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IFN-γ Induction on Carbohydrate Binding Module of Fungal Immunomodulatory Protein in Human Peripheral Mononuclear Cells

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Supporting Information

ABSTRACT: FIP-*fve* is a protein that is isolated from *Flammulina velutipes*. Its known immunomodulatory activities are elicitation of the production of type II interferon from human peripheral mononuclear cells (hPBMCs) and hemagglutination. How the target receptors mediate activation of FIP-*fve*-induced immunomodulatory effects remains to be elucidated. This study postulates the three-dimensional structures to determine whether the carbohydrate binding module family 34 (CBM-34) on FIP-*fve* is conserved to site N of *Thermoactinomyces vulgaris* R-47 α-amylase I. Experimental site-directed mutagenesis data as well as ligand-specific binding competition assay are adopted to identify the key residues W24, T28, D34, T90, I91, and W111 of FIP-*fve* that participate in binding to polysaccharides that are linked to the membrane of immune cells. Treatments of hPBMCs with tunicamycin and deglycosylation enzymes that removed the carbohydrate moieties reduced the secretion of IFN-γ induction from hPBMCs. In conclusion, the experiments herein demonstrated the ligand-binding CBM-34 on FIP-*fve* and ligand-like glycoproteins on the surface of hPBMCs must be required to induce physiological immunomodulatory effects.

KEYWORDS: *fungal immunomodulatory protein, FIP, carbohydrate binding site, protein−carbohydrate interaction, IFN-γ, site-directed mutagenesis, binding competition assay*

INTRODUCTION

Edible golden needle mushroom is a conventional foodstuff that is used extensively in traditional Asian medicine. Its component biomolecules are biological response modifiers exhibiting immunomodulatory,[1] antitumor,[2] antiviral, antifungal, and cholesterol-lowering activities,[3] providing widely accepted health-promoting effects.[4] Fungal immunomodulatory proteins (FIPs) that are isolated from the fruiting body of *Flammulina velutipes* (FIP-*fve*) play an important role in the immunomodulatory activity of mushroom toward human peripheral blood lymphocytes.[1,5]

The FIP-*fve* protein is a 12.7 kDa glycoprotein (Swiss-Prot ID: P80412) that comprises 114 amino acid residues. It is a member of a particular family (Fve superfamily, Pfam ID: 09259) of FIPs. Five mushroom proteins have been identified to date. All have been isolated from fruiting bodies of *Ganoderma lucidum*, *Volvariella volvacea*, *Ganoderma japoncium*, *Ganoderma microsporum*, and *Ganoderma tsugae* and are called LZ-8 (or FIP-*glu*),[6] FIP-*vvo*,[7] FIP-*gja*, GMI (or FIP-*gmi*), and FIP-*gts*,[8] respectively.[9] FIPs exhibit 55−86% sequence identities with the other members of the family. As mentioned elsewhere, several simple sugars do not inhibit FIP-*fve*-induced hemagglutination and cytokine interferon-γ (IFN-γ) secretion.[1] FIPs are members of a small family of fungal lectins that exhibit specificity toward cell-surface complex carbohydrates that perform essential physiological regulation, such as by exhibiting antiallergy and antitumor activities as well as stimulating immune cells to produce a variety of cytokines. [1]

FIP-*fve* induces significant IFN-γ expression and secretion in human peripheral blood mononuclear cells (hPBMCs) and so performs an immune regulation function.[1,10] However, the interaction site and mechanism of action of FIP-*fve* within the mammalian immune system have not yet been conclusively determined and must be clarified. To understand better the structure−function relationship of the family of proteins, bioinformatics tools were adopted to determine the organization of the putative carbohydrate-binding modules (CBMs) in the FIP-*fve* protein C-terminal region. CBMs are typically small and have a physically modular structure, separated from the catalytic domains of hydrolytic enzyme.[11] The known functions of CBMs seem to include supporting interactions between the insoluble substrates and the solubilized enzymes, bringing the substrate to the active site in the catalytic domain and, thereby, improving hydrolysis.[12] To date, 64 CBM families have been defined according to similarities among primary structures, which have been deposited in the CAZy database (http://www.cazy.org).[13] The critical amino acid residues on the FIP-*fve* mimic noncatalytic CBM family 34 (CBM-34), which is a β-sandwich folding family that acts as a granule starch-binding domain.[13] According to the protein structure, FIP-fve has 114 amino acids and its folding is similar to Ig-like

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β-sandwich domains in the CAZy database. Especially, we determined the putative CBM-34 of FIP-*fve* compared with the known N domain of the TVAI gene in its protein size, structural topology, and orientation of key residues in starch binding modules. The binding mechanism on which depend the immunomodulatory functions of recombinant FIP-*fve* (rFIP-*fve*) involves the CBM-34 structure and is responsible for the interaction between glycoprotein on the cells surface of hPBMCs and FIPs protein. This interaction regulates IFN-γ expression and secretion. This investigation confirms the binding domain and targets of FIP-*fve* in the induction of IFN-γ cytokine, which appears to be a promising pharmacological immune-modulating agent in the treatment of allergic diseases.[14]

MATERIALS AND METHODS

Glutathione−Sepharose 4B gel and pGEX-4T1 were purchased from Amersham Biosciences (Uppsala, Sweden). T&A plasmid was obtained from Yeastern (Taipei, Taiwan). M-MLV reverse transcriptase and Taq DNA polymerase were purchased from Promega (Madison, WI, USA). Chemicals for nucleotide sequence analysis were purchased from Applied BioSystems Inc. (Foster, CA). Restriction endonucleases were purchased from Takara (Shiga, Japan). The PNGase F and O-linked glycosidase were purchased from New England Biolabs and dissolved in 50 mM sodium phosphate reaction buffer, pH 7.5. Tunicamycin was purchased from Sigma (St. Louis, MO, USA) and dissolved in DMSO. All other chemicals used were of analytical grade and obtained from Sigma.

Expression and Purification of FIP-*fve*. Total cellular RNA was isolated from the mycelia of *F. velutipes* by homogenization in 4 M guanidium thiocyanate. Poly(A)$^+$ RNA was recovered using an oligo dT column, and total cDNA was synthesized using M-MLV reverse transcriptase, followed by DNA polymerase. Two primers were prepared according to the nucleotide sequence of FIP-*fve* that was isolated from *F. velutipes*. rFIP-*fve* cDNA was synthesized using PCR with primer A (5′-TATGGATCCTCCGCCACGTCGCTCACCTT-3′) and primer B (5′-CGCGAATTCTCAAGTCTTCTTCCACT-CAG-3′) and purified by agarose gel electrophoresis. DNA bands were stained with ethidium bromide and then visualized under ultraviolet light. A 330 bp DNA band was cut out, placed in a dialyzing tube with TAE buffer (20 mM Tris acetate, pH 8.0, 1 mM EDTA), and extracted by electrophoresis at 60 V for 1 h. The solution that contained the DNA fragment was treated with phenol/chloroform (1:1), and DNA was precipitated with 95% ethanol that contained 0.44 M ammonium acetate, pH 5.0. The DNA fragment was ligated into vector T&A plasmid, which had been previously cut with *Bam*HI and *Eco*RI. A ligation mixture was used to transform *Escherichia coli* XL10-GOLD ultracompetent cells that were obtained from Stratagene (La Jolla, CA, USA), and the plasmid was isolated and sequenced (377 DNA Sequencer; Applied Biosystems, Foster City, CA, USA) following the standard protocol. All inserts were sequenced on both strands at least twice, and the fragment was subcloned into pGEX4T1 (Amersham Pharmacia). To evaluate the expression of recombinant GST−FIP-*fve* fusion proteins, recombinant plasmids were introduced into BL21 competent cells of *E. coli* strain, obtained commercially from Stratagene, by $CaCl_2$-mediated transformation. After the cells reached a density of 4×10^8 cells/mL, they were induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and incubated for an additional 3 h. Cells were harvested by centrifugation, and the pellet thus obtained was resuspended in 10 mL of ice-cold resuspension buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM sodium chloride, 1 mM magnesium chloride, and 1 mM dithiothreitol. The cells were then treated with lysozyme (0.2 mg/mL) and lysed by three cycles of freezing and thawing. The cell lysate was centrifuged for 20 min at 20000*g*, and the supernatant was directly applied to a glutathione−Sepharose 4B column (2 mL) that was equilibrated with 10 mM Tris-HCl, pH 8.0. The column was washed using 20 mL of equilibrium buffer and then eluted with 5 mM reduced glutathione in the equilibrium buffer to obtain the fusion protein. FIP-*fve* and rFIP-*fve* were detected to have fewer than 30 endotoxin units (EU)/mL (1 EU = 0.1 ng, Limulus Amebocyte Lysate, Cape Cod, MA, USA).

Structure and Sequence Comparison. The X-ray structure (PDB ID: 1OSY) of the FIP-*fve* monomer was submitted for comparison to the DaliLite v.3.3 server 18. This server provides the significance of a match as a Z-score and a root-mean-square deviation (rmsd) of the three-dimensional alignment. The Z-score is a composite of several evaluations including rmsd and number of residues matched. A score less than 2.0 is not a significant match, while a very strong match is typically indicated by a core of greater than 10.0.

Site-Directed Mutagenesis of rFIP-*fve*. Derivatives of rFIP-*fve* were constructed by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit from Stratagene, according to the manufacturer's instructions. Supplemental Table S1 in the Supporting Information presents primers that were designed in the PCR experiments. Following DNA sequencing verification, the constructed mutated plasmids were transformed into *E. coli* BL21 (DE3) cells to produce recombinant proteins. The procedure for purifying the point mutant proteins was the same as that for purifying the wild-type rFIP-*fve* (Supporting Information, Figure S4).

Preparation of hPBMCs and Assay for Cytokines. hPBMCs were isolated from the heparinized peripheral blood of healthy adults by centrifugation over Ficoll-Paque Premium gradient medium (GE Healthcare, code 17-5442-03). This study was conducted after a human experimentation review by the IRB committee of Chung-Shan Medical University Hospital, and informed consent was obtained for every examined species. All informed consents were completed, signed, and reviewed by the IRB Committee of Chung-Shan Medical University Hospital. hPBMCs (2×10^6 cells/mL) were cultured, with and without stimulus in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS, 100 g/mL streptomycin, 100 units/mL penicillin, and 200 mM L-glutamate in 24-well tissue culture plates (Nunc, Roskilde, Denmark) for 48 h. hPBMCs (2×10^6 cells/mL) were plated into 24-well plates (Nunc) with and without FIP-*fve*. The conditioning medium was assayed to evaluated IFN-γ activity. The solid-phase enzyme-linked immunoabsorbent assay (ELISA) was performed using a kit from R&D Systems (Minneapolis, MN, USA) following the directions of the manufacturer.

Ligand Competition Binding Assay. Determined by pretreated 100 μg/mL FIP-*fve* with a serious concentration ligands of 0, 2, 10, and 30 mM for mono-, di-, and oligosaccharides; 0, 2, 5, and 10 mg/mL for dextrin and soluble starch; 0, 0.1, 0.5, and 1 mg/mL for glycogen. Then, were performed the mixtures co-incubated with 2×10^6 cells/mL hPBMCs in standard buffer for 48 h, and IFN-γ production was measured by ELISA testing.

Statistical Analysis. All values are presented as the mean ± SD. Statistical analysis was performed using one-way ANOVA. In all cases, P values of <0.05 were considered to be statistically significant.

RESULTS

Putative Carbohydrate-Binding Module on FIP-*fve*. FIP-*fve* does not have any homologous proteins with known enzymatic activity. The identification of such a homologue would improve our understanding of the immunomodulatory effects of FIP-*fve*. Because the sequential homologies technical usage did not find a distinct homologue of FIP-*fve*, the use of protein-modeling-based structural reconstitution to reveal particular three-dimensional hidden features was reexamined here. The three-dimensional structure (PDB code 1OSY) of FIP-*fve* is a tertiary structure that consists of an N-terminal α-helix, which undergoes hydrophobic interactions, and an immunoglobulin-like (Ig-like) β-sandwich family with seven strands arranged in two β-sheets to form a "pseudo-h-type" topology as reported previously.[15] To examine the structural homology of FIP-*fve* to other possible folds, the program DaliLite v.3.3 was utilized (http://www.ebi.ac.uk/Tools/dalilite/).[16] FIP-*fve* monomer was tested against all available

structures in the RCSB PDB database, and 901 hits (including protein redundancy) were obtained with a significance (*Z* score) of >2.0. The compared structures included immunomodulatory relative proteins, protective antigens, and HLA class II histocompatibility antigens.[17] These structures matched with average *Z* score between 2.0 and 23.6 and had a range of 2.6−5.2 Å aligned with all backbone atoms according to rmsd criteria. However, only TVAI α-amylase reveals that key residues of the CBM-34 are topologically equivalent in protein size and orientation with FIP-*fve*. Although topologically equivalent, the X-ray structures of the FIP-*fve* molecule and the Ig-like β-sandwich domains do not align well (Figure 1A). Perhaps the FIP-*fve* molecule contains no Cys, Met, or His residues owing to the absence of the intersheet disulfide bond that is present in immunoglobulin domains and is responsible for their compactness. [1]

The FIP-*fve* protein is structurally similar to domain N in the *T. vulgaris* R-47 α-amylase I (TVAI) gene (PDB code 1UH4) with a *Z* score of 3.9 and an rmsd of 3.1 Å over 100 residues that covered all Ig-like β-sandwich domains.[18] A more rigorous examination of the topological similarity between the Ig-like domain on FIP-*fve* and domain N on TVAI α-amylase reveals some interesting characteristics. Figure 1B reveals in detail that the Ig-like folding structure exhibits a strong correlation between the architecture and the numbers of key residues on the CBMs that are responsible for the protein−carbohydrate interactions. Most of the granular starch binding machinery comprises Ile43, Thr44, Trp65, Trp77, Asp75, and Asn65 on domain N of TVAI α-amylase between the Ig-like fold CBM-34 characteristic conserved amino acid residues Ile91, Thr90, Trp111, Trp24, Asp34, and Thr28 identified in the FIP-*fve* C-terminal region, respectively. As described above, examination of the surface of the cleft of CBM-34 reveals the presence of several key residues, which are also conserved in the six FIP members (Figure 1C). Among these conserved amino acids, the only variants in all six sequences were Thr28 in FIP-*fve* and Arg29 in GMI, respectively (Figure 1C).

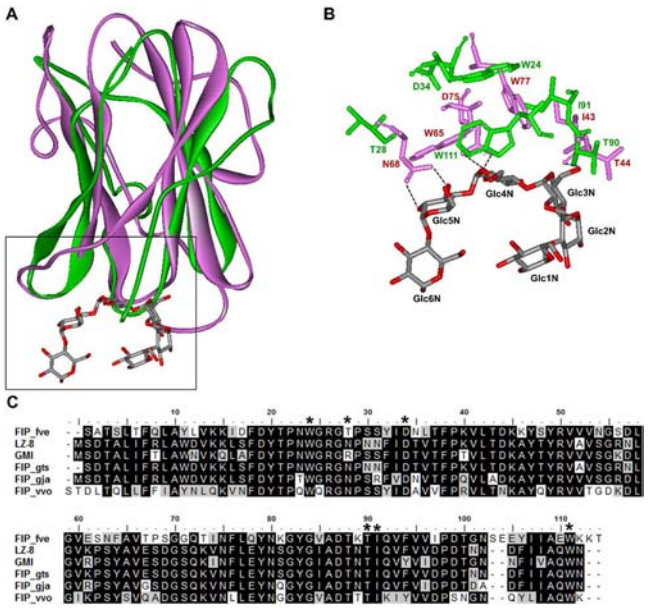

Figure 1. Overall structural comparison between Ig-like domain of FIP-*fve* and domain N of *T. vulgaris* R-47 α-amylase I. (A) Schematic X-ray structures of FIP-*fve* molecule (green, PDB code 1OSY) with domain N of *T. vulgaris* R-47 α-Amylase I (red, PDB code 1UH4). Molecules overlapped in the folds of Ig-like structures, as determined using the "magic fit" algorithm of the SwissPDB viewer program.[31] The box represents the interaction between granule starch and CBM-34 of α-amylase I. The CBM-34 structure on FIP-*fve* and TVAI α-amylase comprises residues 15−114 and 1−121, respectively. The Accelrys ViewerLite 5.0 program was used to render the modeled structure. (B) Closed view of interactions between granule form starch molecule (wire-style model) and molecular surface saccharide-binding site residues of Ig-like domain of FIP-*fve* and domain N of TVAI α-amylase are numbered and shown as green and red sticks, respectively, in an orientation similar to that in (A). Selected hydrogen-bonding interactions between substrates and CBM-34 are marked as dotted lines. (C) Multiple sequence alignments of FIP-*fve* (P80412), LZ-8 (P14945), GMI (3KCW), FIP-*gts* (AA33350), FIP-*gja* (AAX98241), and FIP-*vvo* were calculated using the program CLUSTALW.[32] Numbers above sequences correspond to FIP-*fve*. Asterisks (*) indicate key residues involved in the carbohydrate binding module. This figure was created using BioEdit program; identical amino acids are represented by black shading and similar amino acids by gray shading.[33]

α-Amylase and Starch Affect the Immunomodulatory Activity of FIP-*fve* in hPBMCs. IFN-γ is believed to play a pivotal role in the FIP-*fve*-mediated immune response, leading to subsequent priming of T helper cell type 1 (Th1) differentiation.[1,10] The authors' earlier study established that 100 μg/mL FIP-*fve* stimulates hPBMCs that are composed of heterogeneous immune cells to secrete maximal amounts of IFN-γ cytokine.[10] The structural homology data suggest a strong similarity between FIP-*fve* and TVAI α-amylase molecules in their sharing of similar CBMs that interact with common granular starch-like ligands, whereas the FIP-*fve* protein activates the human peripheral blood lymphocytes (Figure 1B). Figure 2 presents relevant results that were obtained after hPBMCs that had been incubated for 48 h were cotreated with FIP-*fve* and the aforementioned serial concentrations of amylases, inactive amylases, or starch. Supernatants were harvested, and IFN-γ production was analyzed by ELISA. The methylthiazoltetrazolium (MTT) assays provided no evidence of cytotoxic or proliferation effects following treatment of hPBMCs with amylases and starch under experimental conditions (Supporting Information, Figure S2). FIP-*fve* induced the production of IFN-γ in significantly reduced manners and was not affected by *Bacillus* sp. α-amylase at concentrations of <0.01 mg/mL (Figure 2A). In contrast, the in vitro immunomodulatory activity of FIP-*fve* was reduced by 0.01 mg/mL α-amylase, which comprises appended non-catalytic CBM-34 structure, but not by 0.01 mg/mL *Hordeum vulgare* β-amylase, which contains only a single catalytic glycoside hydrolase (GH) family 14 domain (Figure 2B, lanes 4 and 5). The reduction of the immunomodulatory activity of FIP-*fve* by α-amylase is eliminated by autoclave treatment, which perturbs the native structure of the enzyme (Figure 2B, lane 6). The inhibition of immunomodulatory activity of FIP-*fve* by α-amylase when cotreated with starch, which is a potential ligand, but not by β-amylase is consistent with above results (Figure 2B, lane 8 and 9). The results of ligand competitive assay from both α- and β-amylases reveal the enzymatic activity domains not contributing to the effect on FIP-*fve* immunomodulatory abilities; the noncatalytic domain of glycoprotein binding on α-amylase provided a competitor role. On the basis of these observations the putative CBM-34 on FIP-*fve* could be completed by a similar CBM structure, for example, TVAI α-amylase, with substrate binding ability relative to its immunomodulatory activity (Figure 2).

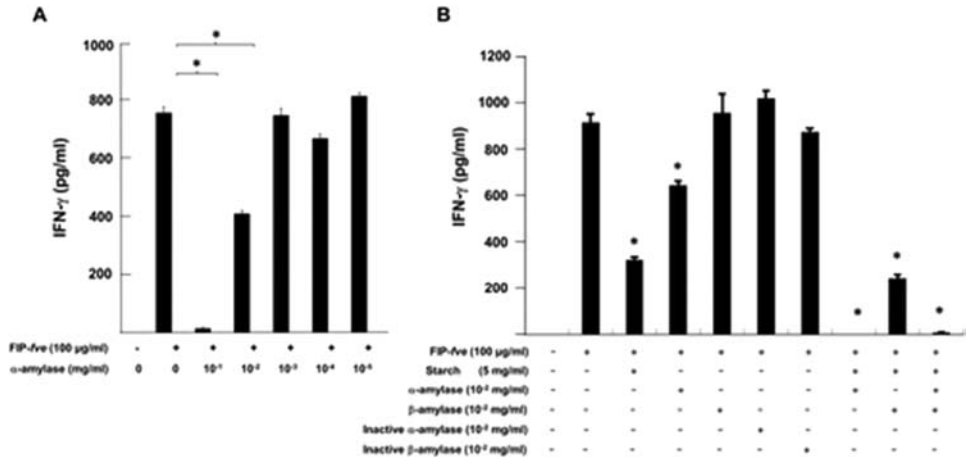

Figure 2. Effects of starch, α-amylase, and β-amylase on native FIP-*fve*-induced secretion of IFN-γ in hPBMCs. Cultured hPBMCs (2×10^6 cells/mL, 1 mL/well) were cotreated with native FIP-*fve* (100 μg/mL), and various concentrations of (A) α-amylase (serial dilutions from 0 to 10^{-5} mg/mL) and (B) were treated with/without active or autoclaved inactive α- and β-amylase (0.01 mg/mL) or with/withou starch (5 mg/mL) in RPMI 1640 that had been supplemented with 5% FBS for 48 h. Conditioned media were subjected to ELISA to measure amounts of secreted IFN-γ. Vehicle controls using distilled water data are represented at each lane 1. The data are represented the mean ± SD from triplicate experiments. Asterisks (*) on top indicate a significant difference from the control group with a calculated $P < 0.05$.

Table 1. Effects of Site-Directed Mutagenesis of the CBM Family 34 Structure of rFIP-*fve* on Immunomodulatory and Hemagglutinating Activities

	IFN-γ production in purified recombinant protein-stimulated hPBMCs[a]		
amino acid substitution in rFIP-*fve*[b]	50 μg/mL	200 μg/mL	hemagglutinating activity of purified protein[c] (μg/mL)
wild type	411.58 ± 11.78 (100)	749.78 ± 2.46 (100)	0.25
W24G	0.00 ± 0.58 (0)	0.00 ± 1.70 (0)	>250
T28G	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)	>250
T28N	249.54 ± 2.66 (61)	676.24 ± 3.89 (90)	0.25
D34G	0.00 ± 2.14 (0)	0.00 ± 3.89 (0)	>250
T90A	148.37 ± 14.10 (36)	251.57 ± 7.08 (34)	0.5
I91A	7.56 ± 2.61 (1.8)	7.72 ± 2.26 (1.0)	>250
W111G	0.00 ± 5.66 (0)	1.56 ± 1.30 (0.2)	>250

[a]Immunomodulatory activity was detected as units of the IFN-γ production from stimulated-hPBMCs (50 and 200 μg/mL) for 48 h. The IFN-γ was measured by ELISA. Data shown are the mean ± SD of triplicate experiments, and a percentage of the wild-type activity is given in parentheses. [b]The CBM-34 structure in rFIP-*fve* comprises residues 15−121. [c]Serially diluted recombinant rFIP-*fve* in 100 μL was added to a mixture containing 100 μL of 2% human red blood cells in PBS. The degrees of hemagglutination were recorded at 1.5 h after incubation. The original data are presented in Figure S1 of the Supporting Information.

Putative CBM-34 on FIP-*fve* Is Essential for Its Immunomodulatory and Hemagglutinaton Activities. The noncatalytic CBMs already signify these key residues belong to the granular starch binding site using the TVAI α-amylase for comparison.[18] On the basis of the three-dimensional structure that is superimposed and with a view to establishing a potential working model, two unique characteristics were evaluated in Figure 1B: (i) Thr28, Asp34, and Thr90 residues on the FIP-*fve* interact with the external surface of the coiled helix of starch via putative hydrogen bonds. (ii) Trp24 with Trp111 hydrophobic residues undergoes putative hydrophobic stacking with the saccharide units for recognizing the sugar units that have the relatively rigid helical structures of starch (Figure 1B). The CBM-34 key residues in FIP-*fve* are essential for the activities of immunomodulatory and hemagglutination. To map the potential binding site of ligands within FIP-*fve*, we examined the substitutions of six amino acid residues in the FIP-*fve* by introducing appropriate mutations using oligonucleotide primers and PCR with pGEX4T-I-FIP-*fve* as a template (Supporting Information, supplemental Table S1). The specificity of the site-directed mutations and the expression of rFIP-*fve* in Stratagene *E. coli* XL10-GOLD ultracompetent cells were verified by DNA sequencing analysis and SDS-PAGE, respectively (data not shown). Replacement of Trp24 by glycine, Thr28 by glycine, Asp34 by glycine, Ile91 by alanine, and Trp111 by glycine in treatments with 50 or 200 μg/mL rFIP-*fve* resulted in the production of little or no IFN-γ in the stimulated hPBMCs and the loss of hemagglutination activity in human red blood cells (Table 1). The replacement of each of these residues considerably weakened the immunomodulatory and hemagglutination activities below those of the wild-type FIP-*fve*. Substitution of alanine for Thr90 yielded a protein with only 35% of the activity of the wild type. However, the replacement of tThr28 with asparagine that was conserved in other FIPs, including LZ-8, had much less effect on the immunomodulatory and hemagglutination activities in vitro (Table 1). These results suggest the key residues of Trp24, Asp34, Ile90, and Trp111 in the CBM-34 domain are highly significant for the substrate binding ability relative to its immunomodulatory activity. It is important to note that in the case of single-residue chemical modifications of Trp24 and Trp111, using 2,4-

Table 2. Ligand Specificity of FIP-*fve* Determined by Binding Competition Assay Using a Library of Mono-, Di-, Oligo-, and Polysaccharides Cotreated with FIP-*fve*-Induced IFN-γ Secretion in hPBMCs

ligand	linkage	branch	dose	IFN-γ release (pg/mL)	binding competition assay[a]
untreated	–	–	0	869 ± 25	–
glucose (mM)	–	–	30	921 ± 40	–
			10	755 ± 27	
			2	800 ± 38	
galactose (mM)	–	–	30	890 ± 27	–
			10	924 ± 51	
			2	888 ± 9	
mannose (mM)	–	–	30	681 ± 24	–
			10	795 ± 4	
			2	842 ± 2	
mannose-6-phosphate (mM)	–	–	30	698 ± 16	–
			10	707 ± 8	
			2	725 ± 13	
glucose-6-phosphate (mM)	–	–	30	1156 ± 98	–
			10	916 ± 37	
			2	725 ± 13	
N-acetylgalactosamine (mM)	–	–	30	372 ± 3	+
			10	703 ± 32	
			2	1007 ± 15	
N-acetylglucosamine (mM)	–	–	30	713 ± 40	–
			10	759 ± 1	
			2	866 ± 16	
N-acetylneuraminic acid (mM)	–	–	30	<16	+++
			10	163 ± 4	
			2	793 ± 4	
maltose (mM)	α-1−4	–	30	67 ± 1	++
			10	435 ± 4	
			2	699 ± 15	
sucrose (mM)	α-1−4	–	30	502 ± 25	–
			10	657 ± 25	
			2	787 ± 32	
maltotriose (mM)	α-1−4	–	30	16 ± 1	+++
			10	361 ± 11	
			2	719 ± 1	
cyclodextrin (mM)	α-1−4	–	30	<16	+++
			10	167 ± 1	
			2	912 ± 2	
dextrin (mg/mL)	α-1−4	0−1 branch/molecule α-1−6	10	<16	+++
			5	335 ± 9	
			2	735 ± 1	
starch (mg/mL)	α-1−4	1 branch/2−30 residues α- 1−6	10	135 ± 5	++
			5	462 ± 11	
			2	812 ± 4	
glycogen (mg/mL)	α-1−4	1 branch/10 residues α-1−6	1	754 ± 20	–
			0.5	923 ± 4	

Table 2. continued

ligand	linkage	branch	dose	IFN-γ release (pg/mL)	binding competition assay[a]
			0.1	731 ± 4	

[a]The following symbols are used: −, no detectable competition; +, weak competition (the highest concentration of carbohydrates inhibits >50%); + +, significant competition (the second higher concentration of carbohydrates inhibits >50%); +++, strong competition (the highest concentration of carbohydrates inhibit >80%).

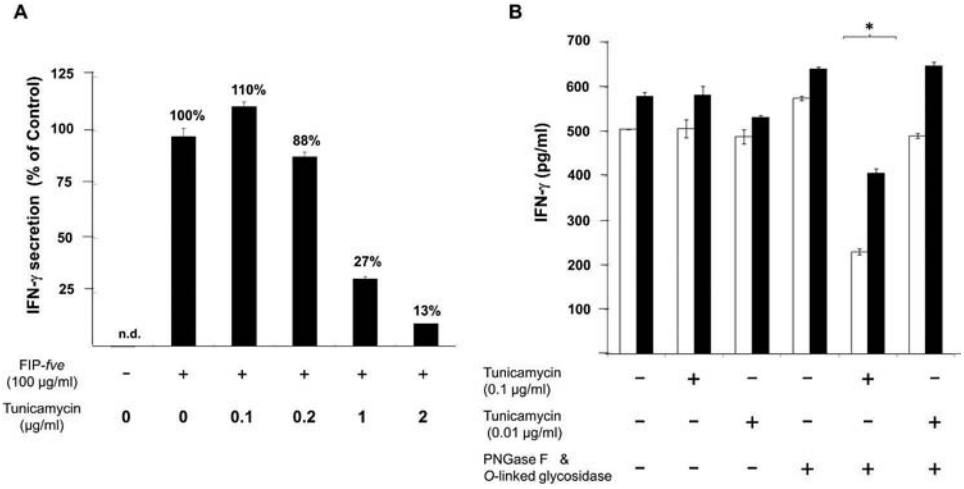

Figure 3. Effects of tunicamycin, *O*-linked glycosidases, or PNGase F on native FIP-*fve*-induced secretion of IFN-γ in hPBMCs. (A) hPBMCs (2×10^6 cells/mL, 1 mL/well) were treated with native FIP-*fve* (100 μg/mL) as 100% of control and various concentrations of tunicamycin 0 μg/mL (lane 2, control), 0.1 μg/mL (lane 3), 0.2 μg/mL (lane 4), 1 μg/mL (lane 5), and 2 μg/mL (lane 6) for 48 h. Results are presented as the mean ± SD form triplicate data. (B) Bars indicate concentrations of IFN-γ (pg/mL) secreted from cultured hPBMCs (2×10^6 cells/mL, 1 mL/well) that had been pretreated with tunicamycin (0, 0.1, or 0.01 μg/mL) for 16 h before they were treated for 4 h with the deglycosylation enzymes *O*-linked glycosidase (8×10^4 units/mL) and PNGase F (10^3 units/mL). Then (□) 50 μg/mL or (■) 100 μg/mL native FIP-*fve* was added to RPMI 1640 that was supplemented with 5% FBS for 48 h. Conditioned media were subjected to ELISA to measure amounts of secreted IFN-γ. Data are presented as the mean ± SD from triplicate experiments. Asterisks (*) on top indicate a significant difference from the control group with a calculated $P < 0.05$.

dihydroxybenzylbromide significantly reduced hemagglutination activity. [15]

Ligand Specificity of FIP-*fve*. The CBMs are classified into three subtypes on the basis of their modes of interaction and ligand specificity.[12] To classify FIP-*fve* further, the potential binding substrates were identified by binding competition assay using the FIP-*fve*-stimulated hPBMCs-induced IFN-γ production (Table 2). Several previous crystallographic characterizations of the substrate-binding sites of CBM-34 have been performed in the loops that connect the two β-sheets.[12] The CBM-34 of TVAI α-amylase was also shown to bind to the granular starch.[18] To characterize in more detail the candidate ligands for binding specificity of FIP-*fve*, the binding of the protein to polysaccharides and oligosaccharides was evaluated using the FIP-*fve* on IFN-γ production. The data presented in Table 2 reveal that the capacities of protein module to bind to a broad range from monosaccharide *N*-acetylneuraminic acid (sialic acid) to oligosaccharides maltotriose, cyclodextrin, and dextrin were assessed as "strong competition" mode by binding competitive assay, proposing that the affinity to terminal sialic acid-containing glycoconjugates, two or more unbranched α-1,4-linked sugar units, and even cyclic form substrate was required for interactions of FIP-*fve*. In the other hands, disaccharide maltose and water-soluble polysaccharide starch also demonstrate "significant competition" mode with affinities to a degree of unbranched α-1,4-linked polysaccharides. In the presence of monosaccharides D-glucose, D-galactose, mannose, mannose-6-phosphate, glucose-6-phosphate, *N*-acetylglucosamine, disaccharide sucrose, or branched polysaccharide glycogen no inhibition effect of immunomodulation was observed. The stoichiometry of binding was unity for multiple residues (in the oligosaccharides), suggesting that a general property of FIP-*fve* is an extended ligand-binding site groove, belonging to "glycan-chain-binding" CBM that comprises several binding sites that can accommodate the individual sugar units of the stretched glucose residues that are linked by a particular combination of β-1,4-glycosidic bonds. Evidence also reveals that polar interactions importantly determine the affinity and enable ligand recognition (Figure 1B). In the CAZy database, one classification based on ligand−substrate binding abilities has been defined, where CBMs are grouped into three subcategories. Type A "surface-binding" CBMs show little or no affinity for soluble carbohydrate. Type B "glycan-chain-binding" CBMs are described as grooves and comprise several subsites able to accommodate the individual sugar units of the polymeric ligands. Type C "small sugar-binding" CBMs revealed a lectin-like property of binding optimally to mono-, di-, or trisaccharides.[19] The data in Table 2 confirm that putative CBM-34 in the FIP-*fve* C-terminal region is a type B CBM on the basis of its substrate specificity. Complex bisected triantennary *N*-glycans on the cell membrane surface may be a candidate molecule for interaction with FIP-*fve*.

FIP-*fve*-Induced IFN-γ Secretion in hPBMCs Requires Cell Surface Glycans. To characterize the ligands on hPBMCs that are used by FIP-*fve* to stimulate the release of IFN-γ cytokine, hPBMCs were treated with tunicamycin to inhibit *N*- and *O*-linked de novo glycosylation combined with PNGase F and *O*-linked glycosidase to remove *N*-linked and *O*-

linked glycans, respectively, from cell surface glycoprotein. Staining with FITC-labeled ConA lectin reproducibly verified the effects of treatment with tunicamycin and glycosidase enzymes on hPBMCs' surface glycoproteins (Supporting Information, Figure S3), to exclude the possibility that tunicamycin exhibits a broadly inhibitive mechanism that interferes with the induction by the original FIP-*fve* of the release of IFN-γ. The data demonstrate clearly that 0.1 μg/mL tunicamycin-treated hPBMCs did not influence the induction of IFN-γ production by FIP-*fve* (Figure 3A). However, inhibiting the de novo synthesis of glycans and cotreatment with PNGase F and *O*-linked glycosidase efficiently reduced the immunomodulatory activity of FIP-*fve* toward hPBMCs (such that the amount of IFN-γ released was 30% that the control; $P < 0.05$). Treatment with tunicamycin or glycosidase alone had no significant effect (Figure 3B). Taken together, these results demonstrated that glycans that are *N*- and/or *O*-linked to membrane receptors are essential to the binding of FIP-*fve* and stimulate the production of IFN-γ cytokine in hPBMCs (Figure 3).

DISCUSSION

The golden needle mushroom is a popular edible mushroom in Asia and is reportly effective in promoting human health. It has great potential for commercial development and use in pharmaceutical products.[1,20] This investigation reveals that a putative CBM-34 on the FIP-*fve* recognizes glycans which are located in surface membranes of the hPBMCs, and such recognition is essential to immunomodulatory activity. Although the ligand-binding site of CBM-34 is historically associated with the loops that connect the β-sheets of these proteins, the association holds only for proteins that recognize the internal regions of rigid helical-coiled polysaccharide.[18] The binding competition assay data herein reveal that FIP-*fve* binds more tightly to complex polysaccharides than to mono- or divalent ligands, and binding is associated with the formation of insoluble polysaccharides that are class features of avidity effects (Table 2). In previous studies not only has sucrose stabilized protein and preserved its immunomodulatory activity against both freezing and dehydration during freeze-drying,[21] but also the disaccharide ligand has been found not to interact with CBM-34 on the FIP-*fve*, eliminating this activity (Table 2). Additionally, sucrose more effectively stabilized at an equivalent sugar-to-protein mass ratio in aqueous solutions and in its lyophilized form.[22] The data together suggest that a sucrose-containing freeze-dried formulation could effectively preserve the immunomodulatory activity and bioactive stability of FIP-*fve* protein when it is used in food or pharmaceutical products for commercial development.

From the FIP structural viewpoint (Figure 1B), the side chain of amino acid provides two hydrogen bonding interactions with starch-like ligand. By combining the protein−ligand modeling hypothesis and experimental outcome (Table I in the Supporting Information), hydrogen bonds play an important role in the interactions between FIP-*fve* protein and carbohydrate, revealing more immunomodulatory activities by IFN-γ released assay in hPBMCs. In this study, the data reveal the binding ability of the putative CBM-34 domain on FIP-*fve* with its substrates relative to stimulated production of IFN-γ in hPBMCs (Figure 3). Previous studies have shown that house dust mites and clinically relevant allergens, the group 4 allergens from *Dermatophagoides pteronyssius* (Der p 4, GenBank ID AAD38942) and *Euroglyphus maynei* (Eur m 4, GenBank ID AAD38943), are 50% identical to both insect and mammalian α-amylases, which also contain noncatalytic starch-binding sites located outside the active site.[23] Furthermore, the enzymes of α-amylases from wheat and fungi have been also shown to be major causes of occupational asthma and dermatitis, particularly in wheat flour, in which the concentrations of these α-amylases are high.[24] This study shows α-amylase has a structure similar to that of noncatalytic CBM-34 and so may significantly interfere with the IFN-γ production by FIP-*fve* stimulated by the hPBMCs (Figure 2). Accordingly, FIP-*fve* induced a Th1-dominated allergen-specific immune response in mice by enhancing transcription of IFN-γ and thus inhibiting the development of a food allergy reaction.[5,10,25] However, α-amylase acts a native allergen to promote Th2 inflammatory response and induces IgE-mediated allergic reactions.[23b] Therefore, the immunomodulatory effects of the two structures of FIP-*fve* and α-amylases compete with each other. Thus, the involvement of structure of CBM-34 in the skewing of the Th2 immune deviation responses to those of Th1 remains to be elucidated in future studies. We noted both the N-terminal dimerization domain and the C-terminal carbohydrate interaction domain are required for FIP-*fve* immunomodulatory function. This phenomenon gives a hint about interaction with complex cell-surface carbohydrates by the C-terminal domain, gathering these immune cells together by dimer form using the N-terminal domain.

TLRs have been shown to sense proximal ligand binding and to recognize patterns in the antibacterial defenses of vertebrates.[26] The direct recognition and uptake of antigens by TLRs may play a critical role in innate host defense.[27] Many papers that address the questions concerning the activation of immunomodulatory pathways focus on the simple protein moiety of FIPs and its essential role in regulating immune responses[1] of proteinase K treated LZ-8[28] *Staphylococcus cerevisiae* that expresses recombinant LZ-8 protein,[29] or rFIP-*fve* as in this study (Table 1). As Figure 11C show, LZ-8 has a 63% protein sequence identity with FIP-*fve*. Following the discovery of Lin et al.[28], LZ-8 is through TLR-4/MD2 in signaling pathways for activation and maturation in human monocyte-derived dendritic cells. Neutralized antibodies could abolish the signaling for TLR-4/MD2-transfected HEK293 cells. However, Yeh et al.[29] demonstrated that rLZ-8 may prime macrophages using the TLR4-independnet route by fluorescence ligand binding to the surface of a TLR4-deficient macrophage. On the basis of these two inconsistent results, we propose herein an alternative hypothesis summarized in Figure 4. FIP-*fve* binds to the cell surface receptors via CBM-34 on the FIP-*fve* and directly interacts with the target substrate by binding to polysaccharides that are in close proximity with the substrates, possibly TLR4 receptor (data not shown). Another study supports the hypothesis that TLR4/MD2 are highly glycosylated proteins and that *N*-linked sites are essential to maintaining the functional integrity of this receptor. [30]

In conclusion, this study not only determined the putative CBM-34 within the C-terminal region of FIP-*fve* but also clarified the specificity of the ligands toward FIP-*fve*. Additionally, FIP-*fve*-induced physiological immunomodulatory effects depend on the interaction between the CBM-34 on the FIP-*fve* and ligand-like glycoproteins on the surface of hPBMCs.

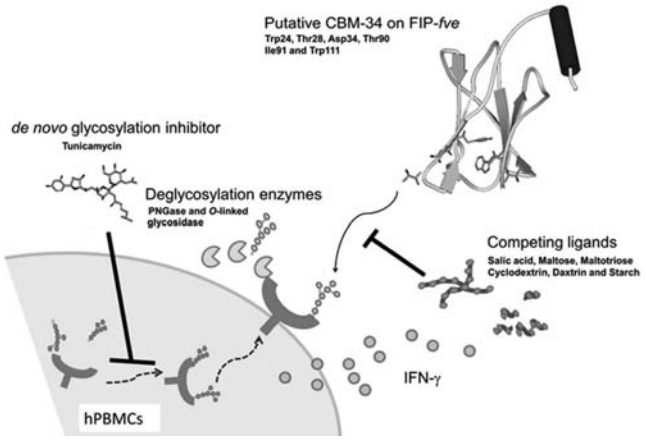

Figure 4. Proposed mechanism regulation of FIP-*fve*-induced secretion of IFN-γ in hPBMCs. The immunomodulatory activities of FIP-*fve* proteins were lost when key residues W24G, T28G, D34G, T90A, I91A, and W111G on putative CBM-34 were disrupted by site-directed mutagenesis, suppressed according to binding competition assay using putative ligands such as salic acid, maltose, maltotriose, cyclodextrin, dextrin, and starch. Tunicamycin inhibited de novo glycosylation and deglycosylation enzymes, such as PNGase and *O*-linked glucosidase. Cotreatment in hPBMCs also eliminated the secretion of IFN-γ that would otherwise have been stimulated by FIP-*fve*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Addition figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

FIP, fungal immunomodulatory protein; TLRs, toll-like receptors; IFN-γ, interferon-gamma; CBMs, carbohydrate-binding modules; hPBMCs, human peripheral blood mononuclear cells; ODTs, orally disintegrating tablets.

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