Genetic immobilization of RNase Rny1p at the Saccharomyces cerevisiae cell surface

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Genetic immobilization of the yeast RNase Rny1p was performed by creating a hybrid protein containing the signal sequence of the S. cerevisiae cell wall protein Ccw12p followed by the catalytic part of the Rny1p (amino acids 19 to 293) and additionally 73 amino acids of the Ccw12p including the GPI-anchoring signal. The construct was expressed in S. cerevisiae VMY5678 and the hybrid protein was secreted through the plasma membrane and incorporated into the cell wall through GPI-anchoring in the same way as the Ccw12p. Thus, it could be released from the wall by β-1,3-glucanase. It retained RNase activity with the optimal pH of about 9 and the optimal temperature at 60°C. It was significantly more stable than the wild type enzyme and retained activity at 50°C for at least 6 hours; at 60°C it maintained full activity for at least 4 h, and at 70°C it lost activity in about 2 h. No DNase activity of the Rny1/Ccw12p was detected. Yeast cells expressing the hybrid protein were successfully used instead of RNase A in a standard procedure for yeast chromosomal DNA preparation with the advantage of quick and easy quantitative removal of the RNase activity from the reaction mixture.

Key Words—Ccw12p; genetic immobilization; Rny1p; Saccharomyces cerevisiae; surface display; yeast cell wall

Introduction

All living cells produce a number of specific ribonucleases that function in a variety of cellular processes, but they also produce general RNases of low specificity typically secreted or targeted to lysosomes or vacuoles. Such enzymes include members of the RNase A, RNase T1 and RNase T2 families. The T2 RNases (EC 3.1.27.1) are found in all organisms so far examined (Deshpande and Shankar, 2002), while RNase T1 exists only in bacterial and fungal organisms, and the RNase A family enzymes are highly represented in animals. The optimal pH range of many T2 ribonucleases is between 4 and 5, in contrast with the alkaline (pH 7–8) or weakly acidic (pH 6.5–7) pH optima of enzymes of the RNase T1 and RNase A families. Furthermore, T2 ribonucleases generally cleave at all four ribonucleotides, whereas RNase A and RNase T1 family members tend to be specific for pyrimidines or guanosine, respectively (Deshpande and Shankar, 2002; Irie, 1999).

The YPL123C/RNY1 gene encodes a 48-kDa protein with an amino acid sequence containing the two active-site motifs characteristic for the T2 RNase family. The N-terminal 293 amino acids of the Rny1p can be aligned to the sequence of T2 RNase from Aspergillus oryzae, which is the archetypal enzyme in this family (Irie, 1997). In addition, amino acids 294–434 of the Rny1p form a C-terminal extension not found in other T2 RNase family members. Rny1p has four potential...
glycosylation sites and a putative secretion signal of 18 amino acids at the N-terminus. The physiological
to this enzyme is still not completely clear although in the last years two interesting functions have been
proposed. MacIntosh et al. (2001) proposed a role of the enzyme in regulation of membrane permeability
and stability, while the more recent results indicate that it may have a role in degradation of cytosolic RNAs in
the state of oxidative stress. Under such conditions Rny1p, which normally accumulates in vacuoles,
seems to be released into cytosol where it promotes cleavage of tRNAs but also triggers signals unrelated
to its RNase activity which lead to cell death (Thompson and Parker, 2009).

Genetic immobilization is a process in which a homo-
logous or a heterologous protein is genetically fused to the whole or a part of a fungal cell wall protein
directing the chimeric product into the fungal cell wall
where it is covalently attached to glucan. In this way
the production of the protein of interest is accompa-
nied with its immobilization to an insoluble matrix of
the yeast cell wall (Murai et al., 1997a, b). Two ways of
covalent attachment of fungal wall proteins to glucan
have been described. About half of so-far-described
yeast cell wall proteins contain C-terminal signals for
the addition of GPI-anchors (Yin et al., 2005). These
proteins are transported to the cell membrane in the
GPI-anchored form after which they are transferred to
β-1,6-glucan moieties attached to β-1,3-glucan (Klis et
al., 2006). It has been shown that the addition of the
GPI-anchoring signal to different proteins from yeast
or other sources could direct them to the cell wall
where they were attached to glucan in the same way
as autochthonous cell wall proteins (Murai et al.,
1997a, b, 1999; Schreuder et al., 1993; Ueda et al.,
1998).

Besides the GPI-anchored proteins there is another
set of yeast proteins covalently attached to glucan. It is
the so-called Pir-protein family which in Saccharomy-
ces cerevisiae consists of four members (Mrša et al.,
1997). Pir-proteins contain a repeating motif at the N-
terminus which serves for their covalent attachment to
β-1,3-glucan by an alkali-sensitive ester bond formed
between a particular glutamine and glucose (Ecker et
al., 2006). Simple fusion of a heterologous protein with
the repeating sequence of a Pir-protein was insufficient
for its incorporation into the cell wall since Pir-
proteins also require other structural elements, and
even the native conformation for the proper attach-
ment (Teparić et al., 2007). However, several proteins
were successfully immobilized at the yeast surface by
fusions with complete Pir-proteins (Andrés et al.,
2005). It is worth noting that the two procedures are
based on fusions at two different sides of the protein of
interest; thus, the immobilization protocol can be cho-
sen according to the importance of the N-terminal or
the C-terminal part for the conformation, activity, and
stability of the protein.

The purpose of this work was to attempt genetic im-
mobilization of the yeast RNase Rny1p at the S. cere-
visiae surface in order to create a potentially valuable
molecular tool with application in different processes
where degradation or depletion of RNA is required.
For this purpose a recombinant protein, Rny1/Ccw12p,
was constructed by introducing the part of the
yeast RNase gene RNY1 coding for amino acids
19–293, containing all motifs required for the enzyme
activity, into CCW12 immediately after the part encod-
ing for the Ccw12p signal sequence (amino acids
1–19). Therefore, the construct contained the up-
stream sequences and the N-terminal signal sequence
of Ccw12p, following by the active domain of the
RNase Rny1p, and finally the C-terminal GPI-anchor-
ing signal and the downstream sequences of Ccw12p.
It could be shown that Rny1/Ccw12p was transported
to the cell surface and that it was covalently incorpo-
rated in the cell wall through the GPI remnant of the
Ccw12p part of the recombinant protein. Properties of
Rny1p/Ccw12p were examined and compared with
those of the RNase A, the most often used RNase with
different applications including isolation of genomic or
plasmid DNA from a variety of biological sources. Re-
combinant Rny1/Ccw12p showed very good activity
and stability in broad pH and temperature ranges. Be-
sides, commercial RNase A used in the soluble form is
quite difficult to inactivate and/or remove completely
from reaction mixtures after digestion, while yeast cells
displaying recombinant RNase are easily and com-
pletely removed by a quick centrifugation step leaving
no detectable traces of RNase activity. Furthermore,
the recombinant RNase can be produced cheaply and
in unlimited quantities.

Materials and Methods

Strains and media. The Saccharomyces cerevisiae
strain used in this work was VMY5678 (MATα ura3-52
leu2-3, 112 his3-∆200 trp1-∆901 lys2-810 suc2-∆9 GAL
Genetic immobilization of RNase Rny1p

ccw5:: (kanMX) ccw6:: (kanMX) ccw7:: (kanMX) ccw8:: (kanMX) URA3). This strain was derived from strain SEY6210 by deleting all four genes coding for Pir proteins. The reason for using this particular strain was our previous observation that it incorporated a somewhat higher amount of other cell wall proteins (not shown). Besides, it had a higher transformation frequency than its wild type. Yeast was grown in standard yeast nitrogen base selective medium (YNB) supplemented with the required amino acids. All cloning and transformations were made in Escherichia coli strain DH5α. For all DNA manipulations and yeast transformations standard procedures were used (Adams et al., 1998).

Construction of recombinant RNY1/CCW12. For the construction of the recombinant gene RNY1/CCW12, plasmid pCCW12R1-2 was used (Ragni et al., 2007). This plasmid contains regions of the CCW12 gene from −282 to +607 amplified from pME11 (Hagen et al., 2004), in which bp 223–309 (amino acids 75–103) coding for the two repeating sequences of Ccw12p were deleted, subcloned in pRS425 as a HindIII–Sacl fragment (Ragni et al., 2007). The strategy for RNY1/CCW12 construction consisted of two steps of PCR amplification. In the first step, the Psfl site was introduced in CCW12 using plasmid pCCW12R1-2 as a template and two pairs of primers: CCW12F1 and CCW12R1; and CCW12F2 and CCW12R2 respectively (Table 1). PCR amplification was performed through 35 cycles as follows: 45 s at 95°C, 90 s at 52°C, and 45 s at 72°C. The Psfl site was introduced directly preceding HA-tag in pCCW12R1-2 to allow in frame insertion of RNY1 into the CCW12 sequence. The part of the RNY1 gene coding for amino acids 19–293 was amplified from yeast genomic DNA with amplification primers CCW12RNY1F2 and RNY1HaΔCR2 (Table 1), containing restriction sites Psfl and NotI respectively. PCR amplification was performed through 35 cycles as follows: 45 s at 95°C, 90 s at 68°C, and 80 s at 72°C. The RNY1 fragment obtained was introduced in plasmid pCCW12R1-2 in a frame with CCW12, between the Psfl and NotI site. The resulting plasmid was named pRS425/RNYCCW12-HA.

Isolation of cell wall proteins. Cells were washed twice with water and twice with 50 mM K-phosphate buffer pH 8.0. After that, cells were resuspended in the same buffer and broken with glass beads using a vortex (5 min, maximal speed), washed 4 times with 50 mM K-phosphate buffer pH 8.0 and boiled 2 times for 10 min in Laemmli buffer (50 mM Tris–HCl buffer pH 6.8, 2 mM Na-EDTA, 2% SDS, 10% glycerol, 0.001% bromphenol blue, 5% β-mercaptoethanol). After that, cell walls were washed 4 times in 50 mM K-phosphate buffer pH 8.0 and once with 50 mM Tris–HCl buffer pH 7.4. Cell walls were then resuspended in 0.1 ml of the same buffer to which 0.1 U of β-1,3-glucanase (Quantzyme ylgTM, Quantum Biotechnologies) per each OD600 unit of cells was added. The suspension was incubated for 2 h at 37°C and centrifuged 3 min at 10,000 rpm. Extracted proteins were subjected to electrophoresis and detected by Western blot using anti-HA-peroxidase antibodies (Roche).

Isolation of proteins from media. Growth media were collected by centrifugation (5 min, 3,000 rpm), and proteins were precipitated with cold acetone (22.5 ml of media: 15 ml of acetone) for 2 h at 4°C. Precipitated proteins were collected by centrifugation for 1 h at 10,000 rpm and dissolved in 50 μl of Laemmli buffer.

Electrophoresis and blotting. Electrophoresis was performed by the method of Laemmli (Laemmli, 1970). To visualize proteins with the HA-tag, proteins were blotted to nitrocellulose which was then incubated for 1 h in 10 ml of blocking buffer (50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 0.1% v/v Triton X-100, 1% w/v non-fat dry milk), and then 1.5 h in the same buffer with anti-HA-peroxidase mouse monoclonal antibodies (Roche) at 1 : 8,000 dilution. Finally, blots were washed three times with the blocking buffer and developed using the ECL kit (PerkinElmer).

Detection of RNase activity by SRED (Single Radial Enzyme Diffusion) method. RNA (RNA from Thorula yeast type VI, Sigma) solution (10 mg/ml in 10 mM Tris–HCl buffer pH=7.5), ethidium bromide (5 g/L in

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>CCW12F1</td>
<td>CCCCTCGAGGCAACAACTATCTGCATAA</td>
</tr>
<tr>
<td>CCW12R1</td>
<td>GGAAGTAGTGATACACTCAACTGCAGA</td>
</tr>
<tr>
<td>CCW12F2</td>
<td>GCGGCAGAGGAC</td>
</tr>
<tr>
<td>CCW12R2</td>
<td>GTACCAGTCGCACTGCTGAGTT</td>
</tr>
<tr>
<td>CCW12RNY1F2</td>
<td>GTCCCTCTCGGCCGTCGAGGCA</td>
</tr>
<tr>
<td>RNY1HaΔCR2</td>
<td>TGGGACATAACC</td>
</tr>
</tbody>
</table>
Prepared. A mixture containing 5.1 ml of reaction buffer (pH = 7.5) and agarose (20 g/L in distilled water) were prepared. A mixture containing 5.1 ml of reaction buffer, 270 µl of RNA solution and 54 µl of ethidium bromide was mixed well and preheated to 50°C. Agarose was melted, cooled to 50°C and 5.4 ml was mixed with the prepared RNA mixture and immediately poured in Petri dishes to prepare gel plates about 2 mm thick. After congelation at room temperature, cylindrical sample wells were punched in the gels. Samples containing 20 OD$_{600}$ units resuspended in 20 µl 10 mM Tris-HCl buffer pH 7.5 were placed in the wells. Gels were incubated at 30°C, and periodically tested on a UV transluminator (Hoefer, Macrovue UVI-20) for appearance of circular dark zones (formed as RNase digested the RNA substrate).

**RNase assay.** Enzymatic assays were done in 10 mM Tris-HCl buffer pH 7.4, for 30 min at 30°C. Cleavage reactions were performed with 10 OD$_{600}$ units of cells with immobilized recombinant RNase, or 10 µg of RNAse A (Roche) and 10 µg/µl of RNA (RNA from *Thorula* yeast type VI, Sigma). After that yeast cells were removed by a short centrifugation step (1 min, 8,000 rpm) and samples were immediately subjected to agarose gel electrophoresis (1.5% agarose in TAE-buffer). The amount of RNA was quantified in electrophoresis gels using the program ImageJ (http://rsb.info.nih.gov/ij/) and the RNase activity was expressed as the difference in the amount of RNA in treated and untreated samples.

**pH optima determination.** In order to determine pH optima the described assay was performed in 10 mM Na-acetate (pH 3.8 and 5.8), Na-phosphate (pH 6.8, 7.4 and 8.0) and Na-carbonate (pH 9 and 10) buffers. Activity was estimated as described above.

**Temperature optima and stability determination.** In order to determine temperature optima enzymatic assays were done in 10 mM Tris-HCl buffer pH 7.4 for 15 min at different temperatures in the range from 30°C to 80°C. For the temperature stability test yeast cells with immobilized RNase were incubated for 2 to 6 h at temperatures of 50°C, 60°C, or 70°C and the enzymatic assays were done as described above.

**Isolation of genomic DNA from yeast cells.** Isolation of genomic DNA from yeast cells was done by the standard procedure described by Adams et al. (1997). In the RNA degradation step commercial RNase A used in the standard protocol was replaced with yeast cells with immobilized Rny1/Ccw12p (10 OD$_{600}$ in 10 µl). After the RNA degradation step (30 min at 50°C) yeast cells were removed from the reaction mixture by centrifugation (1 min, 8,000 rpm), and the procedure was completed following the standard protocol.

**Results**

In this work Ccw12p, one of the most abundant GPI-anchored cell wall proteins in *S. cerevisiae* (Mrša et al., 1999), was used for surface display of the yeast RNase Rny1p. Construction of the recombinant RNY1/CCW12 gene was performed in two steps. First, the part of RNY1 coding for the catalytically active domain of the protein (amino acids 19–293) was amplified by PCR using yeast genomic DNA as the template. In this step appropriate restriction sites were introduced through amplification primers to allow in-frame insertion of the gene into the CCW12 sequence inserted in the plasmid pRS425. CCW12 was previously modified (Ragni et al., 2007) by deleting a part of the gene (bp 223–309) coding for the two repeats in Ccw12p (amino acids 75–103) and inserting the three repeats of the haemagglutinin epitope (HA) immediately after the signal sequence. The deletion made the construct smaller but had no influence on the further construction of RNY1/CCW12. The amplified RNY1 fragment was introduced by replacing the part of CCW12 between the NotI and PstI restriction sites placing the gene between the CCW12 signal sequence and the HA-tag, thus obtaining the plasmid named pRS425/RNYCCW12-HA. The final construct is presented in Fig. 1.

*S. cerevisiae* strain VMY5678 (Mrša et al., 1999) was transformed with the plasmid pRS425/RNYCCW12-HA and cell wall proteins were isolated and analyzed by Western blot using anti-HA antibodies (Fig. 2A). Results showed that the major part of Rny1p/Ccw12p was covalently incorporated in the cell wall and could only be extracted from the wall by β-1,3-glucanase. Only a small portion of the protein was found in the fraction of non-covalently attached proteins extracted by hot SDS and an even smaller fraction was found secreted into the medium. This result confirmed that the recombinant protein was transported to the cell wall and attached to glucan in the way characteristic for Ccw12p. The average molecular mass of Rny1/Ccw12p was about 100 kDa, as estimated by electrophoresis. The calculated mass of the protein part of the molecule was 44 kDa after removal of the signal sequence. The protein, however, had 5 potential N-
glycosylation sites. Thus, its large size could be a result of extensive glycosylation, which is corroborated by the smearing of the band typical for highly glycosylated proteins. The possibility of irregular electrophoretic migration, again typical for glycoproteins with high carbohydrate content, can also not be excluded.

Activity of the recombinant protein was first detected by the “semi quantitative” SRED method (Fig. 2B). A clear halo surrounding the wells containing three clones of RNY1/CCW12 transformants indicated that recombinant Rny1/Ccw12p retained activity in the immobilized form. Since the method was found to be qualitative,
but rather inappropriate for activity quantification, the assay consisting of reaction and subsequent electrophoresis with *ImageJ* quantification of the RNA bands was introduced. Since the aim of the work was to create a tool which could replace commercial RNases used as soluble enzymes, the activity of cells was compared with that of the most frequently used RNase A. Results showed that 1 OD$_{600}$ unit of yeast cells contained about the same activity as 0.1 µg of RNase A (about $5 \times 10^{-4}$ U according to Kunitz). In spite of the fact that a very small fraction of recombinant protein was also detected in the growth media by Western blot, it contained no detectable activity even after a prolonged incubation. This proved also that no autochthonous active RNase(s) were secreted into the medium.

Major enzyme properties of Rny1p/Ccw12p relevant for its application were compared to those of RNase A and the results showed that the optimal pH of the recombinant RNase was about 9, while RNase A was most active at neutral pH (Fig. 3A). Besides, Rny1/Ccw12p showed significantly higher optimal temperature at about 60°C (Fig. 3B). Rny1/Ccw12p was stable at 50°C for at least 6 h, at 60°C it maintained full activity for 4 h, and at 70°C it lost most activity in about 2 h. Specificity of the recombinant RNase was tested by using circular plasmid DNA (pRS425) and linear DNA fragments (λ/HindIII digest). No degradation of either DNA was detected.

To demonstrate the potential application of the recombinant RNase immobilized to yeast cells in protocols usually used for the isolation of genomic DNA from yeast, a typical procedure described in MATERIALS AND METHODS was followed using either RNase A or the yeast cells carrying the recombinant RNase at the surface. The result is shown in Fig. 4 and it can be seen that the cells displaying recombinant RNase completely degraded RNA in the DNA preparation under the conditions of the protocol. However, in contrast to the RNase A, Rny1/Ccw12p could easily be quantitatively removed from the preparation by a short centrifugation step (1 min, 8,000 rpm). To prove that the removal of RNase was quantitative, in another experiment RNA was only partially degraded using immobilized recombinant RNase for 30 min and then cells were centrifuged. Incubation of RNA in the supernatant after removal of cells for an additional 30 min showed no further degradation (Fig. 5).

**Discussion**

Genetic immobilization combines heterologous expression of proteins in fungi with their immobilization at a solid matrix, thus avoiding the often complicated protocols for chemical immobilization frequently ending with a loss of biological activity or increase of $K_m$. In this way the production of immobilized enzymes is simpler and cheaper. Most heterologous proteins constructed so far for yeast surface display have been GPI-anchored to the cell wall (Murai et al., 1997a, b, Fig. 3. A) RNase activity of Rny1/Ccw12p and RNase A at different pH; B) RNase activity of Rny1/Ccw12p and RNase A at different temperatures.

![Fig. 3.](image)

**Fig. 4.** Yeast genomic DNA isolated by a standard procedure (MATERIALS AND METHODS) using: 1. no RNase treatment during the isolation procedure; 2. wild type yeast cells instead of RNase; 3. yeast cells with immobilized Rny1/Ccw12p at the surface; 4. RNase A.
Genetic immobilization of RNase Rny1p

1999; Schreuder et al., 1993; Ueda et al. 1998). This method is based on the fact that the simple addition of the C-terminal GPI anchoring signal is usually a sufficient prerequisite for GPI anchoring of most homologous or heterologous proteins in yeast. GPI signals of different wall proteins have been efficiently used so far. In this work Ccw12p was chosen as the donor not only of the GPI signal, but also of the non-coding promoter and downstream sequences, as well as of the secretion signal sequence. This was motivated by the fact that Ccw12p was one of the most abundant cell wall proteins in cells grown under usual laboratory conditions (Mrša et al., 1999). As the source of the RNase activity we have chosen the catalytic domain of Rny1p/Ccw12p and an additional 30 min at 30°C after removal of cells by centrifugation.

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Fig. 5. A) RNA was treated with yeast cells as described in MATERIALS AND METHODS and subsequently subjected to electrophoresis: 1. RNA incubated 30 min at 30°C in the reaction mixture without yeast; 2. RNA incubated 30 min at 30°C with yeast cells carrying Rny1/Ccw12p; 3. RNA incubated 60 min at 30°C in the reaction mixture without yeast; 4. RNA incubated 30 min at 30°C with yeast cells carrying Rny1/Ccw12p and an additional 30 min at 30°C after removal of cells by centrifugation. B) Percentage of RNA in samples quantified by Image J.
Pir proteins of Saccharomyces cerevisiae are attached to β-1,3-glucan by a new protein-carbohydrate linkage. J. Biol. Chem., 281, 11523–11529.


