ARTICULO

Production and characterization of monoclonal antibodies against association factor and Stm1p of *Saccharomyces cerevisiae.*

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ABSTRACT

Here we report the production and characterization of two monoclonal antibodies (mAbs) against the association factor (2G5B2, 2G5C2) and two others against the Stm1p (1G11D4, 2G1B10) of *Saccharomyces cerevisiae.* Cross-reactivity studies with AF and Stm1p revealed that mAbs against Stm1p also recognized purified AF, but mAbs against AF (2G5B2, 2G5C2) recognized weakly Stm1p. Proteinase K digestion and periodate oxidation studies showed that one mAb (2G5B2) bound a glycoprotein antigenic determinant, while the other three (2G5C2, 1G11D4, 2G1B10) detected peptidic epitopes. One mAb (1G11D4) belongs to the IgG2b class, while the others belongs to the IgG1 class. The specificities of mAbs were evaluated by ELISA, Dot Blot and Western Blot assays. Finally, these results demonstrate that there is antigenic difference between AF and Stm1p.

Key words: monoclonal antibodies, Association Factor, Stm1p, protein synthesis.

Abbreviations: *mAbs: monoclonal antibodies, AF: association factor*

RESUMEN

Producción y caracterización de anticuerpos monoclonales contra el Factor de Asociación y Stm1p de *Saccharomyces cerevisiae*

En este trabajo se reporta la producción y caracterización de dos anticuerpos monoclonales (mAbs) contra el Factor de Asociación de ribosomas (AF) y otros dos contra Stm1p de *Saccharomyces cerevisiae*. Los estudios de reactividad cruzada con AF y Stm1p revelaron que los mAbs contra Stm1p (1G11D4, 2G1B10) también reconocieron AF purificado, pero los mAbs contra AF (2G5B2, 2G5C2) solo reconocieron débilmente a Stm1p. Los ensayos de digestión con Proteína quinasa K y periodato mostraron que el mAb (2G5B2) reconoció una glicoproteína, mientras que los otros tres (2G5C2, 1G11D4, 2G1B10) detectaron epítopes proteicos. Un mAb (1G11D4) pertenece a clase IgG2b, mientras que los otros pertenecen a la clase IgG1. La especificidad de los mAbs fue evaluada por ensayos de ELISA, Dot Blot y Western Blot. Estos resultados demuestran que existe diferencia antigénica ente AF y Stm1p.

Palabras clave: anticuerpos monoclonales, Factor de Asociación, Stm1p, síntesis de proteínas

INTRODUCTION

Previous reports identified Stm1p as a protein that binds G4 quadruplex and purine motif triplex nucleic acid (1); acts with Cdc13p to maintain stability of telomere structure (2); interacts with ribosomes (3) and subtelomeric Y' DNA and acts a multicopy suppressor of tom1 and pop2 mutations. Also, Stm1p has been implicated in cellular apoptosis since cells lacking Stm1 display deficiency in the apoptosis-like cell death process induced by treatment with low concentrations of H_2O_2 (4, 5).

The association factor (AF) was reported as a thermostable protein obtained by a high salt wash of polysomes, which is able to join ribosomal subunits at low Mg^{2+} concentration in the absence of energy or other components of the translational machinery (6). The sequence of an AF fragment obtained by BNPS-skatole, showed a high degree of identity (89%) with Stm1p indicating that there is a close relation between Stm1p and AF (7,8). In previous reports, Stm1p and AF were showed as proteins that share common activities (7) but they have a different relative molecular weight on SDS-PAGE. In order to differentiate AF and Stm1p and investigate if there is an antigenic relationship between Stm1p and AF, we produced monoclonal antibodies (mAbs) against both proteins. In this work we report the production and characterization of two mAbs against the AF (2G5B2, 2G5C2) and two to the Stm1p protein (1G11D4, 2G1B10) of *Saccharomyces cerevisiae* and the possible antigenic relation between these proteins.

MATERIALS AND METHODS

Antigen preparation. AF was obtained by a high salt wash of polysomes of *Saccharomyces cerevisiae* according to Herrera et al. (6). Stm1p was obtained fused to glutathione S-transferase from transformed *E. coli* BL21DE with the plasmid pGEXStm1p that had inserted the STM1 gen from yeast. The recombinant Stm1p was obtained after proteolytic removal of the glutathione S-transferase domain with factor Xa. These procedures were performed as described by Frantz and Gilbert (8). Cell-free yeast S-100' extract was prepared according to Gasior et al (9).

Immunization protocol. 4 Female BALB/c mice (from 6 to 8 weeks old) were immunized with Stm1p of *Saccharomyces cerevisiae* and 4 Female BALB/c mice (from 6 to 8 weeks old) were immunized with association factor (AF) of *S. cerevisiae*. The immunization scheme was as follows: day 1, 50 μg of antigen in complete Freund' adjuvant (Sigma Chemical Company, St. Louis, MO) intraperitoneally; day 15, 50 μg of antigen in incomplete Freund' adjuvant (Sigma Chemical Company, St. Louis, MO) intraperitoneally; day 30, 50 μg of antigen in incomplete Freund' adjuvant intraperitoneally; 15 days after, serum samples were drawn from the mice and the relative serum antibody titers were determined by ELISA. Four days before fusion, the mice that demonstrated the highest level of specific antibodies received an intravenous injection (20 μg) (10). All procedures related to use of animals was conducted in compliance with ethical guidelines guaranteed by the BIOMED ethical committee.

Hybridoma production. Spleen cells from immunized mice were prepared 4 days after the last immunization and were then fused with 2,5 x 107 NSO/2 mouse myeloma cells in 50% polyethylene glycol (Sigma) using the procedure described by Kohler et al. (11). Cells were suspended in hypoxanthine-aminopterin-thymidine (HAT) selecting medium, made with RPMI medium supplemented with 20% Fetal Bovine Serum (FBS) (Hyclone Laboratories, Logan, UT), gentamycin (80μg/mL), 2 mmol/L L-glutamine, 10-2 mol/L hypoxanthine, 4x10-5 mol/L aminopterin and 1,6 x 10-3 mol/L thymidine (Sigma Chemical Co.,St. Louis, MO). Aliquots of 100 μL were seeded on 96-well tissue culture plates. The plates were incubated at 37°C in moisturized atmosphere with 5% CO2 in air. Ten days after fusion, supernatants from cultures exhibiting hybridoma growth were screened by ELISA for the production of

anti-FA and anti-Stm1p antibodies. All positive cultures were expanded to 24-well plates and the cells were frozen under liquid nitrogen in RPMI medium containing 20% FBS and 10% dimethylsulfoxide (DMSO) (SIGMA). Hybridomas of interest were selected and cloned twice by the limiting dilution technique. To induce ascitic fluid, the recloned hybridomas were grown in the peritoneal cavity of BALB/c mice, previously injected intraperitoneally with 0.5 mL of 2,6,10,14 Tetramethylpentadecane (Pristane from Sigma) (10).

Purification of monoclonal antibodies. Immunoglobulins against Stm1p and AF were purified from the ascetic fluid by precipitation with 50% saturated ammonium sulphate. The pellet was dissolved in PBS and dialyzed (10).

Isotype determination. The isotype of monoclonal antibodies were determined with a commercial Kit, (Mouse Typer Isotyping Panel from BioRad Laboratories, Hercules, CA, USA) based on ELISA using HRP-conjugated antibodies to IgG1, IgG2a, IgG2b, IgG3, IgM, IgA and κ and λ light chain.

ELISA. The ELISA assay was performed as previously described by Voller et al.(12). Briefly, 96-well microtitration plates (Dynatech Immunolon 2), were coated with 2 µg/mL of AF and Stm1p, incubated overnight at 4 °C and then washed twice with PBS containing 0,05% tween 20 (PBS-T). The plates were blocked by the addition of PBS containing 1% Bovine Serum Albumin (BSA) (Sigma) and incubated at 37°C for 1h. Following three washes with PBS-T, the hybridoma culture supernatants were added for 1 h incubation at 37 °C. Plates were washed and incubated with 100 µL of goat anti-mouse polyvalent immunoglobulin conjugated with alkaline phosphatase (Sigma). Diluted 1:3000 in PBS for 1 h at 37°C. Plates were washed three times with PBS-T and incubated with 100 µl of p-nitro-phenyl-phosphate (1mg/mL; Sigma) in 0,2 mol/L $NaHCO_3$, 0,2 mol/L $NaHCO_3$, 5 mmol/L $MgCl_2$ (pH 9,5), then incubating at 37°C for 30 min. The reaction was stopped by adding 50 µL of 3 mol/kg NaOH to each well, and read at 405 nm. An absorbance of 0.2 OD or higher was considered positive.

Dot-Blot. The dot blot procedure was described in Bøgh et al. (13). Briefly, 0.2 µg of purified Stm1p, and AF were dotted onto nitrocellulose membranes. After the membranes were blocked with PBS-BSA 3%, they were cut into squares containing the antigen dots. Subsequently, these squares were incubated in 500 µl hybridoma clones supernatant, washed, incubated in goat anti-mouse Immunoglobulin conjugate with alkaline phosphatase (Sigma) washed and finally incubated in carbonate buffer containing 5 bromo-4-chloro 3 indolylphosphate (BCIP) (0,165 mg/mL) and nitroblue tetrazolium (NBT) (0,33 mg/mL).

SDS-PAGE and Immunoblotting. Purified AF, Stm1p and S-100' extract were subjected to SDS-PAGE according to the standard method described by Laemmli (14) and transferred to 0.45 µm nitrocellulose membranes (Bio Rad Laboratories, Hercules, CA) for 1 h (15). The blots were then blocked with 3% (w/v) BSA (Sigma) in PBS, overnight. Nitrocellulose strips were then incubated for 1.5 h with the hybridoma clones supernatants and washed three times for 15 min with 0,05% Tween 20 in PBS. The strips were then incubated for 1h at room temperature with goat anti-mouse immunoglobulin, conjugated with alkaline phosphstase (Sigma). The nitrocellulose strips were developed in carbonate buffer containing 5 bromo-4-chloro 3 indolylphosphate (BCIP) (0,165 mg/mL) and nitroblue tetrazolium (NBT) (0,33 mg/mL).

Detection of mAbs specific for carbohydrate epitopes. Periodate treatment of the antigen was based on the method of Xu et al. (16) with minor modifications. Briefly, NC membranes with dotted antigen and blocked were treated with 100 µL 200 mmol/L sodium acetate buffer (pH 5.5) and 100 µL 30 mmol/L sodium metaperiodate for 1 h at 23 °C in the dark. The reaction was stopped by addition of 100 µL 20 mmol/L sodium metabisulfite. After washing, treatment with mAbs and enzyme-conjugated second antibody was performed as described above.

Detection of mAbs specific for protein epitopes. mAbs with specificity for protein antigenic epitopes were detected using enzymatic digestion with proteinase-K according to the method previously described (17).

RESULTS

Production and selection of mAbs. We conducted somatic fusions to obtain monoclonal antibodies against Stm1p and AF of *S. cerevisiae*. We used the spleens of mice showing the best immune humoral response as determined by ELISA. After cloning and selection we obtained two mAbs against AF (2G5B2, 2G5C2) and two against Stm1p protein (1G11D4, 2G1B10) for further studies. We selected these mAbs on the basis of their high reactivity and the possibility of differentiate AF from Stm1p, and they were characterized. The isotyping studies demostrated that one mAb (1G11D4) belongs to the IgG2b class, while the rest belongs to the IgG1 class (Table 1).

Table 1. Characteristics of Monoclonal antibodies against Stm1p and AF of *Saccharomyces cerevisiae* as determined by ELISA, periodate oxidation, proteinase-K digestion, Dot Blot and Western Blotting.

Monoclonal Antibody	Isotype	Reactivity with		Sensitivity to		Nature of Antigenic epitope
		AF	Stm1p	Periodate	Proteinase-K	
Against AF						
2G5B2	IgG1	+	±	+	+	GP
2G5C2	IgG1	+	±	-	+	P
Against Stm1p						
1G11D4	IgG2b	+	+	-	+	P
2G1B10	IgG1	+	+	-	+	P

++: strong reactivity, ±: weakly reactivity, -: *Lack of reactivity*, GP=glycoprotein, P=protein

All mAbs recognize their purified antigen as were demonstrated by ELISA, and Dot Blot (Figure 1).

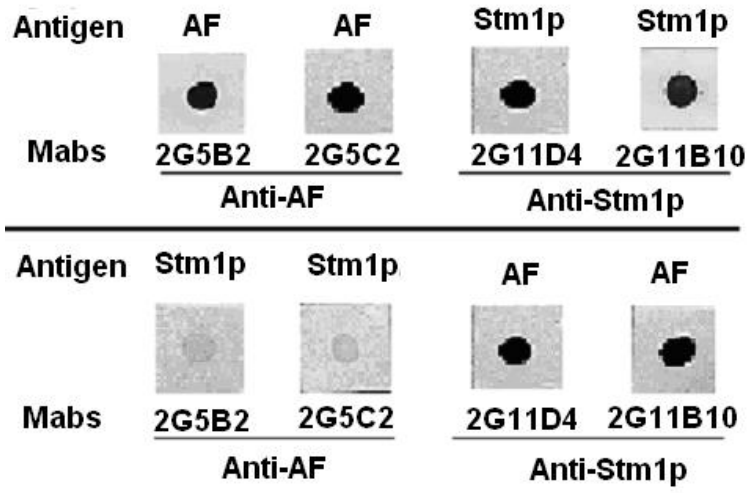

Figure 1. Reactivity and cross-reactivity of monoclonal antibodies against association factor and Stm1p of *Saccharomyces cerevisiae* by dot-blot. Purified AF and Stm1p were doted onto nitrocellulose squares. After blocking, each square was subjected to dot-blot as described.

The Western Blot using S-100' extract of *S. cerevisiae* showed that mAbs against Stm1p recognize two bands (43 and 32 kDa) and mAbs against AF (2G5B2, 2G5C2) recognized strongly the 43-kDa band and faintly the 32 kDa band (Figure 2, Table 1) The molecular weight of the recognized bands correspond exactly to the ones reported for AF (43 kDa) and Stm1p (32 kDa) (6,8).

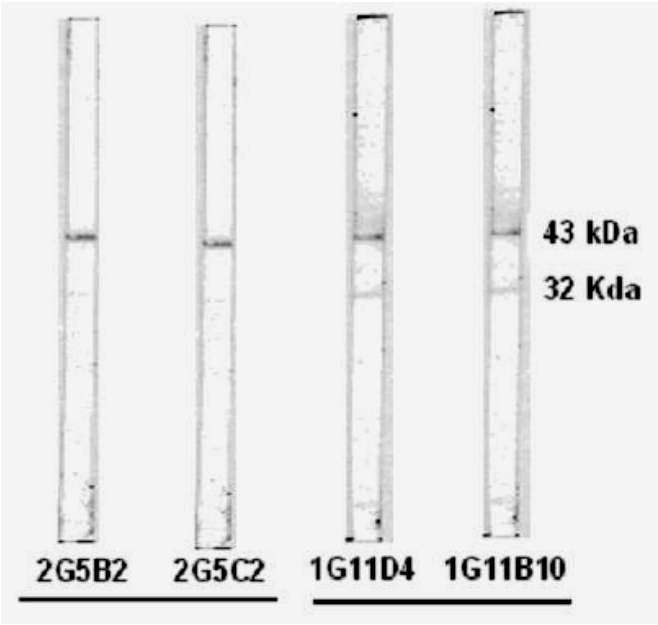

Figure 2. Reactivity of monoclonal antibodies against AF and Stm1p of *Saccharomyces cerevisiae* by western blot. S-100´ extract of *Saccharomyces cerevisiae* was subjected to electrophoresis and transferred onto nitrocellulose membranes. The strips were revealed using the mAbs.

Specificity and cross-reactivity of the mAbs. The specificity of all the mAbs was verified (Western Blot using S-100' extract) by their ability to react with only two protein bands (43 and 32 kDa).

Cross-reactivity studies with AF and Stm1p of *S. cerevisiae* revealed that all mAbs against Stm1p also recognized purified AF, but only two mAbs against AF (2F10D6, 2F10G2) recognize strongly Stm1p. The other two mAbs (2G5B2, 2G5C2) recognize AF strongly and slightly Stm1p, these mAbs could differentiate these proteins, which is not possible with polyclonal antibodies. The cross-reactivity of mAbs were also assayed by western blot using antigens prepared from *Leishmania chagasi, Leishmania amazonensis, Trypanosoma cruzi, Trypanosoma rangeli, Taenia solium* and *Onchocerca volvulus,* and none of the antibodies reacted with these antigens (data not shown).

Biochemical nature of the antigenic epitopes bound by mAbs. Proteinase K digestion and periodate oxidation studies showed that one mAb against AF (2G5B2) bound glycoprotein antigenic determinant, while the rest detected protein epitopes (Table 1, Figure 3).

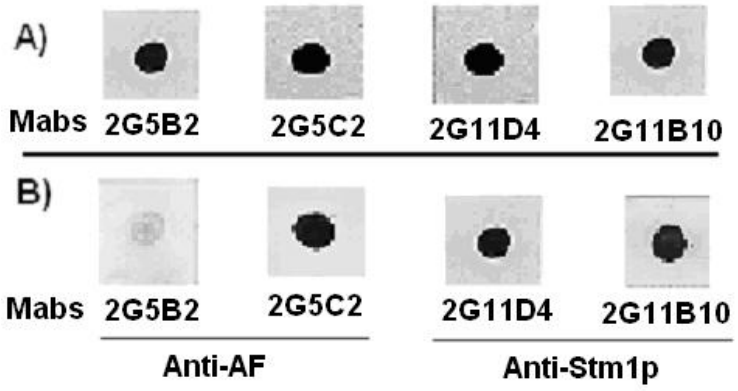

Figure 3. Reactivity of monoclonal antibodies against AF of *Saccharomyces cerevisiae* A) before and B) after periodate treatment. Purified AF was dotted onto nitrocellulose squares. After blocking, each square was subjected to dot-blot as described.

DISCUSSION

The aim of this work was to obtain and characterize monoclonal antibodies against AF and Stm1p of *S. cerevisiae.* We selected two mAbs against AF (2G5B2, 2G5C2) and two others against Stm1p (1G11D4, 2G1B10) for further characterization. Cross-reactivity studies by ELISA and Dot-Blot with AF and Stm1p revealed that mAbs against Stm1p also recognized purified AF, demonstrating that they recognize a peptide epitope that also is present in AF. However, mAbs against AF (2G5B2, 2G5C2) reacted strongly with AF and weakly with Stm1p.

The mAb 2G5B2 has specificity to glycosidic residues which indicates that AF is a glycosylated protein. Therefore, this characteristic could be the cause of the antigenic difference between both proteins. In addition, the glycosylation of AF may explain why it has a molecular weight higher than STM1p (18,19).

In relation to the low reactivity of 2G5C2 to Stm1p could indicate that this protein is present in lower concentration than AF in the yeast extract and as a result there is a weak reaction

with Stm1p although we cannot discard the possibility of the existence of a difference in the peptide epitope recognized by this mAb.

The mAbs herein described could be an important tool in functional and immunolocalization studies of these proteins. Experiments are in progress to resolve these questions.

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