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Isolation and Characterization of a Novel Immunomodulatory α-Glucan−Protein Complex from the Mycelium of *Tricholoma matsutake* in Basidiomycetes

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Tricholoma matsutake, a high-class edible mushroom in Japan, has been reported to have excellent biological activities, but difficulty in cultivating the fruit bodies and limited bulk availability have restricted detailed studies. We have developed a method of culturing in tanks, enabling the bulk supply of the mycelia. The preparation (CM6271) exerts modulative effects on the immune competence of mice and rats. In this study, a sodium hydroxide extract of CM6271 was defatted followed by fractionation with a combination of ion exchange chromatography and gel filtration in order to identify the components involved in the expression of the activity, and a single peak fraction (MPG-1) was obtained with reversed phase chromatography. MPG-1 was a glycoprotein (sugar:protein ratio, 94.3:5.7) with a relative molecular mass of 360 kDa, and the sugar moiety contained about 90% glucose. NMR spectra and methylation analysis revealed that the α-1,4-linkage was the predominant glucan linkage with α-1,6- and α-1,2-linkages in the minority. The amino acid composition in the protein moiety was rich in glutamine, alanine, asparagine, leucine, glycine, valine, serine, threonine, isoleucine, and proline. MPG-1 was resistant to degradation with amylase or protease. The oral administration of MPG-1 promoted, in a dose-dependent manner, the recovery of the mouse natural killer cell activity and serum IL-12 level that had been reduced by the loading of restraint stress. The dose of MPG-1 (25 mg/kg) required for the expression of the effect decreases to 1/12 of that of CM6271 (300 mg/kg). Furthermore, MPG-1 formed a complex with TGF-β1 in vitro, modulating the biological activity of TGF-β1 by binding to its active form. These results indicate that the mycelium of *T. matsutake* contains a novel α-glucan−protein complex with immunomodulatory activities.

KEYWORDS: ***Tricholoma matsutake*; mycelium; MPG-1; α-1,4-glucan−protein complex; NK cell; IL-12; restraint stress**

INTRODUCTION

Many kinds of mushrooms and herbs have been used since ancient times as ingredients in Chinese medicine and folklore treatment because of their diverse biological activities. Their active components are considered to be polysaccharides, low molecular mass (MM) substances, and the like; however, unidentified molecules may also be present. In the process of our search for edible mushrooms and herbs that modulate the immune response by oral administration, we found an active agent in the fruit bodies of *Tricholoma matsutake*, a member of the Tricholomataceae family of basidiomycetes, which serves in Japan as a kind of high-class edible mushroom. Although its constituents have been reported to have excellent antitumor activities (*1*, *2*), difficulty in cultivating the fruit bodies and their limited bulk availability have restricted detailed studies.

Recently, we developed a method of culturing *T. matsutake* in tanks, enabling the bulk supply of the mycelia (*3*). The mycelial preparation (CM6271) consists mainly of sugars, proteins, and lipids, with a similarity in composition to the fruiting bodies. The 90 day safety tests on rats with oral administration showed no abnormalities (*4*). In a study to evaluate the biological properties of CM6271, we found that in addition to having antitumor activity against the in vivo growth of mouse syngeneic fibrosarcoma (*5*) and preventive activity against the formation of azoxymethane-induced precancerous lesions in the colon (*3*), this preparation is effective in improving immunological functions in stressed individuals; the oral administration of CM6271 to mice promoted the recovery of immunocompetence after 18 h of restraint stress in a manner

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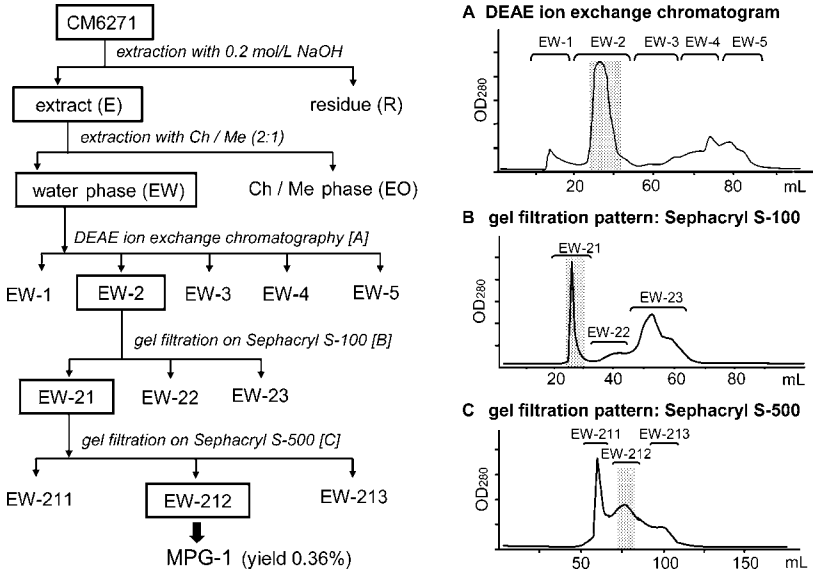

Figure 1. Scheme of extraction and purification of CM6271. The detailed methods of extraction and purification of CM6271-derived components are described in the Materials and Methods section. Slanted lines indicate significant promoting activity. The yield of MPG-1 from CM6271 is 0.36%.

dependent on the timing and dosage (*6*) and prevented the decrease in immunocompetence due to 6 h of restraint stress imposed once daily for 20 days (*7*). The activity was characteristic for CM6271 and the fruit bodies of *T. matsutake* and was not found in some kinds of antioxidants, herbs, or edible mushrooms such as *Agaricus blazei*, *Lentinus edodes*, and *Grifola frondosa* (*6*), suggesting that unidentified structures are involved in the expression of this activity.

Some types of stressors act on the immune system via a network comprising the endocrine−immune−nervous systems and are reportedly responsible for the onset of some diseases, such as autoimmune diseases, allergies, microbial infections, and cancers, in addition to promoting their progression (*8*). Of the cells that constitute the immune system, the activity of natural killer (NK) cells, which play important roles in innate immunity, such as elimination of tumor cells and the prevention of virus proliferation, is markedly affected by stress loading through the host defense network (*6*). Because daily ingestion of CM6271 is considered to impart benefits for the maintenance of health and the promotion of coping with stress-induced changes in immunocompetence followed by prevention of the development of these maladies, we started to investigate its active structure. The results of the study suggest that the component of CM6271 involved in the improvement of NK cell activity in restraint-stressed mice is an α-glucan−protein complex.

MATERIALS AND METHODS

Materials. CM6271, a mycelial preparation derived from the cultured mycelia of *T. matsutake* strain BP-7304 (a member of Tricholomataceae in basidiomycetes), was supplied from Kureha Chemical Industry Co., Ltd. (Tokyo, Japan). The preparation was a white powder containing 73% sugars, 16% proteins, 5% lipids, and 5% ash and was sealed and kept in the dark until use.

Extraction and Purification of Mycelia. Five liters of 0.2 mol/L aqueous solution of NaOH was added to 100 g of CM6271. The mixture was stirred for 1 h at 25 °C and centrifuged at 15000 rpm for 20 min at 4 °C. The supernatant was collected, neutralized with hydrochloric acid, and shaken with an equal volume of a 2:1 mixture of chloroform (Ch) and methanol (Me). The mixture was allowed to stand, and the aqueous and organic solvent layers were collected separately. The aqueous layer was dialyzed against pure water at 4 °C for 3 days and lyophilized. The lyophilizate was dissolved in 50 mmol/L Tris-HCl buffer, pH 7.5, and fractionated with column chromatography as shown with a flowchart in **Figure 1**. The organic solvent layer was evaporated to dryness in an evaporator.

Column Chromatography. For ion exchange chromatography, the sample was applied to a column (22 mm × 200 mm) of DEAE Toyopearl Pak 650M (Tosoh Corporation, Tokyo) equilibrated with 50 mmol/L Tris-HCl buffer, pH 7.5, and eluted with a linear gradient of 50 mmol/L Tris-HCl buffer, pH 7.5, containing 1 mol/L sodium chloride. For gel filtration, the sample was applied to a column (20 mm × 700 mm) of Sephacryl S-100 HR (Amersham Biosciences, Tokyo) or a column (16 mm × 800 mm) of Sephacryl S-500 HR (Amersham Biosciences) equilibrated with 50 mmol/L Tris-HCl buffer, pH 7.5, and eluted with the same buffer. For reversed phase chromatography, the sample was applied to a column (4.6 mm × 250 mm) of Inertsil WP300 C18 (GL Sciences Inc., Tokyo) equilibrated with 0.05% formic acid and eluted with a linear gradient of a 9:1 mixture of 10−50% acetonitrile and pure water. Akta FPLC (Amersham Biosciences) and Shimadzu LC10A series (Shimadzu Corp., Tokyo) were used as analytical systems for monitoring at an absorbance of 280 nm and refractive index (RI). **Figure 1** shows the UV absorption at 280 nm.

Color Reaction. Components in the sample were identified with the phenol−sulfuric acid method, the copper−Folin method, and the iodine−starch reaction method. Bovine serum albumin and glucose were used as the standard protein and glucose, respectively.

Analysis of Neutral Sugars and Amino Sugars. The sample was dissolved in 2 mol/L trifluoroacetic acid and hydrolyzed at 100 °C for 6 h. The hydrolysates were neutralized and analyzed by high-performance liquid chromatography (HPLC) with postcolumn derivatization, using a Shimadzu LC-9A with a TSK-gel Sugar AXG column (4.6 mm × 150 mm, Tosoh Corp.) and a Shimadzu RF-10AXL spectrofluorometer (*9*).

Amino Acid Analysis. The sample was dissolved in 6 mol/L hydrochloric acid, sealed in a vacuum tube, and hydrolyzed at 110 °C for 22 h. For tryptophan analysis, the sample was dissolved in 4.2 mol/L sodium hydroxide containing soluble starch and hydrolyzed in a vacuum-sealed tube at 110 °C for 16 h. The hydrolysates were neutralized and analyzed with a Hitachi L-8500 amino acid analyzer.

Instruments for Structural Analysis. Samples for ^{1}H NMR and ^{13}C NMR spectroscopy were dissolved in deuterium oxide and 0.2 mol/L sodium deuterioxide, respectively, and NMR spectra were measured with a UNITY plus model 500 NMR spectrometer (Varian Inc., CA). In some experiments, to eliminate the effects of peptide-derived signals, hydrazine-decomposed samples were prepared by dissolving in anhydrous hydrazine and heating at 100 °C for 2 h. The chemical shifts were determined referring to the Aldrich Library of ^{13}C and ^{1}H Fourier transform (FT) NMR Spectra (*10*). FT-IR spectra

were measured with the potassium bromide tablet method using a Hyper-IR infrared spectrophotometer. For circular dichroism (CD) analysis, spectra were measured with a JASCO J-500A in the range of 200−250 nm, with a cell length of 1 mm at 23 °C. The amounts of α-helix and β-sheet were calculated according to the method of Chen et al. (*11*).

MM Determination by Gel Filtration. For gel filtration chromatography, a Shimadzu LC-10ADvp system and Shodex Asahipak columns GS-710 7G and GS-310 7G (Showa Denko K.K., Tokyo) were used. The elution pattern was monitored with a Shimadzu RID-6A RI detector. The relative MM was estimated by extrapolating the elution time of 0.5% solution of pullulan standard of known molecular mass P-1600 (1.6×10^6 Da), P-800 (8.5×10^5 Da), P-400 (3.8×10^5 Da), P-200 (2.1×10^5 Da), and P-100 (1.0×10^5Da) (Showa Denko K.K.).

Electrophoresis. The sample was dissolved in sodium dodecyl sulfate (SDS)-containing Tris-HCl buffer (Daiichi Pure Chemicals Co., Ltd., Tokyo), heated at 95 °C for 3 min, and electrophoresed on 4−20% polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd.). Bands were detected by silver staining (Daiichi Pure Chemicals Co., Ltd.).

Methylation Analysis. Sodium hydroxide and methyl bromide were sequentially added to a dimethyl sulfoxide solution of the sample with a modified version of the Hakomori method (*12*), and the mixture was stirred for 1 h for methylation to proceed. The product was subjected to acid hydrolysis followed by reductive acetylation for conversion to the acetyl derivative of partially methylated sugar alcohol, which was measured by electrical ionization−gas chromatography/mass spectrometry using a JMS DX-303 mass spectrometer (JEOL) and an HP5890 gas chromatograph (Hewlett-Packard, United States).

Enzymatic Degradation. To examine the sensitivity to hydrolysis with α-amylase from *Bacillus subtilis* (Wako Pure Chemicals Industry Co., Ltd., Osaka), the enzyme was added to 10 mg/mL solution of the sample in 10 mM phosphate buffer, pH 6.0, to a final concentration of 1.0 U/mL, and the mixture was incubated at 30 °C for 30 min or 24 h. At the end of the reaction, the mixture was placed in a boiling water bath for 10 min to inactivate the enzyme, and the reducing sugar was measured using colorimetry. On the other hand, to examine the sensitivity to hydrolysis with protease from *Streptomyces griseus* (Wako Pure Chemicals Industry Co., Ltd.), the enzyme was added to a 10 mg/mL solution of the sample in 10 mM phosphate buffer, pH 7.5, to a final concentration of 0.5 mg/mL, and the mixture was incubated at 30 °C for 30 min or 24 h. At the end of the reaction, the mixture was placed in a boiling water bath for 10 min to inactivate the enzyme and subjected to SDS−polyacrylamide gel electrophoresis (PAGE).

Animals, Feeding, and Sample Administration. Specific pathogen-free male C57BL/6 mice were purchased from Charles River Japan, Inc. (Kanagawa) and used at 8 weeks of age. The mice were kept in the animal room at 25 ± 0.5 °C at 55% humidity under laminar air flow. To maintain a constant environment, noise was avoided, and only those who kept the animals or performed experiments entered the animal room.

CM6271 and fractionates were suspended in 0.5% aqueous solution of carboxymethyl-cellulose and were orally administered to mice using a probe. As a rule, each experimental group consisted of six mice, and the same experiment was repeated at least twice. The experimental design was reviewed by the Ethics Committee on Animal Experiments of the Biomedical Research Laboratories of Kureha Chemical Industry Co., Ltd., and all experiments were performed in line with the guidelines of the laboratories.

Restraint Stress Loading. The mouse was placed in a 50 mL conical polyethylene tube with more than 30 air holes 5 mm in diameter and subjected to restraint stress for 18 h in the cage in the feeding room, as reported previously (*6*, *7*). While the test mice were restrained, the control mice were kept in the normal environment without access to food or water.

NK Cell Activity. Immediately after the mice were killed by cervical dislocation, their spleens were removed and splenocytes were suspended in RPMI1640 medium with 10% heat inactivated fetal bovine serum so that a single cell suspension was obtained. The NK cell activity of the spleen cells in the restraint-stressed mice was determined by the ^{51}Cr release assay using the YAC-1 mouse leukemia cell line as a target cell, as reported previously (*6*, *7*).

Quantification of Total IL-12 in the Serum. The blood was sampled at 10:00−10:30 a.m. by cardiac puncture within 30 s from each mouse under ether anesthesia, and after collection, the blood was quickly transferred to a refrigerated centrifuge to separate the serum, which was then stored at −80 °C until determinations were made. We confirmed in the previous report (*6*, *7*) that cardiac puncture performed within 30 s under ether anesthesia had little effect on serum levels of cytokines and corticosterone. The serum total IL-12 levels were determined by enzyme-linked immunosorbent assay (ELISA) using an Endogen kit (MN).

Transforming Growth Factor β1 (TGF-β1)-Neutralizing Activity. ^{125}I-recombinant human TGF-β_1 (^{125}I-rhTGF-β_1, Amersham Biosciences) and MPG-1 were mixed in a fixed ratio in 50 mmol/L Tris-HCl buffer, pH 7.5, containing 0.2% bovine serum albumin and 0.2 mol/L sodium chloride, and the mixture was incubated at 37 °C for 3 h. This reaction mixture was fractionated on a Sephacryl S-100HR column to fractionate it into the ^{125}I-rhTGF-β_1−MPG-1 complex and unbound ^{125}I-rhTGF and MPG1, and the radioactivity of each fraction was measured with a COBRA γ-counter (Hewlett-Packard).

To examine the biological activity of the complex, the TGF-β_1 sensitive Mv1Lu mink lung epithelial cell line was plated in a 96 well culture dish at a density of 1×10^4 cells/100 μL/well and cultured in a 5% CO_2 incubator at 37 °C for 24 h and, after the addition of a fixed amount of the complex, for another 24 h. Four hours before the end of culturing, 37 kBq/well of ^{3}H-thymidine (Amersham Bioscience) was added to the wells, and the cells were washed to measure the radioactivity with a scintillation counter. In this experiment, the complex was prepared using nonlabeled rhTGF-β_1 instead of ^{125}I-rhTGF-β_1. Furthermore, to examine the cell-binding ability of the above complex, a fixed amount of the radioactive complex was incubated with Mv1Lu cells at 4 °C for 1 h. After the cells were washed with the medium, the radioactivity was measured with the γ-counter.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD). Statistical differences were tested using Student's *t*-test, a one-way analysis of variance (ANOVA) with posthoc Dunnett test, or a two-way ANOVA. *P* values less than 0.05 were considered significant.

RESULTS

Isolation of the Active Component MPG-1 from CM6271. To examine whether the active component was soluble in water or organic solvents, we added an equal volume of a 2:1 Ch:Me solvent mixture to a 0.2 mol/L NaOH extract of CM6271 for its fractionation. CM6271 (300 mg/kg) or its fractionates corresponding to the dose of CM6271 (30 mg/kg of fraction E, 270 mg/kg of R, 30 mg/kg of EW, or 0.5 mg/kg of EO) were orally administered to C57BL/6 mice daily for 10 days up to the day before restraint stress loading. Then, the mice were subjected to restraint stress for 18 h, and the splenic NK cell activity was measured on the 7 days after release from the restraint stress. As shown in **Table 1**, the biological activity was recovered in the aqueous phase fraction (EW) with little activity in the alkali-extracted residue (R). A reconstituted fraction prepared by the mixture of each fraction in proportion to its yield showed an activity similar to that of CM6271, when it was administered to mice, suggesting that the loss of activity during the isolation process was low. Thus, EW was sequentially fractionated by DEAE ion exchange chromatography and gel filtration chromatography to evaluate the activity at the dose of 30 mg/kg daily for 10 days up to the day before restraint stress loading. As a result, significant enhancement of the recovery of NK cell activity was recovered in EW2, EW21, and EW212 (data not shown), as shown by slant lines in **Figure 1**.

In parallel with biological assays, each fraction was analyzed for purity by reversed phase HPLC and SDS−PAGE, which gave a single peak or spot in the active fraction, EW212 (**Figure 2**). Because repeated reversed phase HPLC showed almost the

Table 1. Effects of CM6271 Fractionates on NK Cell Activity in Mice 7 Days after Release from Restraint Stress[a]

group	stress	preparations	NK cell activity (LU_{30})
1	no	PBS	51.4 ± 3.6
2	yes	PBS	32.1 ± 3.6*
3	yes	CM6271	46.3 ± 4.2**
4	yes	alkaline soluble fraction (E)	43.6 ± 4.1**
5	yes	alkaline insoluble fraction (R)	36.8 ± 4.0
6	yes	water phase fraction (EW)	46.7 ± 3.4**
7	yes	Ch/Me phase fraction (EO)	32.8 ± 3.5
8	yes	reconstitute (EW + EO)	44.1 ± 3.5

[a] C57BL/6 mice were subjected to restraint stress for 18 h, and the splenic NK cell activity was measured on the seventh day after release from the restraint stress. CM6271 (300 mg/kg) or its fractionates corresponding to the dose of CM6271 were orally administered daily for 10 days up to the day before restraint stress loading. Statistically significant at *p < 0.01 (vs group 1) and **p < 0.01 (vs group 2).

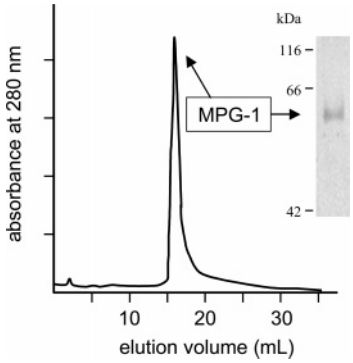

Figure 2. Reversed phase chromatogram and SDS–PAGE pattern of MPG-1. The instruments used and measurement methods are described in the Materials and Methods section.

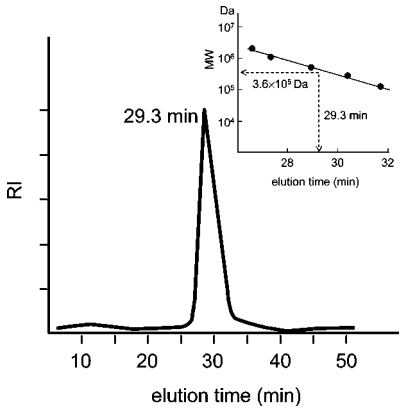

Figure 3. Relative MM of MPG-1 measured by gel filtration. The instruments used and measurement methods are described in the Materials and Methods section.

same elution profile, this fraction was designated MPG-1, and its properties were examined.

Physicochemical Properties of MPG-1. On the basis of colorimetry using the phenol−sulfate method and the copper−Folin method and absorption of FT-IR spectra at 3300−1600, 1650−1500, and 860 cm^{-1}, we confirmed that MPG-1 contained sugars and proteins (data not shown). The relative MM as estimated by gel filtration was 360 kDa, as shown in **Figure 3**.

MPG-1 was rich in sugars, with a sugar:protein mass ratio of 16:1 in terms of hydrolysates (**Table 2A**). Almost the same quantitative values were obtained by the copper−Folin method

Table 2. Sugar and Amino Acid Composition of MPG-1

A: Composition

components	mol %	components	mol %
sugar	94.3	total	100
protein	5.7		

B: Neutral Sugars and Amino Sugars

sugars	mol %	sugars	mol %
glucose	89.4	xylose	0
galactose	6.8	N-acetylmannosamine	0.03
mannose	2.3	N-acetylglucosamine	0.06
fucose	1.6	total	100

C: Amino Acids

amino acids	mol %	amino acids	mol %
asparagine (aspartic acid)	9.65	leucine	9.55
threonine	6.15	tyrosine	2.64
serine	6.83	phenylalanine	4.09
glutamine (glutamic acid)	10.1	lysine	4.99
glycine	8.74	histidine	1.97
alanine	9.84	arginine	5.00
valine	6.94	tryptophan	1.17
cysteine	0.15	proline	5.33
methionine	1.31	total	100
isoleucine	5.47		

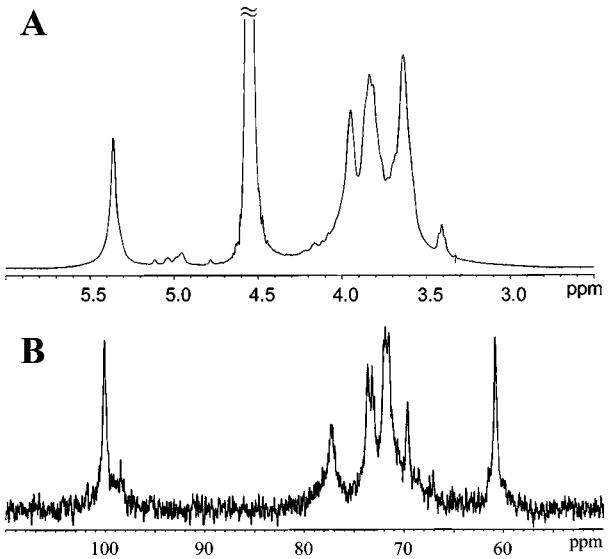

Figure 4. ^{1}H NMR (**A**) and ^{13}C NMR (**B**) patterns of MPG-1. The instruments used and measurement methods are described in the Materials and Methods section.

using bovine serum albumin as the standard and the phenol−sulfuric acid method using glucose as the standard (data not shown). Analysis of neutral sugars by HPLC showed that it contained 89.4% glucose, followed by galactose, mannose, and fucose, at less than 7.0%. Only a small amount of amino sugar was detected (**Table 2B**). On the other hand, the amino acid composition analysis according to HPLC revealed that MPG-1 contained glutamine, followed, in decreasing order, by alanine, asparagine, leucine, glycine, valine, serine, threonine, isoleucine, and proline (**Table 2C**). Furthermore, CD spectral analysis of the protein moiety showed that it contained 21% α-helix, 34% β-sheet, and 45% irregular structures (data not shown).

Next, the mode of glucose linkage in MPG-1 was examined by NMR and methylation analysis. **Figure 4A**,**B** shows ^{1}H NMR and ^{13}C NMR spectra patterns. ^{1}H NMR detected protons at the α-1-position of glucose around 5.4 ppm. ^{13}C NMR detected

Table 3. Methylation Analysis of MPG-1 Sugar Portion[a]

derivatives identified	deduced linkage	mol %
2,3,4,6-tetra-O-methyl-Glc	Glc 1 →	10.5
2,3,6-tri-O-methyl-Glc	→ 4 Glc 1 →	74.5
2,3-di-O-methyl-Hex	→ 4,6 Hex 1 →	7.7
3,6-di-O-methyl-Hex	→ 2,4 Hex 1 →	2.6
2,3,4-tri-O-methyl-Hex	→ 6 Glc 1 →	0.7
3,4,6-tri-O-methyl-Hex	→ 2 Hex 1 →	0.7
2,3,4-tri-O-methyl-Fuc	Fuc 1 →	1.0
2,3,4-tri-O-methyl-Gal	→ 6 Gal 1 →	2.2
total		100

[a] Glc, glucose; Hex, hexose; Fuc, fucose; and Gal, galactose.

peaks at 65−80 and 100 ppm. The peak at 65−80 ppm suggested a glycosyl bond on α-2- and α-4-positions by chemical shift calculation, and the peak at 100 ppm suggested an α-glycoside linkage at position 1. The two-dimensional ^{1}H NMR spectrum was obtained to investigate the presence of overlapping peaks that could not be distinguished by one-dimensional ^{1}H NMR, but no additional information was obtained (data not shown). On the other hand, methylation analysis detected 1,4-linkages of glucose units and also very low levels of 1,2- and 1,6-linkages (**Table 3**). Thus, it was suggested that the predominant linkage of glucose units in the sugar moiety was an α-1,4-linkage and that α-1,2- and α-1,6-linkages were barely present as a branching structure.

Treatment of MPG-1 with amylase caused little or no change in the level of reducing sugars. Treatment with protease produced little or no change in SDS−PAGE patterns. In addition, unlike starch, MPG-1 gave a negative iodine−starch color reaction (data not shown).

Biological Properties of MPG-1. We confirmed that the oral administration of 300 mg/kg/day of CM6271 for 10 days significantly promoted the recovery of NK cell activity that had been reduced by 18 h of restraint stress loading and that the manifestation of the effect of this preparation was marked on the 7 days after release from the restraint, although the CM6271 scarcely affected the NK cell activity of nonrestraint mice (**Figure 5A**). To elucidate the possible action mechanism, we studied changes of the cytokine levels in the mouse blood after the release from restraint stress and the effect of CM6271 on them. The blood IL-12 level in the distilled water-treated restrained group was reduced to about 50% of that in the healthy control group immediately after the release (0.54 ± 0.09 ng/mL), showed a recovery after 5−10 days, and returned to a healthy control level (1.03 ± 0.25 ng/mL). As shown in **Figure 5B**, the administration of CM6271 significantly promoted the recovery of serum IL-12 level and there was a significant difference between distilled water-treated group and CM6271-administered group 7 days after release from restraint stress.

Subsequently, to evaluate the activity of MPG-1 in comparison with that of the starting material, CM6271, by the use of NK cell activity in restraint-stressed mice as a parameter, we orally administered 25 mg/kg of MPG-1 or 300 mg/kg/day of CM6271 to C57BL/6 mice for 10 days before restraint stress loading. The activity on the first day after release from restraint stress in the MPG-1 group and the CM6271 group was reduced at about the same level as that in the control PBS group. On the seventh day, the activity recovery in both the MPG-1 group and the CM6271 group was significantly accelerated as compared to the control PBS group, and on the 14th day, the activity in three groups was recovered to a level almost the same as that in the nonrestraint stress control group (data not shown). There was no significant difference in the recovery pattern

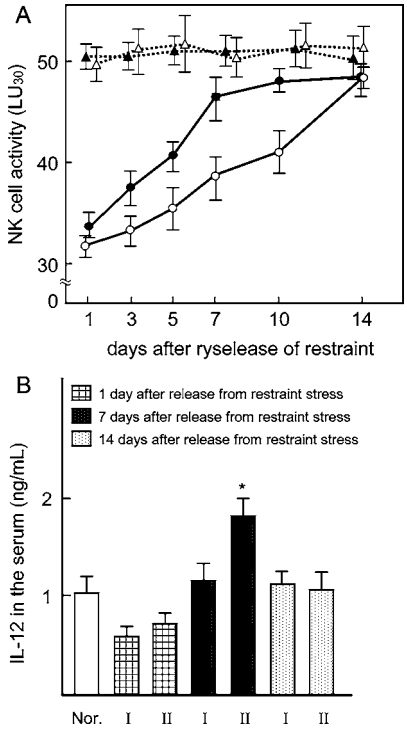

Figure 5. Effects of the administration of CM6271 on the NK cell activity and IL-12 contents of mice after the release of restraint stress. (**A**) C57BL/6 mice were subjected to restraint stress for 18 h, and after being released from the restraint, the splenic NK cell activity was serially measured. CM6271 was orally administered at a dose of 300 mg/kg/day for 10 days up to the day before restraint stress loading. △ indicates the PBS administration group with no restraint stress; ▲ indicates the CM6271 administration group with no restraint stress; ○ indicates the PBS administration group with restraint stress; ● indicates the CM6271 administration group with restraint stress; and a bar indicates the SD. Statistically significant at $p < 0.01$ between ○ and ● by one-way ANOVA. (**B**) The blood was sampled by cardiac puncture under ether anesthesia on each day after their release, and serum was separated and stored at −80 °C until determinations were made. The total IL-12 contents in the serum were determined with ELISA. I, the group administered with distilled water; II, the group administered with CM6271; and a bar, SD. Statistically significant at $*p < 0.05$ (vs the group administered with distilled water, 7 days after release from restraint stress).

between the MPG-1 group and the CM6271 group. Administration of MPG-1 or CM6271 to nonrestraint stress mice did not affect the activity during the observation period.

To clarify the dose dependency of the MGP-1 effect in the restraint-stressed mice, 1, 5, 25, or 50 mg/kg of MPG-1 was orally administered for 10 days before restraint stress loading, and the activities on the seventh day after release of the restraint stress were examined. As shown in **Figure 6A**, the administration of more than 25 mg/kg/day of MPG-1 resulted in significant enhancement of the NK cell, and the level of activity was similar to that in the group administered with 300 mg/kg of CM6271. Furthermore, as shown in **Figure 6B**, the administration of 25 mg/kg of MPG-1 and 300 mg/kg of CM6271 significantly increased the serum IL-12 level. These results suggest that although the pattern of recovery from restraint stress does not significantly differ between the two groups, the dose of MPG-1 required for the expression of the effect decreases to 1/12 of that of CM6271.

Furthermore, we examined the possible involvement of direct interaction of MPG-1 with TGF-β_1, a biomolecule known to inhibit the growth and proliferation of immunocompetent cells

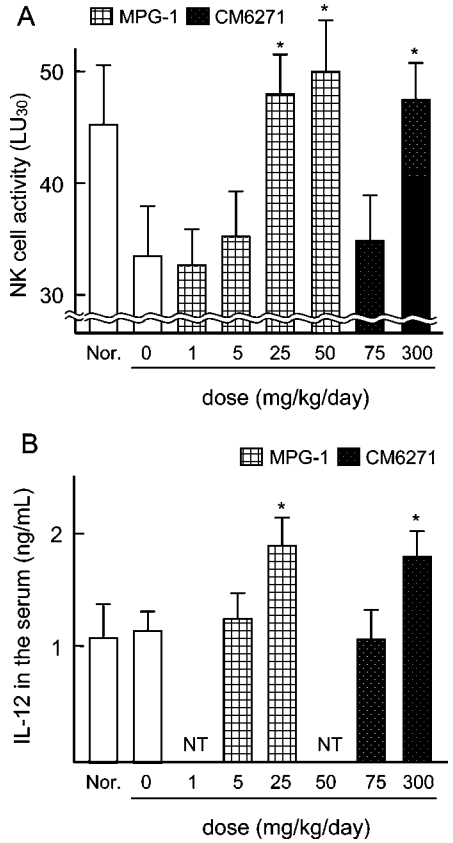

Figure 6. Dose dependency of MPG-1 on the promotion of recovery of NK cell activity and IL-12 contents in restraint-stressed mice. (**A**) C57BL/6 mice were subjected to restraint stress for 18 h, and the splenic NK cell activity was measured on the seventh day after release from the restraint. A fixed amount of MPG-1 or CM6271 was orally administered daily for 10 days up to the day before restraint stress loading. Statistically significant at $*p < 0.01$ (vs 0 mg/kg/day). (**B**) The blood was sampled by cardiac puncture under ether anesthesia on the 7 days after their release, and the serum was separated and stored at –80 °C until determinations were made. The total IL-12 contents in the serum were determined with ELISA. Statistically significant at $*p < 0.05$ (vs 0 mg/kg/day).

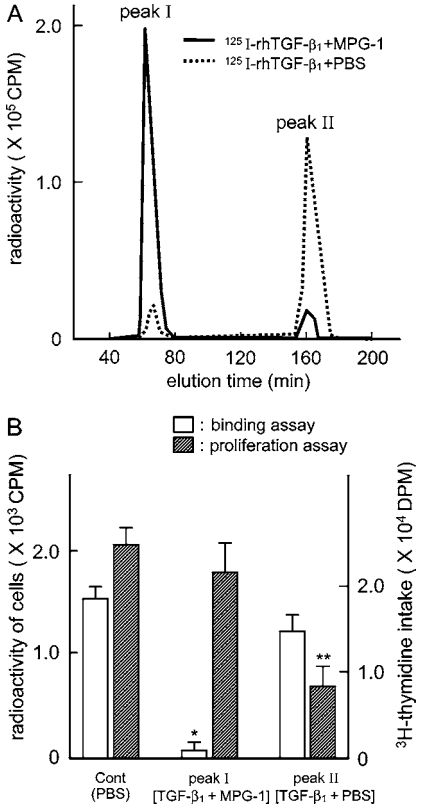

Figure 7. Direct interaction between MPG-1 and rhTGF-β_1 in vitro. A fixed amount of MPG-1 and ^{125}I-rhTGF-β_1 or rhTGF-β_1 was dissolved in PBS containing 0.2% BSA, mixed in a test tube with low protein adsorption, and incubated at 37 °C for 3 h. (**A**) The reaction products of MPG-1 and ^{125}I-rhTGF-β_1 or those of PBS and ^{125}I-rhTGF-β_1 were subjected to gel filtration, and the radioactivity of eluates was measured with a γ-counter. The elution time between 60 and 70 min was designated as peak I, and the time between 155 and 170 min was designated as peak II. The solid line indicates the elution profile of the MPG-1-^{125}I-rhTGF-β_1 reaction products, and the dotted line indicates the PBS-^{125}I-rhTGF-β_1 reaction products. (**B**) The products of reaction of MPG-1 with rhTGF-β_1 or ^{125}I-rhTGF-β_1 were subjected to gel filtration, and elution peak I was collected. Instead of MPG-1, PBS was used as a control, and after the reaction, peaks I and II were collected, respectively. The collected peak fractions were adjusted to contain the same concentration of TGF-β_1 and added to Mv1Lu cells in culture; then, a binding assay or proliferation assay was performed. Statistically significant at * and $**p < 0.05$ (vs control).

and to increase in amount in immunosuppressed individuals, in the expression of the activity of MPG-1 (*13*). In a tube in which little protein was absorbed, ^{125}I-rhTGF-β_1 was dissolved in 50 mmol/L Tris-HCl buffer, pH 7.5, containing 0.2% bovine serum albumin and 0.2 mol/L sodium chloride and adjusted to 10 ng/mL. MPG-1 (100 μg/mL) was added to the tube, and the solution was incubated at 37 °C for 3 h. Then, the reaction mixture was subjected to gel filtration on a Sephacryl S-100HR. Under this condition, the recovery of radioactivity was above 86%. Comparing the elution pattern of radioactivity on gel filtration between the reactant and the ^{125}I-rhTGF-β_1 alone (**Figure 7A**), a decrease in the radioactivity of peak II, the elution position of ^{125}I-rhTGF-β_1, and a marked increase in the radioactivity of peak I, the elution position of MPG-1 or substances with MMs more than MPG-1 were observed in the reactant. These results suggest that MPG-1 and rhTGF-β_1 form a complex.

Subsequently, we examined whether the complex possesses the biological activity of TGF-β_1 by the use of a binding assay and proliferation assay. The addition of the MPG-1−^{125}I-rhTGF-β_1 complex to an in vitro culture of Mv1Lu cells, a cell line sensitive to TGF-β_1, resulted in a clearly lower radioactivity than that of ^{125}I-rhTGF-β_1, suggesting that the complex has a lower affinity to the cells (**Figure 7B**). Furthermore, the addition of the non-RI-labeled MPG-1−rhTGF-β_1 complex to an in vitro culture of Mv1Lu cells for 24 h promoted the uptake of ^{3}H-thymidine in cells more so than that of rhTGF-β_1, suggesting that the complex has a lower growth suppression activity than that of rhTGF-β_1. These results suggest that MPG-1 directly binds to rhTGF-β_1 to inhibit its activity.

DISCUSSION

Glucans are a structurally diverse group of polysaccharides involved in various biological activities. Of the two types of glucan anomers, β-glucan plays important roles mainly in the formation of the structure of cells and tissues, and the α-glucans play important roles in energy storage. For example, the β-glucan cellulose consists of linear β-1,4-linked glucose units with 3000−10000 degrees of polymerization (*14*). The α-glucan starch is composed of two distinct polymers: amylose, which is a straight chain of α-1,4-linked glucose units, and amylopectin, which consists of straight chains of α-1,4-linked glucose units with extensive branching resulting from an α-1,6-linkage

every 24−30 glucose residues. Although glycogen structurally resembles starch, it is more extensively branched, with a branch every 8−10 residues. These α-1,4-linkages make the polysaccharide itself a tight spiral structure with dense granules functioning as an efficient storage system. In addition, microbe-derived pullulan is a structural polysaccharide consisting of α-1,6-linked maltotriose repeat units, frequently containing maltotetrose units (*15*).

The biological activities of *β*-glucans, especially *β*-1,3-glucans, have been well-studied in terms of antitumor activities against animals as well as cancer patients (*16*). In contrast, relatively few studies have reported on α-glucans, including an approximately 8 kDa dextran sulfate with anti-HIV, anticoagulative, and hypolipidemic activities (*17*); α-glucans derived from the fruiting bodies of *A. blazei* (*18*−*20*) and *Agrocybe cylindracea* (*21*), a 150 kDa proteoheteroglycan derived from *Phellinus linteus* (*22*); α-glucans derived from *Mycobacterium tuberculosi* (*23*), mosses (*24*), and herbs (*25*); and α-glucans derived from rice bran (*26*) and synthetic enzyme products (*27*, *28*). The majority of these studies have focused on antitumor and macrophage-enhancing activities, similar to studies of *β*-glucans. MPG-1 in this study exerts little activity on immunocompetent cells, such as macrophages, of healthy individuals and characteristically recovers NK cell activity in stress-loaded individuals. Furthermore, the CM6271-derived M2 fraction reported by Ebina et al. (*5*) is a mixture of α- and *β*-glucans, and its intratumoral injection inhibits the growth of tumors, suggesting that its active component is different from that obtained in this study. Because not only the biological activities but also the MM and protein content differ from those reported previously, we considered that MPG-1 is a glucan that has not been identified to date.

The main physicochemical factors determining the biological activities of glucans are the MM, degree of branching, conformation (the triple helix, the single helix, and the random coil), solubility, and chemical modification. In the case of *β*-1,3-glucan, the triple helix is noted as the active structure (*29*). Studies have investigated the relationship between the structure of α-glucans in solution and their activity (*30*): α-Glucan molecules contain strong hydrogen bonds and relatively weak glucoside linkages, which render the molecules water insoluble. The introduction of sulfuric acid groups and carboxymethyl groups into these polymers not only improves their solubility but also reduces their MMs and enhances their antitumor activity. This is considered to occur through a charge-mediated mechanism, similar to the interaction between antithrombin and heparin in the expression of anticoagulant activity. In the present study, the methylation analysis and NMR spectroscopy of the sugar moiety showed that the α-1,4-linkage was predominant. In addition, unlike starches, the sugar moiety gave a negative iodine−starch color reaction and resisted degradation by amylases and proteases. In a preliminary experiment, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry detected no sugar signals but detected several approximately 2 kDa peptides apparently representing MPG-1 protein fragments that were produced on ionization. These findings suggest that MPG-1 has an α-1,4-glucan as the main chain, with extensive branching by α-1,2- and α-1,6-linkages and interaction with the protein, forming a characteristic stereochemical structure. It is also possible that 1,6-galactose linkages and fucose linkages at position 1, which were detected in small amounts by methylation analysis, make the structure of MPG-1 complex, thereby preventing digestion by various α-glucan degrading enzymes and proteases. The contents of amino sugars revealed low on HPLC analysis of MPG-1 hydrolysate. Furthermore, the patterns of SDS−PAGE and chromatograms before and after treatment with glucosaminoglycan (GAG) degrading enzyme or endoglycsidase or exoglycosidase were similar (unpublished data), and little information supported the GAG chain linkage, suggesting that MPG-1 is more likely to be α-glucan−protein complex than proteoglycan and glycoprotein.

In an experimental model of stress loading in which mice were restrained in tubes, the degree of reduction and the period of recovery of splenic NK cell activity after animals were released from restraint stress loading were dependent on the time for which they were restrained (*6*, *7*). As shown in **Figure 5** in the present study, the splenic NK cell activity was significantly decreased to 65% of activity in healthy control mice, the day after release from restraint when the restraint duration was 18 h, and it took from 10 to 14 days to reach the level of healthy control mice. A reciprocal relation between humoral factors and trafficking of immunocytes has been suggested as the action mechanism of restraint stress on the immune system (*31*). Among humoral factors, glucocorticoids reduce NK cell activity and affect its localization by inhibiting the gene transcription and/or by suppressing expression of cellular adhesion molecules and effector molecules (*32*). The enhancing effect of oral administration of MPG-1 on the recovery of NK cell activity in mice after release of restraint stress was dose-dependent (**Figure 6**). The administration of 25 mg/kg/day or greater of MPG-1 for 10 days before stress loading significantly improved the recovery of NK cell activity 7 days after release. However, on day 1 after release from restraint stress, immunosuppressive effects of stress-induced increases in the serum ACTH and plasma corticosterone levels manifested and avoided exertion of the MPG-1 activity, resulting in the absence of significant difference in the NK cell activity. In contrast, on days 3−5 after release from the stress, these blood hormone levels returned to the healthy levels, which may have decreased the influence on the NK cell activity and induced marked exertion of the action of MPG-1 through IL-12 and TGF-*β*.

Among cytokines, a proinflammatory cytokine, IL-12, appears to be involved in the stress-induced changes in immunocompetence; IL-12 not only induced Th1 responses but also activated and proliferated NK cells (*33*); on the other hand, the action is affected by corticosteroids. The hormone induced by restraint stress reduces IL-12 gene expression in mouse immunocompetent cells and the blood IL-12 level (*33*). On the other hand, TGF-β_1 is a biomolecule that has various biological activities, such as the control of the proliferation and functions of immunocompetent cells, and its overproduction causes immunosuppression (*34*). In addition, the intracellular signal pathway of TGF-β_1 is affected by glucocorticoids produced in stress-loaded individuals (*35*), suggesting that the TGF-β_1-binding activity of MPG-1 is involved in the expression of its effects in vivo. The interaction between MPG-1 and TGF-β_1 was confirmed not only by gel filtration and cell binding assay (**Figure 7**) but also by ELISA, and the 50% binding concentration was 100 μg/mL (unpublished data). Decorin and thrombospondin are also biomolecules that bind directly to TGF-β_1 to modify its activity, but their binding modes appear to differ from that of MPG-1 (*36*, *37*). The protein moiety (representing about 96%) of the immunostimulatory proteoglycan derived from *Coriolus versicolor* in basidiomycetes (*13*) is involved in the binding to TGF-β_1, suggesting that its activity has little in common with that of MPG-1 in terms of protein contents. X-ray analysis of the three-dimensional crystal structure and NMR analysis of

the two-dimensional structure have shown that two identical hydrophobic interfaces of the dimer play an important role in maintaining the active structure of TGF-β (*38*). We deduce that the interaction between the hydrophobic domain of the TGF-β_1 molecule and the active domain of the MPG-1 molecule results in the formation of a complex, modulating the activity of immunocompetent cells such as the NK cells suppressed by stress loading.

When ^{14}C-labeled CM6271 is orally administered to healthy rats, the radioactivity is rapidly absorbed from the intestinal tract into the blood and becomes distributed throughout almost the entire body, including the liver and intestinal tissue, and about 90% of it is excreted from the body in 72 h (*39*). Gel filtration analysis with Superose12 (10−300 mm) (Amersham Biosciences) of the serum, 4 h after its oral administration, showed a radioactivity peak coinciding with the MPG-1 elution peak, with no shift in the peak position, even with analysis under SDS-denatured conditions (unpublished data). Immunostaining with anti-MPG-1 antibody showed that the molecule was distributed in M cells of intestinal Peyer's patches 1 h after oral administration and in parafollicular cells 5 h after oral administration (unpublished data).

Furthermore, treatment with anti-IL-12 antibody attenuated the promotive effects on the recovery of NK cell activity, with a decrease in the serum IL-12 level and the IL-12 p70 productivity of splenocytes. The administration of the preparation promoted the intracellular interferon-γ production of NK cells and increased interferon-γ-producing NK cells in the spleens of restraint-stressed mice (unpublished data). Because IL-12 has promotive effects on the proliferation and activity of NK cells, we speculate that orally administered MPG-1 is taken up by the M cells in the intestine while maintaining its antigenic structure and exerts its activity via IL-12. Further studies using MPG-1 as a model not only to determine its structure but also to identify its receptors will clarify in more detail the biological activities of bioactive α-glucans derived from food.

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