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Identification and functional characterization of a novel fungal immunomodulatory protein from *Postia placenta*

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ABSTRACT

In this study, a previously unknown fungal immunomodulatory protein (FIP), here called FIP-ppl, was identified from the basidiomycete fungus *Postia placenta* by searching its genome sequence database using known FIPs as baits, which was the first basidiomycete FIP to be identified outside the order of edible macro fungi. The gene *FIP-ppl* was synthesized and expressed in *Escherichia coli* to produce a glutathione S-transferase (GST) fusion protein. The fusion protein was purified on a GST affinity column and the protein tag was removed using *in situ* thrombin cleavage. The purified recombinant protein (rFIP-ppl) displayed hemagglutination activity toward rabbit red blood cells but not against human red blood cells. RFIP-ppl stimulated mouse splenocyte cell proliferation and enhanced interleukin-2 (IL-2) release. Antitumor assays indicated that rFIP-ppl had significant cell proliferation inhibitory activity and apoptotic effects in human tumor cells with more pronounced inhibiting proliferation and inducing apoptotic effects on gastric tumor cells (MGC823) than against hepatoma (HepG2) cells. This study confirms an alternative means of identifying, producing, and isolating new FIPs. It may provide convenient access to FIP-ppl with potential human therapeutic applications.

1. Introduction

Fungal immunomodulatory proteins (FIPs) are a class of small proteins that share considerable sequence, structural, and functional similarity (Wang et al., 2012). As such, FIPs are classified in their own family. These proteins exhibit 57–100% sequence identity, consist of 111–114 amino acid residues with a molecular weight of 12.4–15.0 kDa. FIPs lack His, Cys and Met residues, but are rich in Asp and Val, and the N-terminal amino acids are acetylated (Li et al., 2011; Wang et al., 2012). The crystal structures of FIP-fve of *Flammulina velutipes* (PDB entry: 1OSY) (Paaventhan et al., 2003), LZ-8 of *Ganoderma lucidum* (PDB entry: 3F3H) (Huang et al., 2008) and FIP-gmi of *Ganoderma microsporum* (PDB entry: 3KCW) display an N-terminal α-helix followed by a C-terminal fibronectin III (FNIII) domain. The N-terminal α-helix stabilizes FIPs by hydrophobic interactions. The C-terminal FNIII domain belongs to the immunoglobulin-like β-sandwich fold, with two β-sheets packed tightly with seven β-strands. Structural comparison revealed that significant changes in conformation occur in the two loop regions (loop DE and loop FG) of the FNIII domain that may make up the active site (Huang et al., 2008).

FIPs have several *in vitro* biological activities. FIPs can agglutinate erythrocytes (Kino et al., 1989; Ko et al., 1995), stimulate lymphocyte mitogenesis (Haak-Frendscho et al., 1993; Murasugi et al., 1991; Van der Hem et al., 1996), promote the release of T_H1-specific cytokines (interleukin-2, IL-2; interferon-γ, IFN-γ; and tumor necrosis factor β, TNF-β) (Hsu et al., 1997), interrupt tumor cell proliferation (Chang et al., 2013) and induce tumor cell apoptosis (Liao et al., 2006, 2007; Lin et al., 2009). *In vivo*, FIPs also inhibit systemic anaphylaxis via reduction of antibody production during the Arthus reactions (Hsieh et al., 2003; Kino et al., 1991; Ko et al., 1995). FIPs show a very broad application prospect in future medical care and pharmaceutical products.

Despite their considerable primary sequence and structural conservation, FIPs are quite varied in their biological activities (Hsu et al., 1997). FIPs from *Ganoderma* spp. have been reported to display strong antitumor activities (Liao et al., 2006, 2007; Lin et al., 2009). However, FIP-fve from *F. velutipes* inhibits the anaphylactic reaction. Hsieh et al. reported that oral administration of FIP-fve can induce a T_H1-predominant allergen-specific immune response in mice and protect mice from systemic anaphylaxis-like symptoms (2003). FIP-fve may be suitable for oral immunoprophylactic applications. FIP-vvo, purified from *Volvariella volvacea*, induces one T_H2-specific cytokine interleukin-4 (IL-4) and also induces most T_H1-specific cytokines in mouse splenocytes. IL-4 induces the activation and differentiation

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of B cells, leading to the production of IgE (Hsu et al., 1997; She et al., 1998). This renders the ability of FIP-vvo to prevent systemic anaphylaxis less pronounced than that of other FIPs. Huang et al. reported that the bioactivity diversity of FIPs was caused by the structural differences in loop DE and loop FG regions (2008).

Until now, five FIPs have been extracted from *Ganoderma* spp. and identified (Bastiaan-Net et al., 2013; Kino et al., 1989; Lin et al., 1997, 2010). Other six have been found in *F. veltipes* (Ko et al., 1995), *V. volvacea* (Hsu et al., 1997), *Auricularia polytricha* (Sheu et al., 2004), *Pleurotus citrinopileatus* (Sheu et al., 2007), *Poria cocos* (Chang et al., 2009) and *Trametes versicolor* (Li et al., 2011). FIPs exhibit important bioactivities and favorable application foregrounds; nevertheless, it is time-consuming, expensive, and inefficient to extract FIPs from wild or even cultivated mushrooms. For this reason, researchers have shifted away from optimizing extraction technology and studying the functions and mechanisms of the known FIPs toward investigating and exploring new methods of obtaining previously unknown and high-producing FIPs. The burgeoning collection of genomes provides a platform by which previously unknown genes can be produced by genome mining. In order to quickly identify new FIPs from the burgeoning genome databases and produce sufficient FIPs for the function studies and clinical applications, sequence similarity searches and recombinant expression were used in this work. Finally, a previously unknown FIP gene was identified from the basidiomycete fungus *Postia placenta* using homological alignment. The gene was custom-synthesized and expressed in *Escherichia coli*, and the purified FIP was subjected to bioassays to evaluate its immunoregulatory and antitumor activity.

2. Materials and methods

2.1. Protein expression, purification and identification

A new FIP gene (**KJ818121**), designated *FIP-ppl*, was identified in the fungal genomic database of *P. placenta* Mad-698-R (**ABWF01004372**) via BLAST searches using the amino acid sequence of FIP-fve as a bait. After optimizing the codon usage for *E. coli*, the putative FIP-encoding gene was synthesized by GenScript (Nanjing, China). The pGEX-4T-1 expression system (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used to produce recombinant FIP-ppl (rFIP-ppl) in *E. coli* Rosetta cells (Novagen, Schwalbach/Ts., Germany), initially as an *N*-terminal GST fusion protein. The fusion protein was purified from the cell lysate by affinity chromatography on a glutathione-sepharose column (Pharmacia, Uppsala, Sweden). The fusion protein was treated with thrombin, and the cleaved protein tag was removed with Benzamidine sepharose 6B (GE Healthcare, Fairfield, CT, USA). The purity of the resultant rFIP-ppl protein was evaluated by Tricine-SDS-PAGE. Protein quantitation was performed using a NanoDrop-1000 Spectrophotometer (Thermo Scientific, Rockford, IL, USA). RFIP-ppl was further purified by Tricine-SDS-PAGE and subjected to MALDI-MS analysis using a 4700 Proteomics Analyzer (ABI, Foster City, CA, USA) at the Tianjin Biochip Corporation (Tianjin, China).

2.2. Modeling the tertiary structure (3D) structure

Discovery studio 2.5.5 was used for protein 3D modeling. The crystal structures of FIP-fve (1OSY), LZ-8 (3F3H), and FIP-gmi (3KCW) served as templates.

2.3. Hemagglutination test

The hemagglutination activity of rFIP-ppl was assayed as described previously (Qin et al., 2008). Cells from rabbit blood and four types of human blood (A, B, AB, and O) were collected by centrifugation at $1000 \times g$ for 5 min, washed three times with phosphate buffer solution (PBS, 10 mM, pH 7.4), and resuspended to 1.5% (g/v) in PBS. Twenty-five microliters of rFIP-ppl at different concentrations was added to 25 μl aliquots of blood solution (1.5%) in 96-well V-bottom microtiter plates and incubated at 25 °C for 2 h. Concanavalin A (ConA, 5 μg/ml in PBS) was used as the positive control.

2.4. Mouse splenocyte proliferation assay

The 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to evaluate the cell proliferation stimulatory effect of rFIP-ppl on mouse splenocytes (Strong et al., 1973). Splenocytes were isolated from six-week-old BALB/c mice (Vital River Laboratories, Beijing, China) and suspended to 1×10^6 cell/ml in RPMI1640 medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The splenocytes (100 μl) and different concentrations of purified rFIP-ppl (1, 2, 4, 8, 16 and 32 μg/ml) were seeded into a 96-well microtiter plate. The sample was incubated at 37 °C under 5% CO_2 for 72 h. Then 20 μl of MTT (5 mg/ml) was added and the mixture was incubated for another 4 h. The culture supernatant was carefully aspirated, and then 100 μl dimethyl sulfoxide was added to the cells, which were gently agitated for 10 min. Absorbance (OD) was measured at 570 nm using a Multiskan MK3 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). ConA (5 μg/ml) and lipopolysaccharide (LPS, 2 μg/ml) served as positive controls.

2.5. IL-2 release assay

IL-2 production was used to estimate the immunomodulatory activity of rFIP-ppl using mouse splenocytes. The mouse splenocytes were prepared as described above and adjusted to 1×10^7 cells/ml in complete RPMI1640 medium. Aliquots of 100 μl splenocytes and 100 μl rFIP-ppl (1, 2, 4, 8, 16 and 32 μg/ml) were seeded in a 96-well microplate. ConA was used as the positive control. After incubation at 37 °C under 5% CO_2 for 48 h, the culture supernatant was collected by centrifugation at $1000 \times g$ for 5 min. The amount of IL-2 in the supernatant was detected using an ELISA kit for mouse IL-2 (Uscn Life Science Inc., Wuhan, China).

2.6. Cell proliferation inhibitory and apoptosis assay

The cultured tumor cell line gastric cancer MGC823 and hepatoma HepG2 (Cell Resource Center of Peking Union Medical College Hospital, Beijing, China) were suspended to 5×10^5 cells/ml in complete DMEM and RPMI1640 medium, respectively. Aliquots of 100 μl cell suspension and 100 μl rFIP-ppl (2, 4, 8, 16, 32 and 64 μg/ml) were seeded into a 96-well microplate. PBS served as a negative control. The cells were cultured at 37 °C under 5% CO_2 for 24 h and assayed using MTT method. OD was measured at 570 nm using a Multiskan MK3 (Thermo Scientific). The IC_{50} value was determined as the concentration that caused 50% inhibition of cell proliferation (Peng et al., 2008).

To detect apoptosis precisely, flow cytometer was used to analyze the tumoricidal effect of rFIP-ppl. Eight-hundred microliters of tumor cell suspension (1×10^6 cells/ml) and 200 μl rFIP-ppl (32 and 64 μg/ml) were added to a 24-well microplate and cultured as described above for 24 h. Cells were harvested by centrifuging at $1000 \times g$ for 5 min and washed twice with PBS. The apoptotic cells were detected using a flow cytometer and Annexing-V-EGFP apoptosis detection kit (BD FACSCalibur, San Jose, CA, USA; Vigorous Biotechnology Beijing Co. Ltd., Beijing, China).

2.7. Statistical analysis

All values are presented as the mean ± SD of triplicate performed in three independent experiments. Statistical comparisons were carried out by analysis of variance (ANOVA) using SPSS 19 (PASW statistics, IBM, New York, NY, USA). Differences were considered to be statistically significant at $p < 0.05$, and extremely significant at $p < 0.01$.

3. Results

3.1. Heterologous expression, purification and identification of rFIP-ppl

Searching the NCBI fungal genomic databases with the protein sequence of FIP-fve from *F. velutipes* as bait revealed two small predicted proteins with significant sequence similarity to FIP-fve. Characterization of the FIP-like sequence from the ascomycete *Nectria haematococca* was reported (Bastiaan-Net et al., 2013), while the manuscript was being prepared (Li et al., 2014). The other FIP-like sequence was found in the genome database of the basidiomycete *P. placenta* Mad-698-R (http://www.jgi.doe.gov/Postia). This protein was annotated as a new FIP gene (**KJ818121**). It was then predicted that this protein has 52% sequence identity with FIP-fve. This predicted protein, here named FIP-ppl, contains 125 amino acid residues with a calculated molecular weight of 14,738 Da. The deduced FIP-ppl lacks Met and His but is rich in Val, Asp, Thr, and Tyr residues with the typical sequence characters of FIPs. Sequence comparisons revealed that the deduced amino acid sequence of FIP-ppl shares 54–58% identity with other FIPs (58% with FIP-gmi, 56% with FIP-gja and 54% with LZ-8, respectively; Supplementary Fig. S1).

The gene encoding FIP-ppl was custom-synthesized and expressed in *E. coli* as a GST fusion protein. It was purified on a GST column and then digested with thrombin to release rFIP-ppl. The corresponding 17 kDa protein was observed in Tricine-SDS-PAGE (Fig. 1A). Eight peptide fragments corresponding to 87 residues,