Dimerization of the N-terminal Amphipathic α-Helix Domain of the Fungal Immunomodulatory Protein from *Ganoderma tsugae* (Fip-gts) Defined by a Yeast Two-hybrid System and Site-directed Mutagenesis

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A fungal immunomodulatory protein (Fip-gts) was purified from *Ganoderma tsugae*. The DNA encoding Fip-gts was isolated from a cDNA library of *G. tsugae* by reverse transcriptase-polymerase chain reaction. The complete amino acid sequence of Fip-gts, deduced from the nucleotide sequence of the cDNA, was the same as the N-terminal sequence of *Ganoderma lucidum*. Recombinant Fip-gts was expressed as a glutathione S-transferase fusion protein in *Escherichia coli* with a yield of 20 mg/liter of culture. Recombinant Fip-gts, purified to homogeneity, had the same blast formation stimulatory activity to human peripheral blood lymphocytes as native Fip-gts.

The yeast two-hybrid system and site-directed mutagenesis were used to determine whether dimerization of Fip-gts occurred. Deletion analysis of the N-terminal amphipathic α-helix domain of Fip-gts identified a sequence of about 10 amino acids responsible for inducing immunomodulatory activity. Non-functional Fip-gts deletion mutants did not form dimers, whereas wild type Fip-gts did as determined by gel filtration. A mutant with deletions at Leu-5, Phe-7, and Leu-9 lost the amphipathic characteristics of the N-terminal domain and the ability to form dimers as well as its immunomodulatory activity.

Fusion of Fip-gts with the DNA binding and the transactivation domains of GAL4 resulted in the activation of the *lacZ* activator gene, indicating the interaction of Fip-gts with it itself. The dimerization domain was further defined by analyzing the ability of the N-terminal 13 amino acids or Leu-5, Phe-7, and Leu-9 deletion mutants of Fip-gts to interact with the wild type Fip-gts. These experiments confirmed the N-terminal amphipathic α-helix as the dimerization domain and suggest that the dimerization of Fip-gts may play an important role in Fip-gts immunomodulatory activity.

A new family of fungal immunomodulatory proteins (Fips) has recently been established. Four Fips have been isolated and purified from *Ganoderma lucidum, Flammulina velutipes, Volvariella volvacea*, and *Ganoderma tsugae* and designated as LZ-8, Fip-fve, Fip-ovo, and Fip-gts, respectively (1–3).

Fips are mitogenic in vitro for human peripheral blood lymphocytes (hPBLs) and mouse splenocytes, and induce a bell-shaped dose-response curve similar to that for lectin mitogens. Activation of hPBLs with Fips results in the increased production of IL-2, IFN-γ, and tumor necrosis factor-α molecules associated with ICAM-1 expression (2, 3). Fips can also act as immunosuppressive agents; in vivo these proteins can prevent systemic anaphylactic reactions and significantly decrease footpad edema during the Arthus reaction (1, 2). LZ-8 can also suppress autoimmune diabetes in young female non-obese diabetic mice (4). Furthermore, LZ-8 has a significant effect on cellular immunity, as shown by the increase of graft survival in transplanted allogeneic mouse skin and allogeneic pancreatic rats (5) without producing the severe toxic effects on pancreatic islets associated with prednisolone and cyclosporin A treatment (6, 7).

The Fips identified to date have a molecular mass of 13 kDa and share high amino acid sequence homology. Alignment of these proteins revealed 44% identity and 42% homology for approximately 110 amino acid residues. The Fips are rich in β-structure by secondary structure prediction, and contain seven β-strands, two α-helices, and one β-turn.

The amphipathic α-helix is a common structural motif, which is found in a number of functional proteins or peptides and is involved in various functions such as glucon binding to its receptor, plasma apolipoproteins solubilization of lipids, anti-microbial peptide disintegration of bacterial cells, and signal peptide targeting to mitochondria (8). In the present study, we isolated a fungal immunomodulatory protein, Fip-gts. The cloned cDNA of Fip-gts was expressed in *Escherichia coli*, and a putative amphipathic α-helix was identified at the N-terminal 13 amino acid residues, which would be essential for the formation of the active Fip-gts dimer. We employed a yeast two-hybrid system (9, 10) and site-directed mutagenesis to examine Fip-gts dimerization. Plasmids in which Fip-gts was fused with both the GAL4 DNA binding domain and transactivation domain were constructed, and these plasmids were transformed together into yeast to activate the *lacZ* indicator gene, to examine the interaction of Fip-gts with itself. We also assayed the ability of Fip-gts deletion mutants to interact with the wild type Fip-gts. These studies map the dimerization domain to the amphipathic N terminus of Fip-gts, which is responsible for inducing immunomodulatory activity. The dimerization of wild type Fip-gts was verified by chemical cross-linking with glutaraldehyde.
EXPERIMENTAL PROCEDURES

Materials—Matchmaker Two-Hybrid System 2 was purchased from CLONTECH (Palo Alto, CA). Yeast strain Y187 (MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met+, gal80Δ, URA3: GAL1USA-GAL1TATA-lacZ) was used for assaying protein-protein interactions. Y187 has the upstream activating and TATA sequences of the GAL-1 promoter fused to the lacZ gene such that LacZ is responsive to GAL4 transcriptional activation. The enzyme-linked immunosorbent assay kits for measuring human IL-2 and IFN-γ were used to amplify Fipt-gts and sequence on both strands at least twice. E. coli previously with DNA fragment was ligated into vector pBS(1). Lithium acetate method (16, 17). Colonies of this transformant were isolated from the mycelia of G. tsugae by homogenization in 4 M guanidium thiocyanate. Poly(A)+ RNA was recovered with messenger affinity paper, and total cDNA was synthesized by using avian myeloblastosis virus reverse transcriptase followed by DNA polymerase (11). Two primers were prepared based on the amino acid sequence of LZ-8 isolated from G. lucidum (12). Primer A encodes the first 8 N-terminal amino acid residues of LZ-8, and primer B encodes the last 8 C-terminal amino acid residues.

Primer A: 5′-TCCGACCACCTGCTTATGCTTCAG-3′ (forward)

Primer B: 5′-TTAGTTCCACCTGGGGCAGATGAGA-3′ (reverse)

PCR was carried out to synthesize the Fipt-gts cDNA by using primer A and primer B. The amplified DNA was purified by agarose gel electrophoresis, and DNA bands were stained with ethidium bromide and then purified by agarose gel electrophoresis at 60 V for 1 h. The solution containing the DNA fragment was treated with calf intestine phosphatase. The DNA fragment was precipitated by phenol/chloroform (1:1), and the DNA fragment was precipitated by poly(A)+ RNA was isolated from the mycelia of G. tsugae by homogenization in 4 M guanidium thiocyanate. Poly(A)+ RNA was recovered with messenger affinity paper, and total cDNA was synthesized by using avian myeloblastosis virus reverse transcriptase followed by DNA polymerase (11). Two primers were prepared based on the amino acid sequence of LZ-8 isolated from G. lucidum (12). Primer A encodes the first 8 N-terminal amino acid residues of LZ-8, and primer B encodes the last 8 C-terminal amino acid residues.

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Cloning and Nucleotide Sequencing of Fipt-gts cDNA—Total cellular RNA was isolated from the mycelia of G. tsugae using TRIZOL reagent. 50 μg of total RNA were used for cDNA synthesis, followed by PCR amplification. The PCR products were sequenced using the Sequenase kit (U. S. Biochemical Corp.).

Construction and Expression of Fipt-gts Deletions—Various primers were used to amplify Fipt-gts deletion mutants. All forward primers contained BamHI sites and the reverse primers contained EcoRI sites for ligation into the expression vector, pGEX-2T (14). The resulting construct, pGT-Fipt-gts, contained both the GST and Fipt-gts genes. The mutant primers are shown below.

Primer C (forward): 5′-AGGATCTCGAGACCTGCGCTTTC-ATCTCCAGG-3′

Primer D (reverse): 5′-AGGATCCTTTAGTTCCACCTGGGGCAGATGAGA-3′

Primer F ΔN1–13 (forward): 5′-AGGATCTTGCGCTTACCTGCTG-3′

Primer F ΔN1–13 (reverse): 5′-AGGATCTTGCGCTTACCTGCTG-3′

Primer G ΔL5/P7/L9 (forward): 5′-AGGATCTCGAGACCTGCGCTTTC-ATCTCCAGG-3′

Primer H ΔL5 (forward): 5′-AGGATCTCGAGACCTGCGCTTTC-ATCTCCAGG-3′

Primer I ΔF7 (forward): 5′-AGGATCTCGAGACCTGCGCTTTC-ATCTCCAGG-3′

Primer J ΔL9 (forward): 5′-AGGATCTCGAGACCTGCGCTTTC-ATCTCCAGG-3′

Primer K ΔL5/F7 (forward): 5′-AGGATCTCGAGACCTGCGCTTTC-AGGTCCTCGG-3′

Primer L Δ5–7 (forward): 5′-AGGATCTCGAGACCTGCGCTTTC-CTGCGCTTGGAG-3′

For the expression of recombinant GST-Fipt-gts and mutant fusion proteins, the recombinant plasmids were introduced into E. coli strain T101 by CaCl2-mediated transformation. When the cells reached a density of 4 × 10⁹ cells/ml, they were induced by adding 0.5 mM isopropyl-1-thio-β-d-galactopyranoside and the culture was incubated for an addition 3 h. The cells were harvested by centrifugation and resuspended in 10 ml of ice-cold resin suspension buffer containing 10 mm Tris-HCl, pH 7.5, 100 mM sodium chloride, 1 mM magnesium chloride, and 1 mM dithiothreitol. The cells were treated with lysozyme (0.2 mg/ml) and then lysed by three cycles of freeze/thawing. The cell lysate was clarified by centrifugation at 20,000 × g for 20 min, and the supernatant was directly applied onto a glutathione-Sepharose 4B column (2 ml), which was equilibrated with 100 mM Tris-HCl, pH 8.0. The column was washed with 20 ml of equilibration buffer and then eluted with 5 mM reduced glutathione in the equilibration buffer to obtain the fusion protein (15). The active fractions were identified by the blast transformation stimulatory activity assay and then pooled. The fusion protein was treated with thrombin at an enzyme to substrate molar ratio of 1:100 in 50 mM Tris-HCl buffer, pH 8.0 at 25 °C for 2 h. The reaction products were applied onto a Mono Q column (1.6 mm × 50 mm), which was equilibrated with 50 mM Tris-HCl buffer, pH 8.0, and then eluted with a linear gradient from 0 to 0.3 M sodium chloride in the same buffer. The active fractions were detected in the first peak as assayed by the blast transformation stimulatory activity described previously (2).

Construction of pAS2-1-Fipt-gts and pACT2-Fipt-gts—Yeast shuttle vectors pAS2–1 and pACT2, containing the GAL4 DNA binding domain and GAL4 activation domain, respectively; pVA3 (the p53 gene) and pT7D (SV40 large T antigen) were obtained from CLONTECH. Fipt-gts cDNA was amplified by PCR using pcFip-gts as template, primer M encoding the first 8 N-terminal amino acid residues of a BamHI restriction site, primer N encoding the last 8 C-terminal amino acid residues with a PstI restriction site, and primer O encoding the last 8 C-terminal amino acid residues with an EcoRI restriction site. To obtain the Fipt-gts gene without its N-terminal 13 amino acid residues or Leu-5, Phe-7, and Leu-9, primers P and Q with a BamHI site were used as forward primers. PCR products run on an 1% agarose gel, eluted from the gel by electrophoresis, and ligated to the vectors, pAS2–1 and pACT2, respectively.

Primer M (forward): 5′-GGGATCCGTTACCGACCTGCGCTTTC-ATCTCCAGG-3′

Primer N (reverse): 5′-GGGATCCGTTACCGACCTGCGCTTTC-ATCTCCAGG-3′

Primer O (reverse): 5′-GGGATCCGTTACCGACCTGCGCTTTC-ATCTCCAGG-3′

Primer P ΔL5/F7/L9 (forward): 5′-GGGATCCGTTACCGACCTGCGCTTTC-ATCTCCAGG-3′

Primer Q ΔN1–13 (forward): 5′-GGGATCCGTTACCGACCTGCGCTTTC-ATCTCCAGG-3′

Primer R ΔN1–13 (forward): 5′-GGGATCCGTTACCGACCTGCGCTTTC-ATCTCCAGG-3′

All constructs were sequenced to confirm the fidelity of the wild type Fipt-gts in pAS2–1 and wild type Fipt-gts or various deletion mutants in pACT2. Sequencing was performed using the Sequenase kit (U. S. Biochemical Corp.).

Transformation and Positive Clone Assay—pAS2–1-Fipt-gts, the two-hybrid DNA binding vector, was transformed into Y187 cells by the lithium acetate method (16, 17). Colonies of this transformant were Trp+ . The Y187 transformant was grown overnight in SD/Tryptophan selection medium to ensure the presence of pAS2–1-Fipt-gts in every cell. The overnight culture was transformed with 0.1 μg of wild type or Fipt-gts mutant inserted into the pACT2 two-hybrid activation vector. Double transformed cells were incubated in SD/Tryptophan plates at 30 °C for 5 days.

Yeast containing both GAL4 binding and activation domain fusion proteins were analyzed for β-galactosidase activity using filter and liquid assay methods. For the filter assay method, the positive yeast colonies were transferred to nitrocellulose filter and submerged in liquid nitrogen for 10 s to permeabilize the cells. The nitrocellulose filter was then placed on filter paper, which had been treated with Z-buffer.
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FIG. 1. Amino acid sequence and secondary structure of Fip-gts. The 344-bp fragment was sequenced by the dyeodeoxy chain termination method. The amino acid sequence was deduced from the cDNA nucleotide sequence using the single code for amino acids. The secondary structure of Fip-gts was predicted by the method of Garnier et al. (21).

FIG. 2. SDS-PAGE analysis of GST fusion protein and recombinant Fip-gts. Samples of purified fusion protein and recombinant Fip-gts were analyzed by 12% SDS-PAGE and Coomassie Blue staining. Lane M, molecular size markers from Pharmacia: bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lactoalumin (14.4 kDa); lane 1, crude extract; lane 2, recombinant GST-Fip-gts fusion protein; lane 3, recombinant GST-Fip-gts fusion protein after hydrolysis with thrombin; lane 4, purified recombinant Fip-gts; lane 5, GST; lane 6, native Fip-gts. The culture was harvested and the amounts of IFN-γ or IL-2 were determined by enzyme-linked immunosorbent assay.

RESULTS

Cloning and Nucleotide Sequence of Fip-gts cDNA—A 330-bp DNA fragment from the PCR amplification of G. tsugae cDNA was ligated into SmaI-linearized pBS (+). Three positive clones containing the 330-bp DNA fragment were isolated. The vector was transformed into E. coli strain TG1, and the purified recombinant plasmids were used as template for direct DNA sequence analysis. All three clones contained an open reading frame of 330 bp, which encoded 110 amino acids. The complete amino acid sequence of Fip-gts was deduced from the nucleotide sequence of Fip-gts cDNA (Fig. 1). Fip-gts has the same amino acid sequence as LZ-8.

Expression and Purification of Recombinant Fip-gts and Mutants—To study the structure and function of Fip-gts, we expressed the Fip-gts in E. coli. The soluble recombinant fusion protein of the expected molecular mass was purified on a glutathione affinity column. The GST portion of the recombinant Fip-gts fusion protein was cleaved with thrombin, and Fip-gts was purified on a Mono Q column. The yield of recombinant Fip-gts was about 20 mg/liter of induced culture. The recombinant Fip-gts and its mutant proteins contain two extra amino acid residues, Gly-Ser, at their N termini, which were part of the thrombin-sensitive linker. Recombinant Fip-gts and the mutant proteins appeared homogeneous on 12% SDS-PAGE gels (Fig. 2).

Yeast Two-hybrid System—Wild type Fip-gts cDNA was fused with the DNA binding domain and the activation domain of GAL-4 to examine whether homodimers could form. When these plasmids were co-transformed into Y187 cells, the lacZ indicator gene was activated as shown by increased β-galactosidase activity (Table 1). β-Galactosidase activity was not detected when yeast cells were transformed with the DNA binding domain of GAL-4 fused to Fip-gts (pAS2–1-Fip-gts) or the activation domain of GAL-4 fused to Fip-gts (pACT2-Fip-gts). Activation of lacZ was observed in positive control Y187 cells in which p53 was fused to the GAL-4 binding domain and the SV40 large T cell antigen was fused to the activation domain.

To prove the structure that is essential for the self-interaction of Fip-gts, the ability of the various Fip-gts mutant proteins to interact with wild type Fip-gts was evaluated. Fip-gtsΔN1–13 fused to the transactivation domain (pACT2-Fip-gtsΔN1–13) was unable to associate with the wild type Fip-gts mutant proteins to interact with wild type Fip-gts was evaluated. Fip-gts, pACT2-Fip-gtsΔN1–13 was incapable of association with the wild type Fip-gts (pAS2–1–Fip-gts). Yeast
β-Galactosidase activity and filter color of fusion constructs

| Protein fused to GAL4 domain | DNA binding | β-galactosidase activity | Filter color |
|-----------------------------|-------------|-------------------------|-------------|
| pVA3—a                      | pTD1—*a     | 20.8 ± 1.5 Blue         |             |
| pAS2–1–FIP-gts              | None        | 0.1 ± 0.01 White        |             |
| None                        | pACT2-FIP-gts| 0.1 ± 0.01 White        |             |
| None                        | pACT2-ΔN1–13| 0.2 ± 0.02 White        |             |
| None                        | pACT2–ΔL5/F7/L9| 0.2 ± 0.01 White    |             |
| pAS2–1–FIP-gts              | pACT2-FIP-gts| 1.9 ± 0.2 Blue         |             |
| pAS2–1–FIP-gts              | pACT2-ΔN1–13| 0.2 ± 0.02 White        |             |
| pAS2–1–FIP-gts              | pACT2–ΔL5/F7/L9| 0.3 ± 0.02 White    |             |

* The plasmids pVA3 and pTD1 contain murine p53 and SV40 large T-antigen, respectively, interact strongly, and serve as a positive control.

transformed with the activation domain of GAL-4 fused to Fip-gtsΔN1–13 or Fip-gtsΔL5/F7/L9 by themselves failed to induce β-galactosidase activity. These results show that the N-terminal 13 amino acid residues of Fip-gts contain the essential elements necessary for the formation of homodimers.

**Chemical Cross-linking—** Chemical cross-linking experiments were carried out to demonstrate the presence of Fip-gts homodimers. Various concentrations of glutaraldehyde were added to Fip-gts for 2 h at room temperature. The reaction products were analyzed by SDS-PAGE (Fig. 3). When wild type Fip-gts was incubated with buffer alone, only monomeric Fip-gts was observed. In the presence of glutaraldehyde at concentrations higher than 20 μM, a new band was observed corresponding to a homodimer of about 26 kDa. Most Fip-gts appeared in the dimeric form at 200 μM glutaraldehyde. For the N-terminal deletion mutant, the Fip-gtsΔN1–13, only the monomeric 13-kDa species was observed at 200 μM glutaraldehyde (Fig. 3A). Cross-linked dimeric products were also not detected for Fip-gtsΔL5/F7/L9 (Fig. 3B). The formation of dimeric species was further demonstrated by gel filtration (Fig. 4). The molecular mass of wild type Fip-gts was shown to be 26 kDa, while that of the deletion mutants, Fip-gtsΔN1–13 or Fip-gtsΔL5/F7/L9, was 13 kDa.

**Induction of Cytokines—** The induction of cytokines from hPB-Ls by wild type Fip-gts or the deletion mutants was used to evaluate the effects of the deletions on immunomodulatory activity. The deletion mutants, Fip-gtsΔN1–13, Fip-gtsΔL5/F7/L9, and Fip-gtsΔ5–7 did not significantly induce IL-2 and γ-IFN, whereas mutant Fip-gtsΔN1–6 displayed 86% of the wild type Fip-gts activity. Other Fip-gts deletion mutants such as ΔL5, ΔF7, ΔL9, and ΔL5/F7 all exhibited the same activities as wild type Fip-gts.

**DISCUSSION**

Three Fips have been isolated from *F. veltipes* (2), *V. volvacea* (3), and *G. tsugae* by our laboratory and named Fip-fve, Fip-vvo, and Fip-gts, respectively. These Fips exhibit high homology in their amino acid sequences, and alignment of their sequences revealed 51 invariant amino acid residues among the three Fips (Fig. 5) (3). The amino acid sequence of Fip-gts cDNA was identical to LZ-8 isolated from *G. lucidium* (1). We demonstrated that Fip-gts can be produced as a GST fusion protein in soluble form with a relatively high yield. Pure recombinant Fip-gts was obtained by treating the fusion protein with thrombin, followed by purification on a Mono Q column based on the different pi values of GST and Fip-gts. The yield of Fip-gts was relatively high with about 20 mg/liter of culture obtained.

To study the contribution of the N-terminal 13 amino acids to the structure and function of Fip-gts, the secondary structure of Fip-gts was predicted by the method of Garnier et al. (21). Fip-gts was predicted to contain two α-helices, seven β-sheets, and one turn. The N-terminal 13 amino acid residues included
10 amino acids of the α-A-helix. Based on the method of Eisenberg et al. (22), an amphipathic structure could be constructed for Fip-gts but not for the inactive Fip-gtsΔN_{1-13} mutant. In addition, an amphipathic structure could not be drawn for the inactive Fip-gtsΔL5/F7/L9 mutant. The amphiphilicity perpendicular to the helical was quantitated by calculation of the hydrophobic moments (μH) of the wild type and mutant helices of Fip-gts (23). The μH values for the α-A-helix in the N-terminal 13 amino acids of the active mutants ranged from 0.54 (ΔL5) to 0.21 (ΔL9); for the wild type α-A-helix, the μH value was 0.43. In contrast, the μH values for inactive mutants were less than 0.1. Therefore, the amphiphilicity of the α-A-helix correlated with function and the maximum μH was at least 0.54, while the minimum ranged between 0.00 and 0.10.

Dimerization is an important process for hormones and growth factors to bind to their receptors on the cell surface and exert their activity. For example, insulin and epidermal growth factors to bind to their receptors on the cell surface and exert their activity. For example, insulin and epidermal growth factors are hormones that form homodimers for binding to cell surface receptors (8). The N-terminal α-A-helix of Fip-gts may play an important role in the formation of homodimers for binding to cell surface receptors to exert its immunomodulatory activity. The formation of homodimers could be attributed to the interaction of the hydrophobic faces of the helices. Because most of the 13 amino acid residues of the α-helix could be deleted in one or another while maintaining activity, the hydrophobic interaction may not depend on specific amino acid side chains or a specific sequence. Two-amino acid deletions (ΔL5/L7) were also dispensable for activity. However, removal of three amino acids (ΔS–7) disturbed the amphiphilicity of the α-A-helix and led to the loss of activity.

The information from the present study may be applied to the design of proteins containing a N-terminal α-helix of 10 amino acid residues to form homodimers with higher activity than the monomeric proteins. Protein engineering for the rational design and efficient preparation of homodimers will allow us to extend our understanding of the structure and function of homodimers.

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