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Inhibition of β-1,3-Glucan Synthase and Cell Growth of *Cryptococcus* species by Recombinant Single-chain Anti-idiotypic Antibodies

Dakshnamurthy Selvakumar, Masahiko Miyamoto, Yasuhiro Furuichi, Tadazumi Komiyama

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Abstract Recombinant single-chain fragment variable (scFv) anti-idiotypic antibodies were produced to represent the internal image of a HM-1 killer toxin, which is characterized by a wide spectrum of anti-fungal activity through inhibiting β-1,3-glucan synthase (GS). We examined if scFv antibodies are active against *Cryptococcus* species, a human pathogen of increasing medical importance. The anti-cryptococcal activity of scFv antibodies and HM-1 were assessed by MIC analysis for *C. neoformans* IFM 40215 and *C. albidus* NBRC 0612 cells. The scFv antibodies had strong anti-cryptococcal activity *in vitro* with IC_{50} at 1.07×10^{-7} to 2.60×10^{-7} M for *C. neoformans* and *C. albidus*. Furthermore, the scFv antibodies potentially inhibited GS of *C. neoformans* with IC_{50} at 1.27×10^{-7} to 2.27×10^{-7} M. Both the anti-fungal and anti-GS activities of the scFv antibodies were markedly neutralized by the monoclonal antibody that neutralizes HM-1 killer toxin.

Keywords HM-1 killer toxin, recombinant anti-idiotypic antibodies, scFv antibodies, fungicidal protein, β-glucan synthase inhibitors, *Cryptococcus neoformans*, *Cryptococcus albidus*

T. Komiyama (Corresponding author), **D. Selvakumar, M. Miyamoto:** Department of Biochemistry, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, 5-13-2 Kamishinei-cho, Niigata 950-2081, Japan, E-mail: tkomiyam@niigata-pharm.ac.jp
Y. Furuichi: GeneCare Research Institute Co. Ltd., 200 Kajiwara, Kamakura 247-0063, Japan.

Introduction

Opportunistic infections are becoming increasingly common because of the growing number of individuals immunocompromised by chemotherapy or immunosuppressants [1]. Recently, fungal infections have especially become a problem [2,4]. *Cryptococcus neoformans* causes a serious infection in immunocompromised individuals, such as AIDS or transplant patients [5]. This fungus is not eradicated with conventional therapeutic approaches, and survivors need lifelong suppressive therapy [6]. The immunosuppressive effects of some cryptococcal capsular compounds, such as glucuronoxylomannan, released during infection, could be an additional deleterious, life-threatening factor [7].

The gene encoding β-1,3-glucan synthase (EC 2.4.1.34) has been cloned from *C. neoformans* and has proved essential for viability and growth of fungal cells [8]. β-1,3-Glucan synthase (GS), an enzyme localized in the plasma membrane, catalyzes the synthesis of β-1,3-glucan, a major polymer component of the cell wall of *Candida albicans* and other fungi [9]. The cell wall serves as a protective barrier for the fungal cell and is essential for fungal viability and shape. Fungal cell wall synthesis enzymes are proven to be effective targets for anti-fungal drugs, in particular, GS is essential for fungal growth and has no mammalian equivalent [10]. A GS inhibitor caspofungin acetate, as a semisynthetic analogue of pneumocandin B_o, was developed as a broad-spectrum parenteral agent for the treatment of aspergillosis and candidiasis [11]. In general, the most common treatment for fungal infections is based on the use of amphotericin B, 5-flucytosine and

fluconazole. However, these drugs have repeatedly failed against infections caused by *Cryptococcus* species [12]. Therefore, the development of novel molecules and alternative therapeutic strategies for the battle against fungal infections is becoming a topical and widely recognized need.

HM-1 killer toxin (HM-1) is produced by *Williopsis saturnus* var. *mrakii* IFO 0895 and is characterized by a wide spectrum of anti-fungal activity through inhibiting GS [13～17]. Recently, we developed single chain fragment variable (scFv) anti-idiotypic antibodies with anti-fungal activity by using recombinant DNA technology with the monoclonal antibody that neutralizes the killing activity of HM-1 killer toxin (nmAb-KT). These scFv antibodies have the internal image of HM-1, are characterized by candidacidal activity (Selvakumar *et al.*, unpublished), and kill the various pathogenic strains of *Candida albicans* and *Saccharomyces cerevisiae*, through inhibiting GS activity. To our knowledge, no anti-fungal antibody that inhibits GS activity has been previously reported. In this study, we discovered that selected scFv anti-idiotypic antibodies kill *Cryptococcus neoformans* through inhibiting GS activity *in vitro* dose dependently. We believe this is the first study to show recombinant anti-cryptococcal antibodies that potentially inhibit GS activity.

Materials and Methods

Preparation of scFv Anti-idiotypic Antibodies

HM-1-like scFv anti-idiotypic antibodies used in this study were produced according to a procedure using the Recombinant Phage Antibody System (Amersham Biosciences) (Selvakumar *et al.*, unpublished). Briefly, female BALB/c mice were immunized subcutaneously and intraperitoneally (booster injection) with 50 μg of nmAb-KT. Three days after final booster injection the mice were killed, their spleens were removed and the mRNA was isolated from the splenic lymphocytes. The purified mRNA was primed with random hexamers and reverse transcribed. The genes encoding the antibody-variable region of heavy and light chains were amplified, then assembled as scFv genes using a linker fragment, and finally cloned into a specific phagemid vector pCANTAB 5E. Recombinant phages produced in *Escherichia coli* TG1 were repeatedly panned against nmAb-KT and were screened by using conventional enzyme-linked immunosorbent assay with nmAb-KT. The selected recombinant phages were infected a non-suppressor *E. coli* HB2151 to produce soluble recombinant scFv anti-idiotypic antibodies that were purified by using affinity chromatography. Finally, based on amino acid sequence, four scFv anti-idiotypic antibodies were selected (designated as scFv-A1, -A2, -A3 and -A4) and used throughout this study.

Measurement of Minimum Inhibitory Concentration (MIC)

MICs were determined by the standardized protocol for yeasts developed by the National Committee for Clinical Laboratory Standards [18]. Briefly, *C. neoformans* IFM 40215 and *C. albidus* strains NBRC 0612 fungal cells were suspended in sterile normal saline and diluted to a concentration of 5×10^5 cells/ml. The suspension were diluted 1 : 1000 in RPMI 1640 medium with L-glutamine, without bicarbonate, which had been buffered to pH 7.0 with 20 mM HEPES (Sigma, St. Louis, MO). Tubes containing 0.1 ml aliquots of scFv antibodies or HM-1 at 10 times the final drug concentration were inoculated with 0.9 ml of the diluted suspensions. The tubes were incubated at 30°C for 72 hours with shaking. The MIC endpoints were read visually as the lowest concentration at which there was an absence of growth.

In Vitro Anti-fungal Assay

The qualitative antifungal assay against the fungal isolates was done by using a conventional colony forming unit (CFU) assay, as described previously [19]. Briefly, approximately 5×10^2 cells of *C. neoformans* IFM 40215 or *C. albidus* NBRC 0612 were suspended in 10 μl of phosphate buffered saline (PBS) and were incubated with 100 μl scFv antibodies or HM-1 at various concentrations for 16 hours at 37°C. PBS was used as a control; as a further control, similar fungal cells were prepared by the adding scFv antibodies that were preincubated overnight at 4°C with 20 μl of nmAb-KT. The fungal cells were incubated with the scFv antibodies or HM-1, spread on the surface of Sabouraud dextrose agar plates and subsequently incubated at 30°C. The cells were incubated for 48 hours and their fungal CFU was enumerated. The concentration of 50% inhibition (IC_{50}) was measured by using semi-logarithmic graphs. Each experiment was performed in triplicate.

Preparation of Membrane Fraction Containing GS Activity

The membrane fraction was prepared by using the method described previously [20] with some modifications. *C. neoformans* IFM 40215 cells in mid-exponential phase were collected by centrifugation and washed with 1 mM EDTA. The collected cells were suspended in breaking buffer (consisting of 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA and 1 mM phenylmethanesulfonyl

fluoride), disrupted by vortexing with glass beads, and then collected by centrifugation (5 minutes at 1,000×g at 4°C). The supernatant was centrifuged for 30 minutes at 100,000×g at 4°C. The resulting membrane fraction was homogenized in membrane buffer (50 mM Tris-HCl (pH7.5), 10 mM EDTA, 1 mM β-mercaptoethanol, 33% glycerol) and stored at −80°C.

Measurement of GS Activity

The assay of GS activity was performed using the method of Cabib and Kang [20]. The reaction mixture consisted of 5 mM UDP-D-[U-^{14}C]glucose, 75 mM Tris-chloride (pH 7.5), 0.75% bovine serum albumin, 25 mM KF, 0.75 mM EDTA, 20 μM guanosine 5′-[γ-thio]triphosphate and 20 μl membrane fraction, in a total volume of 40 μl. The reaction was started by adding the membrane fraction, and the mixture was incubated at 30°C for 60 minutes. To measure the inhibitory effect of scFv antibodies and HM-1 on GS activity, various concentrations of scFv antibodies or HM-1 were added to the reaction mixture. To examine the neutralization effect of nmAb-KT on GS activity, 10 μg/ml of scFv antibody or 20 μg/ml of HM-1 was incubated with 10 μg/ml of nmAb-KT. The reaction was stopped by adding 250 μl of 10% (w/v) TCA. After a 10 minutes quenching period, the mixture was filtered through glass microfibre filters (Whatman GF/B). The filters were washed four times with 250 μl of 10% (w/v) TCA and were further washed twice with 250 μl of 95% ethyl alcohol. The radioactivity retained on the filters was counted using a liquid scintillation counter. The IC_{50} values were measured by using semi-logarithmic graphs. Each experiment was performed in triplicate.

Table 1 MICs for *in vitro* susceptibilities of *C. neoformans* IFM 40215 and *C. albidus* NBRC 0612 to scFv anti-idiotypic antibodies and HM-1

Anti-fungal agents	MIC (μg/ml)	
	C. neoformans	*C. albidus*
scFv-A1	12.5	25.0
scFv-A2	6.25	12.5
scFv-A3	6.25	25.0
scFv-A4	12.5	12.5
HM-1	75.0	100

MICs measurements of cell growth of *C. neoformans* and *C. albidus* are described in Materials and Methods.

Results

MICs for *In Vitro* Susceptibilities of *Cryptococcus* Species to scFv Anti-idiotypic Antibodies and HM-1

MICs for *in vitro* susceptibilities of *C. neoformans* IFM 40215 and *C. albidus* NBRC 0612 against scFv anti-idiotypic antibodies and HM-1 were summarized in Table 1. Among the scFv antibodies, scFv-A2 and -A3 at MIC 6.25 μg/ml were more potent than scFv-A1 and -A4 against *C. neoformans* and scFv-A2 and -A4 at MIC 12.5 μg/ml were also more active than scFv-A1 and -A3 against *C. albidus*. Overall, scFv-A2 was the most active agent for both *C. neoformans* and *C. albidus*. HM-1 showed MICs at 75 and 100 μg/ml for *C. neoformans* and *C. albidus*, respectively.

Anti-fungal Activity of scFv Anti-idiotypic Antibodies and HM-1 against *Cryptococcus* Species *In Vitro*

Figs. 1A and B show the anti-fungal activity of scFv-A2 anti-idiotypic antibody against the pathogenic strain of *C. neoformans* IFM 40215 by adding PBS as a control. At a concentration 4 μg/ml scFv-A2, the growth of *C. neoformans* was strongly inhibited. However the activity of 10 μg/ml HM-1, which was isolated as the original

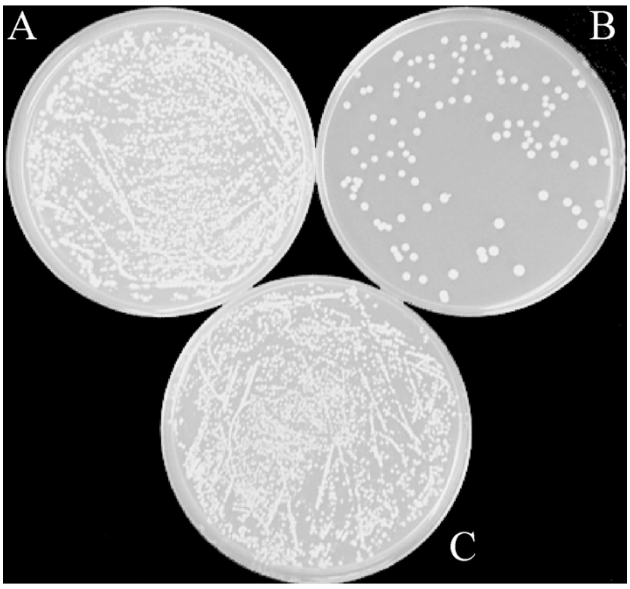

Fig. 1 Anti-cryptococcal activity of scFv-A2 anti-idiotypic antibody.

Effect of scFv-A2 anti-idiotypic antibody on the growth of *C. neoformans* IFM 40215 cells in a CFU assay. A, standardized fungal inocula treated with PBS; B, fungal cells treated with 4 μg/ml of scFv-A2; C, fungal cells treated with scFv-A2 neutralized with nmAb-KT. One of the three plates used for each sample is shown.

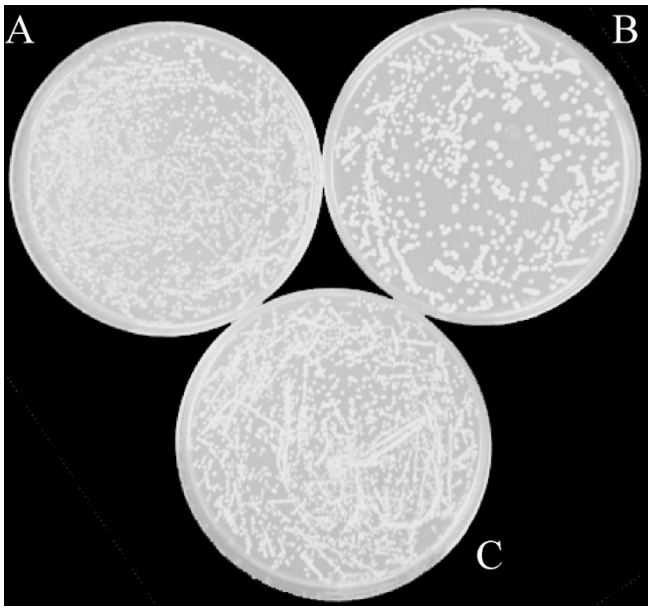

Fig. 2 Anti-cryptococcal activity of HM-1.

Effect of HM-1 on the growth of *C. neoformans* IFM 40215 cells in a CFU assay. A, standardized fungal inocula treated with PBS; B, fungal cells treated with 10 μg/ml HM-1; C, fungal cells treated with HM-1 neutralized with nmAb-KT. One of the three plates used for each sample is shown.

anti-fungal protein, was significantly less than the activity of the same concentration of scFv-A2 (Figs. 2A, B). To measure the potency of scFv anti-idiotypic antibodies and HM-1 against *C. neoformans* and *C. albidus* NBRC 0612, CFU were determined after incubation with increasing concentrations of scFv antibodies or HM-1. The IC_{50} values of scFv antibodies were 3.2 to 5.4 μg/ml (1.07×10^{-7} to 1.80×10^{-7} M) against *C. neoformans*, and 6.4 to 7.8 μg/ml (2.13×10^{-7} to 2.60×10^{-7} M) against *C. albidus*; both results showed strong anti-cryptococcal activity (Table 2). The IC_{50} of HM-1 was 15.2 μg/ml (1.60×10^{-6} M) against *C. neoformans*, and 15.8 μg/ml (1.66×10^{-6} M) against *C. albidus* (Table 2). The lower concentration of scFv antibodies and HM-1 showed no marked effect. The anti-cryptococcal activities of scFv antibodies and HM-1 were markedly neutralized by nmAb-KT (Figs. 1C, 2C).

Table 2 IC_{50} values of scFv anti-idiotypic antibodies and HM-1 against *C. neoformans* IFM 40215 and *C. albidus* NBRC 0612 cell growth

Anti-fungal agents	IC_{50} of cell growth	
	C. neoformans	*C. albidus*
	M	M
scFv-A1	1.80×10^{-7}	2.47×10^{-7}
scFv-A2	1.07×10^{-7}	2.13×10^{-7}
scFv-A3	1.27×10^{-7}	2.60×10^{-7}
scFv-A4	1.67×10^{-7}	2.20×10^{-7}
HM-1	1.60×10^{-6}	1.66×10^{-6}

IC_{50} measurements of cell growth of *C. neoformans* and *C. albidus* are described in Materials and Methods.

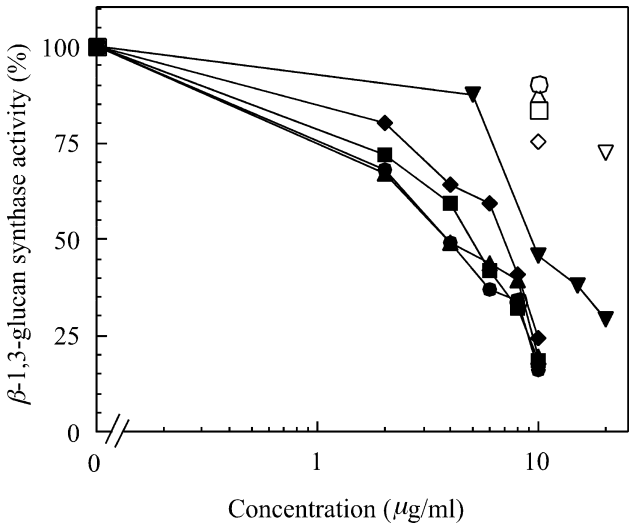

Fig. 3 Inhibition of GS by scFv anti-idiotypic antibodies and HM-1.

The membrane fraction prepared from the cells of *C. neoformans* IFM 40215 and the method of activity measurement were described in Materials and Methods. The activity was expressed by using the sample without scFv antibodies and HM-1 as 100%. Anti-idiotypic antibodies (scFv) or HM-1 was added to the reaction mixture. ■, scFv-A1; ●, scFv-A2; ◆, scFv-A3; ▲, scFv-A4 and ▼, HM-1. The scFv anti-idiotypic antibodies and HM-1 that were neutralized by nmAb-KT are represented by open symbols: □, scFv-A1; ○, scFv-A2; ◇, scFv-A3; △, scFv-A4; and ▽, HM-1.

Inhibitory Activity of scFv Anti-idiotypic Antibodies and HM-1 against GS from *C. neoformans*

To determine if scFv anti-idiotypic antibodies and HM-1 inhibit GS activity of *C. neoformans* IFM 40215, freshly prepared membrane fractions were incubated with different concentrations of scFv antibodies or HM-1. UDP-D-[*U*-^{14}C] glucose was used as the GS substrate. The scFv antibodies and HM-1 inhibited GS in a concentration-dependent manner (Fig. 3). The inhibition was expressed as a percentage decreases in GS activity. The IC_{50} values were 3.8 to 6.8 μg/ml (1.27×10^{-7} to 2.27×10^{-7} M) for scFv antibodies and 11.2 μg/ml (1.18×10^{-6} M) for HM-1 (Table 3). Inhibition of GS by both scFv antibodies and HM-1 was markedly neutralized by nmAb-KT at specified

Table 3 IC_{50} values of scFv anti-idiotypic antibodies and HM-1 against GS activity of *C. neoformans* IFM 40215

scFv antibodies and HM-1	IC_{50}
	M
scFv-A1	1.67×10^{-7}
scFv-A2	1.27×10^{-7}
scFv-A3	2.27×10^{-7}
scFv-A4	1.27×10^{-7}
HM-1	1.18×10^{-6}

IC_{50} measurements of GS activity of *C. neoformans* membrane fractions are described in Materials and Methods.

concentrations (Fig. 3, open symbols). Because both the anti-fungal and anti-GS IC_{50}s of scFv antibodies and HM-1 were on the same order of magnitude, there is an implication that these anti-fungal agents may act through inhibition of cell membrane GS.

Discussion

C. neoformans is an important fungal pathogen that produces firmly established infections in immunocompromised patients. After inhalation of spores into the lung, infection spreads by the bloodstream to the brain where it causes life-threatening infection of the central nervous system [21]. Cryptococcosis is the fourth most common cause of mortality in patients with AIDS [22, 23]. Currently, 4% of AIDS patients in the United Kingdom [24], 5～10% of AIDS patients in the United States [25], and an increasing percentage in the developing countries have cryptococcosis [26]. Even after successful treatment of acute infection with currently available anti-fungal drugs (*e.g.*, amphotericin B, 5-flucytosine or fluconazole), the recurrence rate is high, and patients require life-long suppressive therapy [27]. The therapeutic options are still unsatisfactory because of the toxicity of available drugs, the inability of these drugs to eradicate the fungus, and the emergence of drug resistance [28]. Therefore, novel and more effective molecules against *C. neoformans* are needed in the clinic.

In related work, we found that scFv anti-idiotypic antibodies derived from HM-1 killer toxin have strong candidacidal activity *in vitro* through inhibiting GS activity (data not shown). However, analysis of the cryptococcal cell walls from *in vitro* cultures showed that both β-1,3-glucan and β-1,6-glucopyrans are in fungal cells [29]. Nonetheless, production of cryptococcal spheroplasts requires β-1,3-glucan, which suggest that these linkages are required for cell wall integrity [30]. Therefore, as glucans are critical structural components for *C. neofromans*, we felt encouraged to assess if scFv anti-idiotypic antibodies derived from HM-1 affect cryptococcal cells. In this study, we showed that scFv anti-idiotypic antibodies have strong, dose-dependent anti-cryptococcal activity *in vitro* through inhibiting GS activity, which is particularly relevant because glucan is a major structural component of the cell wall. The gene encoding GS is essential for yeast viability and growth [8], and thus the fact is conceivable that scFv anti-idiotypic antibodies interfere with regular cell wall formation of *C. neoformans*.

Molecules able to selectively interact with fungal cell wall components, which are not present in mammalian cells, should be logically considered as putative anti-fungal drugs [31]. The scFv anti-idiotypic antibodies derived from HM-1 killed *Cryptococcus* cells through inhibiting GS activity. *Cryptococcus* is unique among fungal pathogens for its major virulence factor, a complex polysaccharide capsule. The scFv-A2 and -A3 MIC at 6.25 μg/ml was active followed by scFv-A1, -A4 and HM-1 against *C. neoformans*. The scFv-A2 and -A4 MIC at 12.5 μg/ml was potent followed by scFv-A1, -A3 and HM-1 against *C. albidus* (Table 1). The scFv antibodies had a strong anti-cryptococcal activity against *C. neoformans* and *C. albidus* *in vitro* with IC_{50} at 1.07×10^{-7} to 2.60×10^{-7} M and were neutralized by adsorption with nmAb-KT. All four scFv antibodies potentially inhibited GS activity of the membrane fraction of *C. neoformans* at IC_{50} 1.27×10^{-7} to 2.27×10^{-7} M. But in case of HM-1, the IC_{50} of cell growth and GS activity was ten times higher than for scFv antibodies (Tables 2, 3). The above results suggested that scFv-A2 which was the most potent inhibitor because of its lowest MIC and IC_{50} values against both *C. neoformans* and *C. albidus* may be useful in the treatment of cryptococcosis.

All four scFv anti-idiotypic antibodies potently inhibited the growth of *C. neoformans* and *C. albidus* to the same extent as their inhibition of GS in the membrane fraction of *C. neoformans*. These results strongly suggest that inhibition processes by scFv antibodies and HM-1 are similar. Both the killing of fungal cells and the inhibition of GS by scFv antibodies and HM-1 were markedly neutralized by nmAb-KT (Figs. 1, 2, 3). Therefore, we speculate that scFv antibodies and HM-1 share common target molecule(s) that are most likely the cell surface GS. This concept supports the idea that scFv antibodies may be cytocidal against a wide range of cell wall microorganisms that have glucan, including clinically important opportunistic pathogens.

In conclusion, scFv anti-idiotypic antibodies showed

strong, marked anti-fungal activity against *Cryptococcus* species, the data are consistent with the hypothesis that these scFv antibodies act by inhibiting the fungal GS, which is the specific target of broad-spectrum anti-fungal drugs. This is particularly relevant, because cryptococcosis is still incurable in immunocompromised patients. These results strongly suggesting a reasonable basis for a novel GS inhibitory agent to cure pathogenic fungal disease like cryptococcosis.

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