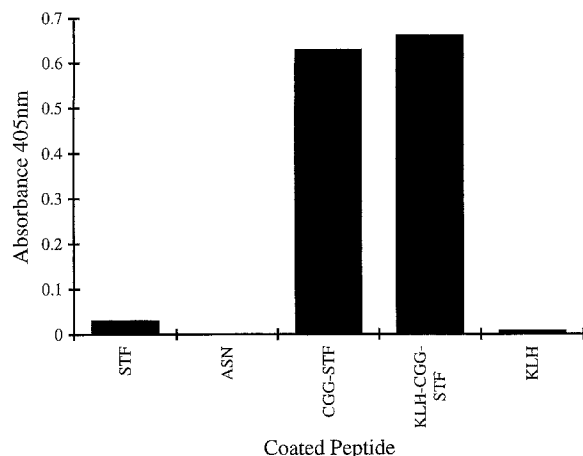


FIG. 3. Standard ELISA with immobilized peptide. STF, ASN, CGG-STF, and KLH-CGG-STF, and KLH were coated onto a microtiter plate and probed with mAb 24.64. Results are expressed as absorbance at wavelength 405 nm.

RESULTS

Isolation of the site D peptide

Meadow fescue GpI was purified and digested as described. The mapping of site A to the carboxyl terminus of GpI utilized trypsin digests of GpI that had been reduced, alkylated, and then treated with citraconic anhydride to prevent cleavage after lysine residues. In that case, citraconylation was necessary to retain the integrity of the site A epitope. Despite the fact that site D possesses an internal lysine residue, this epitope remained intact after 5 hours of trypsin digest of reduced, alkylated, noncitraconylated GpI. It is possible that site D would have been disrupted by cleavage at the lysine had the digest been allowed to proceed longer. A preliminary inhibition ELISA showed that the unfractionated digest was able to inhibit mAb 24.64 from binding to wells coated with GpI antigen. The digest was then fractionated by HPLC, and individual fractions were screened for blocking activity by inhibition ELISA. In this assay, 1% of each fraction was preincubated with mAb 24.64 as a potential inhibitor of the antibody. Fractions 19 and 20 inhibited mAb 24.64 from binding to ELISA plates coated with GpI. Those fractions were pooled and reinjected for HPLC fractionation with a modified gradient. One-minute fractions were again collected and screened by the inhibition ELISA. Fractions 11, 12, and 13 of this second fractionation displayed inhibitory activity. Because fraction 12 exhibited the greatest amount of inhibition, it was selected for amino acid sequence analysis. Edman degradation of this fraction revealed a 13-residue peptide: STFYGKPTGAGPK. This corresponds to residues 23-35 of the previously published perennial rye GpI sequence.^{2,3}

Inhibition ELISA

Several peptides were produced for use in inhibition assays. The site D peptide (STFYGKPTGAGPK, or STF), an analog of the site D peptide (CGGSTFYGPKTGAGPK, or CGG-STF), and a control peptide

TABLE I. ELISA with immobilized STF

	mAb 24.64	mAb 16.9
GpI	+++	+++
KLH-CGG-STF	+++	---

GpI and modified STF conjugated to keyhole limpet hemocyanin (KLH-CGG-STF) were coated onto microtiter wells.

+, Significant binding; −, negligible binding.

(ASNPNYLAILK, or ASN) were synthesized and purified, and their masses were verified by MALDI-TOF mass spectrometry. CGG-STF was used in a standard ELISA to be described in the next section. ASN represents residues 154-165 of GpI, a region known not to bind to mAb 24.64. STF, CGG-STF, GpI, and ASN were each evaluated for their ability to inhibit the binding of mAb 24.64 to grass GpI in an ELISA. A series of quantities of each inhibitor (ranging from 5×10^{-5} to 5 μ g) was incubated with a dilution of mAb 24.64 overnight at 4°C, and the incubation solution of each dilution was applied to GpI-coated plates. After the application of secondary antibody and substrate and appropriate washings, absorbances were read. The curves in Fig. 1 were generated from averages of quadruplicate screenings and represent the absorbance readings 2 hours after the addition of substrate. As seen here, STF exhibits dose-dependent inhibition comparable to that of GpI itself, while ASN shows negligible inhibition.

Monoclonal antibody 24.64 was previously shown to inhibit human IgE binding to GpI allergens of five different grass species.⁷ Inhibition ELISA was performed to determine whether STF is an important peptide in the GpIs of seven species of grass (MF, PR, JU, RT, SV, OR, TI). Lyophilized grass extracts (Greer Laboratories) were suspended in H₂O to a concentration of 10 mg/ml. These were diluted 1:10⁴ and coated onto microtiter plates. Inhibitions were then performed as previously described with the use of STF, GpI, and VDK (a 15-residue peptide unrelated to GpI) as inhibitors. Fig. 2 shows that STF inhibits mAb 24.64 binding to all seven GpI-containing extracts in a dose-dependent manner. Inhibitions with GpI were very similar for all seven coated extracts, and are represented by a single example in Fig. 2. Whereas 1 μ g of STF inhibited all binding, ten times this amount of the unrelated 15-residue peptide VDK displayed no inhibition; this demonstrates that the inhibition is specific and not simply due to steric hindrance from too much peptide.

Direct bind ELISA

To provide more direct evidence that the STF peptide binds to mAb 24.64, ELISAs were performed in which synthetic peptides were coated directly onto microtiter plates and probed with mAb 24.64. Such an assay is useful to establish the direct binding of antibody to peptide, but can present technical problems due to the potentially low binding efficiency of relatively short

TABLE II. N-terminal sequences of GpI from seven species of grass

					5				10					15				20				25				30				35					
MF	I	A	K	V	<u>P</u>	P	G	<u>P</u>	N	I	T	A	E	Y	G	D	K	W	L	D	A	K	S	T	<u>F</u>	Y	G	K	P	T	G	A	G	P	K
SV	—	—	—	—	—	—	—	—	—	—	—	—	T/D	—	—	—	—	—	—	—	—	—	—	W	—	—	—	—	—	—	—	—	—	—	
OR	—	P	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	—	W	—	—	—	—	—	—	—	—	—	—	
JU	—	—	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	—	W	—	—	—	—	—	—	—	—	—	—	
RT	—	—	—	—	—	—	—	—	—	—	—	—	T/D	—	—	—	—	—	—	—	—	—	—	W	—	—	—	—	—	—	—	—	—	—	
PR ^{2,3}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	W	—	—	—	—	—	—	—	—	—	—	
TI ⁴	—	P	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	—	W	—	—	—	—	—	A	—	—	—	—	

MF, Meadow fescue; SV, sweet vernal grass; OR, orchard grass; JU, june grass; RT, redtop grass; PR, perennial rye; TI, timothy grass.

The STF region is shown in bold letters. Hydroxyprolines are underlined. The glycosylated asparagine is italicized.

—, Residue is the same as that of analogous MF residue.

peptides to the ELISA plate. We found that, when compared with a GpI-coated microtiter well, a well coated with a comparable amount of STF yielded much lower readings when probed with mAb 24.64 followed by anti-mouse IgG. Presumably, this is due to low coating efficiency of the 13-residue peptide. A slightly modified peptide consisting of STF with a 3-residue N terminal extension (CGGSTFYGKPTGAGPK, or CGG-STF) was synthesized and conjugated to keyhole limpet hemocyanin (KLH) by the use of *m*-maleimidobenzyl-*N*-hydroxysuccinimide ester (Pierce Chemical, Rockford, Ill.). This covalent coupling of the peptide to a large protein was performed to ensure that the peptide would be adsorbed to the plate. A quantity of 0.05 mg each of STF, the unrelated GpI peptide ASN, CGG-STF, KLH-CGG-STF, and KLH was applied to a microtiter plate in Tris coating buffer and incubated overnight at 4° C. Wells were probed with mAb 24.64 at 1:10⁶, followed by anti-mouse IgG at 2.5×10⁴. Fig. 3 shows that, as expected based on preliminary results, wells coated with the STF peptide showed only mild binding above background (ASN-coated control). The modified STF peptide coupled to KLH (KLH-CGG-STF) displayed binding to mAb 24.64 demonstrably higher than that of its KLH-only counterpart. The 3-residue extension of CGG-STF is apparently sufficient to render the peptide "coatable," because unconjugated CGG-STF binds mAb 24.64 strongly, indicating again that STF binds mAb 24.64.

An alternative explanation for the ability of STF to bind to mAb 24.64 is that the peptide might bind directly to the Fc region of immunoglobulins. To exclude this possibility, an ELISA was performed in which GpI and KLH-CGG-STF were coated on a plate and probed with mAb 24.64 and with mAb 16.9, an anti-GpI antibody that does not recognize Site D. Table I shows that, whereas immobilized KLH-CGG-STF binds to mAb 24.64, it does not bind to mAb 16.9. The fact that this peptide does not bind to either mAb 16.9 or to the secondary antibody suggests that STF indeed binds to the mAb 24.64-specific idiotope.

Amino terminal sequence analysis

GpI proteins were purified from several species of grass by use of a simplified purification procedure. Pollen extracts were fractionated on a Sephadex G75 fine molecular sieve column. GpI-containing fractions from OR and JU were subsequently run on SDS-PAGE

and transferred to PVDF membranes. Staining of the membrane with Ponceau S revealed single bands of approximately 30 kDa. These bands were excised from the membrane and subjected to 35 cycles of Edman degradation. When GpI-containing fractions from SV and RT were processed this way, multiple sequences were found that precluded positive identification of the GpI sequence. These GpI-containing fractions were therefore further purified by use of a mAb 24.64 affinity column. Eluents from the column (10% acetic acid) yielded single bands when analyzed by SDS-PAGE. These eluents were applied directly to PVDF membranes for sequencing, as was the previously purified Fes e 1. Direct and deduced amino acid sequences from PR and TI have been previously published²⁻⁴ and those sequences were not repeated here. Table II shows the N-terminal sequences for GpIs from seven species of grass. The sequence is highly conserved throughout the entire amino terminus of the molecule, and only two substitutions have been noted within the site D epitope: These are the very conservative substitutions of a hydrophobic F for W at position 25 of MF, and of an A for G (both small, neutral amino acids) at position 31 of TI. Hydroxyproline residues were found at residues 5 and 8 of all molecules sequenced. Position 9 of all GpI proteins sequenced was presumed to be a glycosylated asparagine because it was recovered in extremely low yield, as is typical of glycosylated residues, and because it fits the 'N X T' motif characteristic of N-linked glycosylation. Position 9 was reported to be an asparagine in the sequence deduced from cDNA.

DISCUSSION

Great interest continues in the development of immunotherapeutic strategies for the long-term management of allergic diseases. Proposed therapeutic intervention can be conveniently divided into two major categories. The first is global therapy, in which either the synthesis and secretion of all IgE or the function of the high-affinity IgE receptor are ablated or modified.¹² A second approach is allergen-specific therapy, in which only the IgE response to a specific allergen is modified. In the latter category, several potential levels of intervention might give rise to an altered antibody response. Immunotherapeutic protocols in which the route and dose of crude or purified, chemically or physically modified, allergen have been established and are in current clinical

use. More recent developments, following our increasing understanding of the role of T cells and cytokines on the development of IgE secretion by B cells,¹³ focus on small peptides that are thought to interact with class I and class II major histocompatibility complex antigens and might therefore alter the T cell response to specific allergens.^{14, 15}

Cross-reactive allergy-related B cell epitopes, when used for the production of protective antibodies, might provide the design for yet another refined immunotherapeutic strategy for hypersensitivity. Because of this, and because more remains to be discovered about allergenic structure in general, these epitopes continue to be worthy of investigation. In the case of the highly homologous GpI allergens, some, but not all, of the antigenic epitopes are cross-reactive.⁷ We have mapped site D, an allergenically important cross-reactive epitope, to residues 23-35 of GpI (STFYGKPTGAGPK). A synthetic version of this peptide inhibits GpI/mAb 24.64 binding in ELISA, although it coats ELISA plates poorly and could only be weakly demonstrated to bind the antibody directly. An analog, composed of residues 23-35 of Fes e 1 with a 3-residue amino terminal extension (CGG), is able to coat ELISA plates efficiently and has been shown directly in ELISA to bind mAb 24.64 significantly. We have also performed preliminary inhibition experiments in which a shortened peptide (STFYGKPTGA) was tested for its ability to inhibit GpI/mAb 24.64 binding. This peptide inhibited binding as well as an equal amount of full-length STF peptide, suggesting that site D is located within the first 10 residues of STF, residues 23-32 of GpI.

The cross-reactivity of STF was demonstrated in inhibition ELISAs showing that STF inhibits mAb 24.64 interaction with GpIs of seven different species of grass. The amino terminal sequence of all of these was determined and proved to be highly conserved. One of the two sequence variations found in the STF region was F for W at position 3 (GpI residue 25) in our prototype, Fes e 1. Although the site D homologs used in our experiments have F at position 3, they functioned very well in the cross-reactive experiments. Two explanations are possible: this residue is not important to mAb 24.64 binding, or the relatively conservative substitution is not sufficient to disrupt mAb 24.64 binding. Only one other substitution was found in the site D region, a conservative A for G at position 9 (residue 31 of GpI). Another notable result from protein sequence analysis is the presence of hydroxyproline residues in positions 5 and 8 of all GpIs that were sequenced. This finding, which is in agreement with previously published data,^{16, 17} could not be predicted by the sequence deduced from data derived by nucleic acid analysis. Glycosylation at asparagine 9 could be predicted from the deduced sequence and has been confirmed here by direct amino acid sequence analysis.

Direct demonstration of IgE from patients allergic to

grass binding to the site D peptide has not been conclusive (data not shown), suggesting that the STF peptide reported here may not represent the precise human IgE binding epitope. Whether site D is a part of the human IgE binding site, or whether it is involved in the conformation of the protein such that mAb 24.64 bound to it alters a distinct IgE binding epitope, is not known at this time. Regardless of its role, the identification of site D provides another detail in the structure of GpI allergens, and offers potential improvements in the treatment of patients with grass allergy.

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