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Ray38p, a Homolog of a Purine Motif Triple-Helical DNA-Binding Protein, Stm1p, Is a Ribosome-Associated Protein and Dissociated from Ribosomes prior to the Induction of Cycloheximide Resistance in *Candida maltosa*

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Cycloheximide (CYH) resistance in *Candida maltosa* is dependent on the induction of a ribosomal protein, Q-type L41, the 56th residue of which is glutamine, not proline as in ordinary P-type L41. We found that a 38-kDa protein in a wild-type *C. maltosa* ribosomal fraction became undetectable upon CYH treatment but detectable again with the establishment of CYH resistance by the induction of Q-type L41. We cloned a gene coding for this protein and named it *RAY38* (ribosome-associated protein of yeast). Ray38p is a homolog of a purine motif triple-helical DNA-binding protein, Stm1p, and has a putative RNA-binding motif RGG. The ribosome-associated Ray38p was phosphorylated at serine and threonine residues, and Ray38p that was dissociated from ribosome by CYH treatment was highly phosphorylated in threonine residues. A *ray38* null mutant recovered faster from CYH-caused growth stasis than the wild-type strain, suggesting that the dissociation of Ray38p from ribosome facilitates the induction of CYH resistance in *C. maltosa*.

Cycloheximide (CYH) is an antibiotic that inhibits peptidyl transfer on the eukaryotic ribosome. Some Eumycetes are intrinsically resistant to CYH, and their resistances are divided into two classes, constitutive resistance and inducible resistance, in which cellular growth is arrested after the addition of CYH but resumes later (1). CYH-sensitive yeasts have only ribosomes with P-type L41, the 56th amino acid residue of which is proline, whereas CYH-resistance yeasts have ribosomes with Q-type L41, where the 56th amino acid residue is replaced with glutamine (2).

In our previous papers, we showed that the yeast *Candida maltosa* was inducibly resistant to CYH (1). *C. maltosa* has two types of multiple L41 ribosomal protein genes, *L41-Ps* and *L41-Qs*, which encode P-type L41 and Q-type L41, respectively. The expressions of *L41-Ps* are constitutive, whereas the expressions of *L41-Qs* are induced by the addition of CYH (3). By *in vivo* and *in vitro* experiments using mutant strains in which either all *L41-Qs* or all *L41-Ps* were knocked out, we also showed that ribosomes with only Q-type L41 were resistant to CYH, whereas those with only P-type L41 were sensitive to CYH but had a higher translational rate than those with only Q-type L41 under the condition without CYH (4). The promoter of *L41-Q2a* has a GCRE (Gcn4p-responsive element of *Saccharomyces cerevisiae*)-like element as an essential element for the induction by CYH. Amino acid starvation that induces the expression of many amino acid synthetic genes through the action of *GCN4* in *S. cerevisiae* also induced the expression of *L41-Q2a*. *C-HIS5*, one of the amino acid synthetic genes of *C. maltosa*, has a GCRE-like element in its promoter and is also induced by CYH. We suggested that *L41-Q2a* is induced in a Gcn4p-dependent manner by the addition of CYH or the starvation of amino acids (5).

In this paper, we report on finding a ribosome-associated protein that became undetectable in a ribosome fraction after the addition of CYH to *C. maltosa*

The nucleotide sequence reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence database with Accession No. AB057768.

Abbreviations used: CYH, cycloheximide; Δ, deletion; L41, L41 ribosomal protein; P-type L41, L41 which has proline as the 56th amino acid; *L41-Ps*, genes encoding P-type L41; Q-type L41, L41 which has glutamine as the 56th amino; *L41-Qs*, genes encoding Q-type L41.

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wild-type cells. This protein, named Ray38p (38-kDa ribosome-associated protein of yeast), is a serine/threonine phosphoprotein with an RGG motif. Its threonine residues are highly phosphorylated after the release from ribosomes by the addition of CYH. Ray38p was also released from ribosomes with P-type L41 by the addition of anisomycin, which had no effects on protein synthesis, and by a nutrient shift-down. Both conditions also induced Q-type L41. The dissociation of Ray38p from ribosomes suggests that the induction of CYH resistance in *C. maltosa*.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this work are *C. maltosa* IAM12247 (the prototrophic wild-type strain), CHAU1 (*his5, ura3, ade1*), CHA1 (*his5, ade1*), CMT100 (*his5::HIS5, ura3::URA3, ade1::ADE1*), ΔRAY38 (*RAY38a::HIS5, RAY38b::URA3, ade1::ADE1*), ΔL41-Qs (*L41-Q2a::HIS5, L41-Q2b::URA3, L41-Q3::ADE1*), and ΔL41-Ps (*L41-P1a::HIS5, L41-P1b::URA3, L41-P2::ADE1*). Minimal medium or YPD medium (1% yeast extract, 2% polypepton, and 2% glucose) was used as culture medium. CYH or anisomycin was added to the medium at the final concentration of 50 μg/ml or 1 mg/ml. The ΔRAY38 strain was constructed by sequential two-step gene replacements (6, 7) of the chromosomal *RAY38* genes using the *Pvu*II fragment of the RAY38 deletion cassette and was confirmed by Southern blot analysis. Isolation of total DNA from yeast cells was done according to the method previously described (8). All DNA enzymes were purchased from Takara Shuzo Co. Transformations of *C. maltosa* strains were performed by the modified lithium acetate method (9).

Construction of RAY38 deletion cassette. A 2.9-kbp *Xho*I–*Eco*RI fragment bearing *RAY38* was inserted between *Xho*I and *Eco*RI sites of pUC19. The *RAY38* open reading frame was deleted by removing a *Hin*dIII–*Hpa*I fragment. A blunt-ended *Sal*I fragment containing the *C-HIS5* (10) gene or a *Xho*I fragment containing the *C-URA3* (11) gene was then inserted between the blunt ended *Hin*dIII and *Hpa*I sites.

Ribosome preparation and Western blot analysis. Ribosomes were prepared as previously described (9), except that 0.5 M KCl was replaced with 0.25 M KCl. Samples were separated by 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and were transferred to a Hybond-C (Amersham) membrane in a transfer buffer, 25 mM Tris–HCl (pH 8.3) containing 0.15 M glycine and 20% methanol, by Electrophoretic Transfer (Bio-craft) at 20 mA for 12 h. After rinsing the blot with PBS (10 mM Na_2HPO_4, 10 mM $NaHPO_3$, 150 mM NaCl), the blot was blocked in PBS containing 5% skim milk and 0.1% Tween 20 for 1 h at the room temperature. Then the blot was incubated for 1 h at room temperature with anti-Ray38p antiserum diluted in 0.5% skim milk in PBS. After three 5 min washes with 0.5% Tween 20 in PBS, the blot was incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit immunoglobulin G (CAPPEL) diluted in 0.5% skim milk in PBS and washed three times with 0.5% Tween 20 in PBS. An anti-Ray38p antiserum was prepared from rabbits immunized with purified histidine-tagged Ray38p that was overproduced in *Escherichia coli* by the BL21/SET system. Specific recognition of the anti-Ray38p antiserum was confirmed by Western blot analysis on the extracts from ΔRAY38 strain (negative) and the wild type strain (positive). The protein-antibody complexes were visualized by ECL Direct System (Amersham).

Two-dimensional gel electrophoresis. A mixture of ribosomal proteins and ribosome-associated proteins was prepared from the ribosomal fraction by acetic acid method (12). Samples were analyzed by RFHR (radical-free and highly reducing method)–PAGE according to the Wada procedure (13).

Gel-filtration chromatography. Ribosome fraction of wild-type cells was applied on a Sephadex G-250 (Amersham Pharmacia Biotech) that was equilibrated with buffer [50 mM Tris–HCl (pH 7.6), 30 mM KCl, 10 mM $MgCl_2$]. Proteins were eluted with the same buffer at a flow rate of 1.0 ml/min.

Determination of peptide sequences of Ray38p. The 38-kDa protein was separated by 15% SDS–PAGE and transferred onto the Immobilon-Psq (Millipore) membrane in the transfer buffer [25 mM Tris–HCl (pH 8.3), 150 mM glycine, 20% methanol, 0.1% SDS]. The 38-kDa protein band was cut out after staining Ponceau 2R (Sigma). Sequencing of the protein was performed in a protein sequencer (Applied Biosystems Model 492) after lysyl-endopeptidase digestion and separation of the peptides by HPLC. Three peptide sequences, ELEGEVEGAEDAEAE, QQEVFFASTHAK, and AAATGSTKPEVNDKNFP, were obtained.

Screening of Ray38p-encoding gene. Three oligodeoxynucleotide primers (primer 1, 5′-CAACAAGAAGTNTTY-TTYGCN-3′; primer 2, 5′-YTTRTCRTTNACTTCWGGYTTN-3′; and primer 3, 5′-GAAGTNGAAGGNGCNGAAGAYGCN-3′) were synthesized on the basis of the above peptide sequences. PCR products by primer 2 and 3, and *C. maltosa* genomic DNA as a template, was used as a template for next PCR by primers 1 and 2. The resultant PCR product was used as a probe for screening of *C. maltosa* genomic library.

Immunoaffinity purification. Anti-Ray38p antiserum was bound to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech), put into columns, and washed with buffer [50 mM Tris–HCl (pH 7.6), 30 mM KCl, 10 mM $MgCl_2$]. Samples containing Ray38p were loaded onto the column, washed with a buffer [50 mM Tris–HCl (pH 7.6), 500 mM KCl, 10 mM $MgCl_2$], and then eluted with 100 mM glycine buffer (pH 2.0).

Detection of phosphorylated amino acids by anti-phosphoamino acid antibodies. Ray38p was first purified by the immuno affinity purification using anti-Ray38p antiserum from a 100,000*g* supernatant of 0.5 M KCl, washed ribosomes or S-100 fraction of CYH-treated cells. Western blotting was performed as described above using anti-phosphoserine, anti-phosphothreonine, or anti-phosphotyrosine monoclonal antibody (Sigma) as primary antibodies and peroxidase-conjugated goat anti-mouse immunoglobulin G (Sigma) as a secondary antibody.

Treatment of ribosomes with alkaline phosphatase. Ribosomes of 10 OD units were dialyzed against a buffer [100 mM Tris–HCl (pH 9.8), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$] and incubated with various amounts of alkaline phosphatase (Sigma P-5521 from bovine intestinal mucosa) for 30 min at 30°C. After treatment of alkaline phosphatase, samples were separated into ribosomal pellets and supernatant by ultracentrifugation (100,000*g*/4.5 h).

Measurement of growth curve. The growth of *C. maltosa* was monitored by an automatically recording incubator TN1506 (Advantec). CYH was added when OD_{660} was 0.1.

RESULTS

Finding of a Ribosome-Associated 38-kDa Protein and Its Dissociation from Ribosomes upon CYH Treatment of C. maltosa

To know the effect of CYH treatment on the protein profile of ribosomes, we analyzed ribosomal fractions of CYH-treated *C. maltosa* with SDS–15% polyacrylamide gel electrophoresis. Figure 1A shows that a 38-kDa protein band was detected in the samples from cells not treated with CYH but not in those from CYH-treated cells. Since wild-type cells not treated with CYH have ribosomes that contain P-type L41 and are

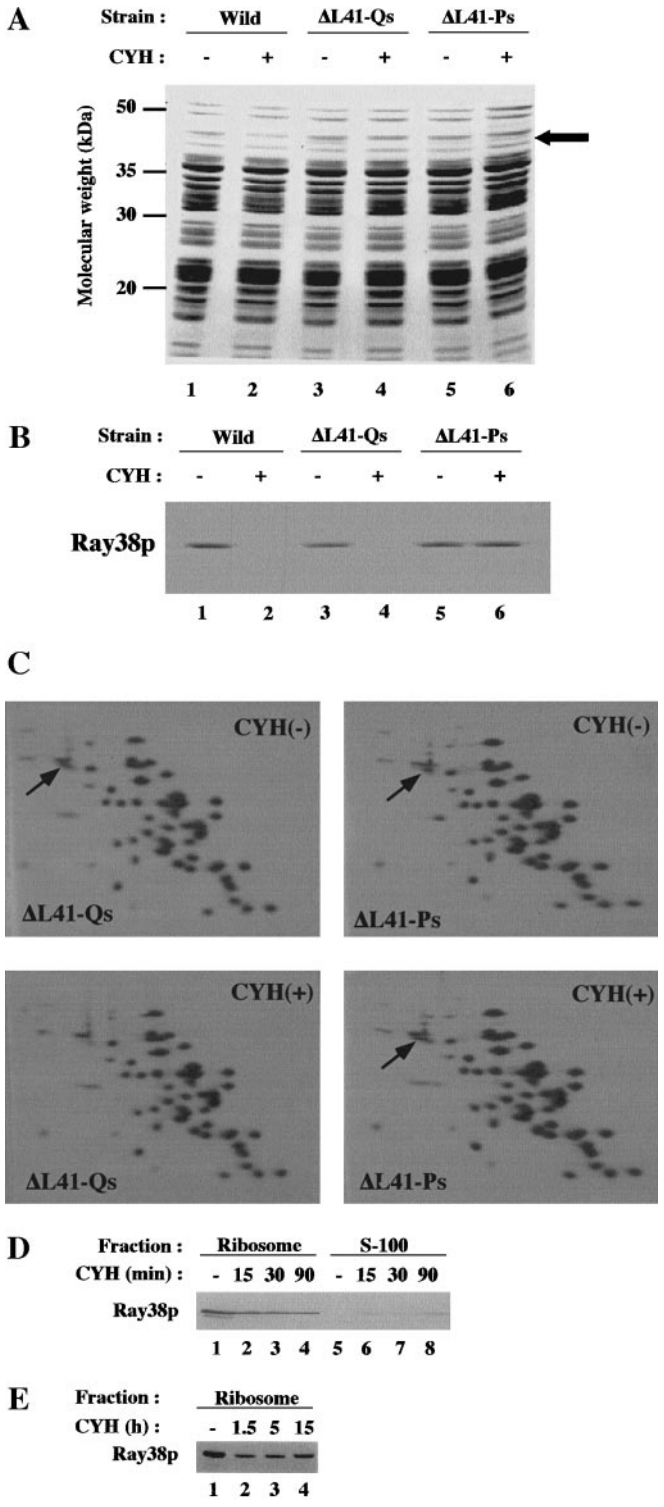

FIG. 1. A 38-kDa protein dissociates from P-type ribosomes containing P-type L41 protein, but not from Q-type ribosomes containing Q-type L41 protein, by the addition of CYH. (A) Ribosome fractions from the wild-type, ΔL41-Qs and ΔL41-Ps strains were separated by 15% SDS–PAGE followed staining with Coomassie brilliant blue. Samples were prepared from the wild-type (lanes 1 and 2), ΔL41-Qs (lanes 3 and 4), ΔL41-Ps (lanes 5 and 6) strains. Strains were grown in the absence of CYH (lanes 1, 3, and 5) and in the presence of 50 μg/ml CYH 1.5 h before harvest (lanes 2, 4, and 6). The position of Ray38p is indicated by an arrow. (B) Western blot analysis using anti-Ray38p antiserum. Each lane was the same as above. (C) Ribosome fractions from respective strain grown in the absence of CYH [CYH(−)] or in the presence of CYH [CYH(+)] were separated by two-dimensional gel electrophoresis and stained with Amide Black (Wako). The position of Ray38p was determined by Western blot analysis and is indicated by arrow. (D) The released Ray38p was found in S-100 fraction. The ribosome fractions of wild-type strain are lanes 1, 2, 3, and 4 and S-100 fractions are lanes 5, 6, 7, and 8. In the absence of CYH: lanes 1 and 5. After the addition of CYH: 15 min, lanes 2 and 6; 30 min, lanes 3 and 7; 1.5 h, lanes 4 and 8. (E) Change in the amount of ribosome-associated Ray38 after the addition of CYH. In the absence of CYH: lane 1. After addition of CYH: 1.5 h, lane 2; 5 h, lane 3; 15 h, lane 4.

sensitive to CYH until L41 is replaced with Q-type L41, we did similar experiments using either CYH-sensitive ΔL41-Qs strain that carried only *L41-Ps* or CYH-resistant ΔL41-Ps strain that carried only *L41-Qs*. The 38-kDa protein band was detected in the sample from the CYH-treated ΔL41-Ps strain but not in the one from the CYH-treated strain with only P-type L41. The same protein band was detected in the samples from both strains when they were not treated with CYH. A clearer view of the ribosomal protein profile and the behavior of the 38-kDa protein was given by using two-dimensional polyacrylamide gel electrophoresis (Fig. 1C). This disappearance of the 38-kDa protein is most likely a result of its dissociation from ribosomes, because, after the CYH treatment, the ribosome fraction of wild-type cells contained a smaller amount of the 38-kDa protein (Fig. 1B and 1D) and some of the protein appeared in the ribosome-free supernatant fraction (Fig. 1D). The amount of the ribosomal 38-kDa protein in the wild-type strain seems to have been restored when CYH resistance was established after 15 h (Fig. 1E, lane 4).

These results suggest that the 38-kDa protein is dissociated from ribosomes upon CYH treatment of wild-type *C. maltosa* and that this dissociation could be related to the induction of CYH resistance.

The 38-kDa Protein Is Not a Ribosomal Protein but a Ribosome-Associated Protein

To confirm ribosomal localization, we fractionated a crude ribosome preparation of wild-type cells by gel filtration and examined the presence of the 38-kDa protein among the fractions. The distribution profile of the 38-kDa protein was very similar to that of L41 (Figs. 2A–2C), and hence most cellular 38-kDa protein was binding to ribosomes unless CYH was added to the culture medium. This ribosome association of the 38-kDa protein was resistant to washing with 0.25 M KCl but not to washing with 0.5 M KCl (data not shown), indicating that the 38-kDa protein is a novel ribosome-associated protein but not an intrinsic ribosomal protein.

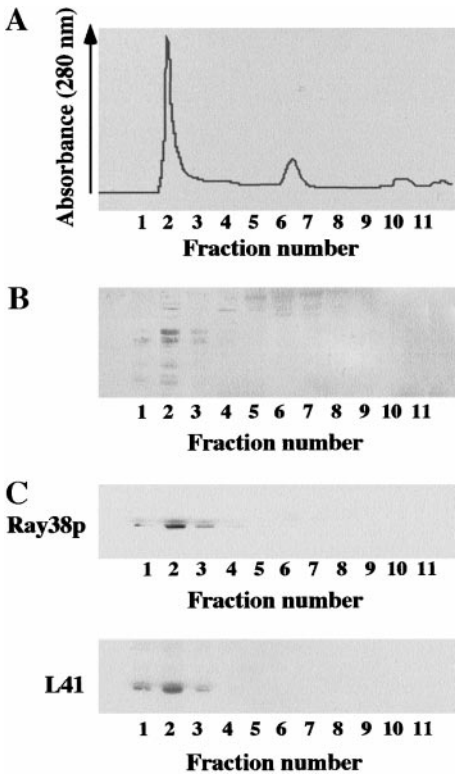

FIG. 2. Cytoplasmic Ray38p is colocalized with ribosomes. (A) Gel-filtration column chromatography of the ribosome fraction of wild-type cells. (B) Collected fractions were separated by 15% SDS–PAGE, blotted to the membrane and stained with Amido black. (C) The same membrane in B was treated with anti-Ray38p antiserum and anti-L41 antiserum.

Identification of a Gene Coding for the 38-kDa Protein

The 38-kDa protein was also dissociated from ribosomes by the action of alkaline phosphatase. We purified ribosomes by washing with 0.25 M KCl and treated them with alkaline phosphatase as described under Materials and Methods. Ribosomes were removed by ultracentrifugation, and the resultant supernatant containing the 38-kDa protein was concentrated by ultrafiltration. Proteins were separated with SDS–15% polyacrylamide gel electrophoresis. The 38-kDa protein was transferred to Immobilon membrane (Millipore) and digested with lysyl-endopeptidase. The resultant polypeptides were separated with high-pressure liquid chromatography (HPLC) and subjected to amino acid sequencing. Three sequences were obtained, as shown under Materials and Methods. Based on this sequence information, we polymerase chain reaction (PCR)-amplified a DNA fragment from a *C. maltosa* genome and cloned a gene from the *C. maltosa* genomic library by the colony hybridization method using the amplified DNA as a probe.

Figure 3A shows the entire nucleotide and deduced amino acid sequences of a gene coding for the 38-kDa protein. All three peptide sequences are present in its coding region and are indicated with bold letters. We named the gene RAY38 (38-kDa ribosome-associated protein of yeast). RAY38 encodes a protein with a calculated molecular mass of 29 kDa and has a repetitive sequence of Arg, Gly and Gly (RGG motif) at the carboxyl end. The RGG motif is known in relation to RNA binding. The discrepancy between the observed and calculated molecular masses may be due to the gene's abundance in basic charges (pI = 9.8). The deduced amino acid sequence of Ray38p is 40% identical and 76% similar to Stm1p (14) of *Saccharomyces cerevisiae* and 33% identical and 67% similar to Spbc16a3.08cp of *Schizosaccharomyces pombe* (Fig. 3B). The function of the two proteins is not well known.

Dissociation of Ray38p Is Dependent on Temperature but Not on Energy Depletion

To characterize the dissociation of Ray38p from ribosomes, we tested the effect of energy depletion by 10 mM sodium azide. This concentration of azide efficiently inhibits protein synthesis of *C. maltosa,* as judged by a pulse-labeling experiment (data not shown), but it did not dissociate Ray38p from ribosomes (Fig. 4A, lane 3). We also examined the effect of this drug on the CYH-induced dissociation of Ray38p from ribosomes. The addition of 10 mM azide 1 h prior to CYH treatment did not influence the dissociation of Ray38p from ribosomes (Fig. 4A, lane 4). These results suggest that inhibition of protein synthesis by azide does not result in the release of Ray38p from ribosomes and that its CYH-induced dissociation is not necessarily dependent on energy generation and new protein synthesis.

We next examined the effect of low temperature, which we expected would block protein synthesis. A culture of *C. maltosa* at 30°C was transferred into ice water and continued to aerate. Under this condition, protein synthesis was halted (data not shown), but Ray38p was not dissociated from ribosomes (Fig. 4A, lane 2). This result also supports the notion that the inhibition of protein synthesis does not necessarily cause the dissociation of Ray38p from ribosomes. In contrast to the case of sodium azide, however, low temperature suppressed the CYH-induced dissociation of Ray38p from ribosomes, suggesting that the dissociation process is dependent on temperature.

Anisomycin, another glutarimide antibiotic, induces the expression of *L41-Qs* but has no effect on the growth or protein synthesis of *C. maltosa* (data not shown). This drug released Ray38p from ribosomes (Fig. 4A, lane 5), which also proved that the inhibition of protein synthesis is not a prerequisite for Ray38p

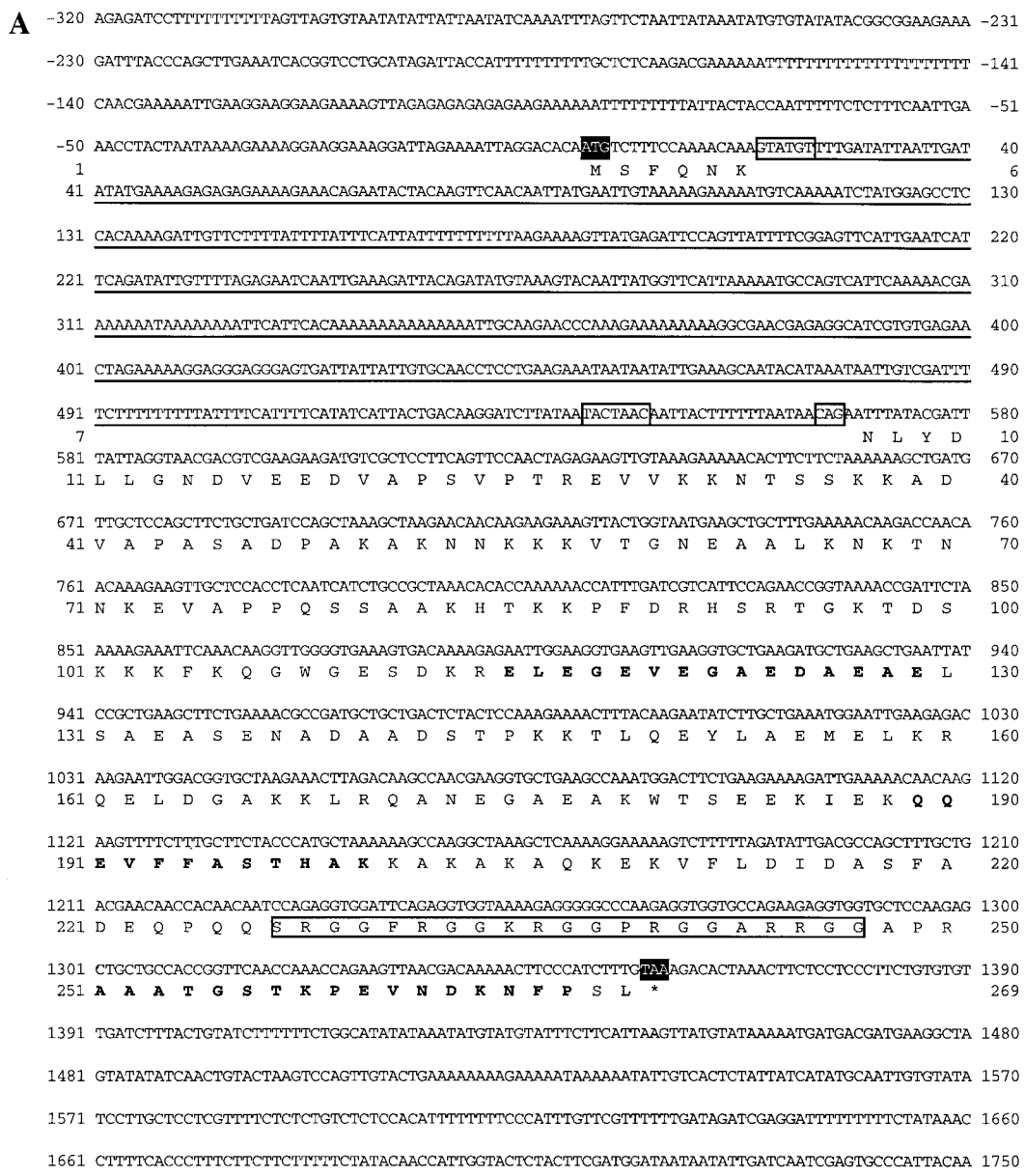

FIG. 3. Gene structure of *RAY38*. (A) The nucleotide and amino acid sequences. Boxed amino acids indicate the RGG motif. Initiation and termination codons are indicated by white letters. The predicted intron is underlined, and boxes in the intron show the consensus sequences for splicing in *C. maltosa*. Bold letters indicate the amino acid sequences determined by peptide sequencing. (B) Comparison of the deduced amino acid sequences of *RAY38*, *STM1*, and *SPBC16A3.08C* (Clustal X). Amino acids identical and conserved among all sequences are indicated by asterisks and dots, respectively.

release and the induction of *L41-Qs*. A general nutrient shift-down that induced *L41-Qs* resulted in the release of Ray38p from ribosomes (Fig. 4A, lane 6), but histidine starvation, which also induces *L41-Qs*, did not release Ray38p from ribosomes (Fig. 4B, lane 4 and 6). These results suggest that CYH, anisomycin and nutrient shift-downs work through a common pathway that might involve the release of Ray38p from ribosomes, whereas histidine starvation induces *L41-Qs* through another pathway in which there is no release of Ray38p.

Ray38p Is a Serine/Threonine Phosphoprotein

Ray38p was purified by washing the ribosome fraction with 0.5 M KCl and analyzed using antibodies against phosphoserine, phosphothreonine or phosphotyrosine. The results indicate that the ribosome-bound Ray38p is phosphorylated at serine and threonine but not at tyrosine residues (lane 1 of Figs. 5A–5C). Since alkaline phosphatase treatment of ribosomes results in the release of Ray38p in a dose-dependent manner (Figs. 5D and 5E), this level of

B

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Ray38p          MSFQNKNLYDLLGNDVEEDVAPSVP---------------TREVVKKNTSSKKAD--VAP
Stm1p           MS----NPFDLLGNDVEDADVVVLP---------------PKEIVKSNTSSKKAD--VPP
Spbc16A3.08cp   MSVASKNLFDLLGEETPAATTTEKKTAASRDKKRSDSPPVPRELVAQSTTSRKRDPNQPT
                **    * :****::.    .                   .:*:* ..*:*:* *   ..

Ray38p          ASADPAKAKNNKKK-----VTGNEAALKNKT----NN--KEVAPPQSSAAKHTKKP----
Stm1p           PSADPSKARKNRPR-----PSGNEGAIRDKTAGRRNNRSKDVTDSATTKKSNTRRA----
Spbc16A3.08cp   PRERTVNKKADQPRRRRQAPQGNEAFAREGKEARANNAAHPVDATGAPSNRRNARARRGR
                .   . : : :: :       ***.  :: .    **  : *  . :.   .. :.

Ray38p          -FDRHSRTGKTDSKKKFKQGWGESDKRELEGEVEGAEDAEAELSAEASENADAADS-TPK
Stm1p           -TDRHSRTGKTDTKKKVNQGWGD-DKKELSAEKEAQADAAAEIAEDAAEAEDAGKPKTAQ
Spbc16A3.08cp   EFDRHSQTGRVDTKKATERGWGD-----LVNSAANPDVAENEGNTPSGAQTPAAEE-ENV
                  ****:**:.*:**  ::***:     *  .      *  *    :.    *..

Ray38p          KTLQEYLAEMELKRQELDGAKKLRQANEGAEAKWTSE--EKIE-KQQEVFFASTHAKKAK
Stm1p           LSLQDYLN-----QQ----AN--NQFNKVPEAKKVELDAERIETAEKEAYVPATKVKNVK
Spbc16A3.08cp   KTLDEYLS----ERKS-AAKPVGRTVEKLENATKVEK--SAPEELFASLKKSASQKKSAA
                 :*::**      ::        .  ::  :*. ..   .  *    .   .::: *..

Ray38p          AKAQKEKVFLDIDASFADEQPQQ--------SRGGFRGGKRGGPRGGARRGG----APRA
Stm1p           SKQLKTKEYLEFDATFVESNTRKNFGDRNNNSRNNFNN-RRGG-RG-ARKGNNTANATNS
Spbc16A3.08cp   KESKPKKVLLDIEQTFTARPARG--------GRPNRAP-RRGP-------------SETA
                 :    *  *::: :*.   .:         .* .    :**             :  :

Ray38p          AATGSTKPEVNDKNFPSLF
Stm1p           ANTVQKNRNIDVSNLPSLA
Spbc16A3.08cp   SKTQQAPPTLSETDFPALA
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FIG. 3—*Continued*

phosphorylation might be required for its ribosome binding.

Next, we purified Ray38p from ribosome-free supernatant of CYH-treated cells using anti-Ray38p antiserum, as described under Materials and Methods, and analyzed its phosphorylation. Unexpectedly, Ray38p was hyperphosphorylated at threonine (Fig. 5C, lane 2). Although the exact sites and stoichiometry of the phosphorylation are not clear, the difference of the phosphorylation state of Ray38p may be related to its cellular localization.

Effect of Disruption of RAY38 Alleles

Two alleles of genomic *RAY38* gene were disrupted, as described under Materials and Methods. We compared the growth properties of disruptant with CMT100, which was made identical by transforming CHAU1 to Ade, His, and Ura. The growth of the ΔRAY38 strain was not different from that of the CMT100 strain, even in the presence of 50 μg/ml of CYH. When the CYH concentration was raised to 1 mg/ml, growth of the CMT100 strain was immediately arrested and did not recover until after 25 h. ΔRAY38, however, did not show complete growth arrest in response to the addition of CYH and recovered from the effect of CYH 6 h earlier did the CMT100 cells (Fig. 6). This rather unexpected result suggests that the *RAY38* gene product is involved with strictly halting cellular translational activity upon treatment by the translation-inhibitory agents, and its release from ribosomes allows leaky translation for the synthesis of Q-type L41.

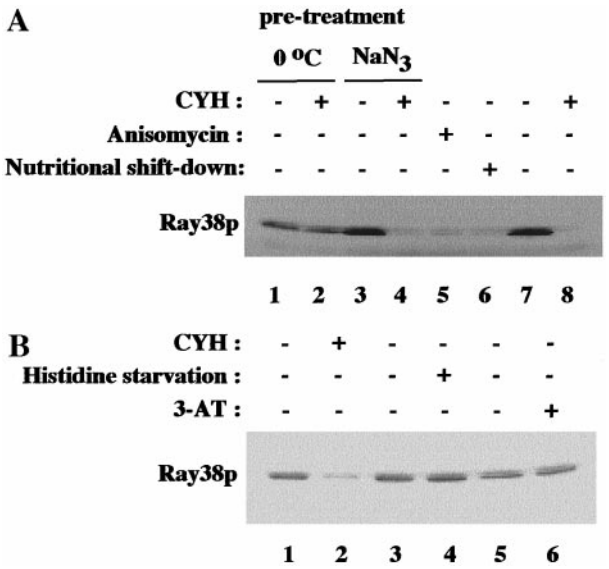

FIG. 4. Ray38p was released from ribosomes by conditions other than the inhibition of protein synthesis by CYH. Ribosome proteins were separated by 15% SDS–PAGE and blotted with anti-Ray38p antiserum. (A) Ribosomes from wild-type cells were pretreated at 0°C for 1 h (lanes 1 and 2) or for 1 h in 10 mM NaN_3 (lanes 3 and 4), then grown in the presence of CYH (lanes 2 and 4) or in the absence of CYH (lanes 1 and 3) for 1.5 h. Cells were grown in the presence of 50 μg/ml anisomycin for 1.5 h (lane 5), under nutritional shift-down for 1.5 h (lane 6), in the absence of CYH (lane 7) or in the presence of 50 μg/ml CYH for 1.5 h (lane 8). (B) Ribosomes from strain CHA1 before (lane 3) and after (lane 4) the histidine starvation. Ribosomes from wild-type cells grown in the absence of CYH (lane 1), in the presence of 50 μg/ml CYH for 1.5 h (lane 2), in the absence of 3-AT (lane 5), or in the presence of 20 mM 3-AT for 1.5 h (lane 6) which causes histidine starvation.

DISCUSSION

In this paper, we report a novel ribosome-associated protein, Ray38p, of *C. maltosa*. This protein is released from P-type ribosomes, not from Q-type ribosomes, upon the addition of the potent translation inhibitor CYH to the culture medium. Ray38p has a possible RNA binding motif RGG at its carboxyl terminus, which was initially described as an RNA-binding motif in hnRNP U, hnRNP A1 and nucleolin, and it has usually been found in association with other RNA-binding domains (15). Ray38p is also a serine/threonine phosphoprotein that is released from ribosomes by treatment with alkaline phosphatase, suggesting that phosphorylation of Ray38p or of any ribosomal components is required for its binding to ribosomes. The Ray38p dissociated by CYH treatment, however, was hyper-phosphorylated at the threonine residues. Although we cannot currently explain this apparent discrepancy, we speculate that properly phosphorylated Ray38p binds to ribosomes through the RGG motif but is dissociated by hyperphosphorylation at the threonine residue(s) upon CYH treatment. Threonine hyper-phosphorylation might reduce the affinity of Ray38p to binding sites on the ribosomes.

The dissociation of Ray38p from ribosomes occurred under Q-type L41-inducing conditions except for the case of histidine starvation, and the dissociation was not necessarily caused by the inhibition of *de novo* protein synthesis. Anisomycin treatment had no visible effect on the growth of *C. maltosa*, but the treatment dissociated Ray38p from ribosomes and induced *L41-Qs*. In mammalian cells, anisomycin or CYH stim-

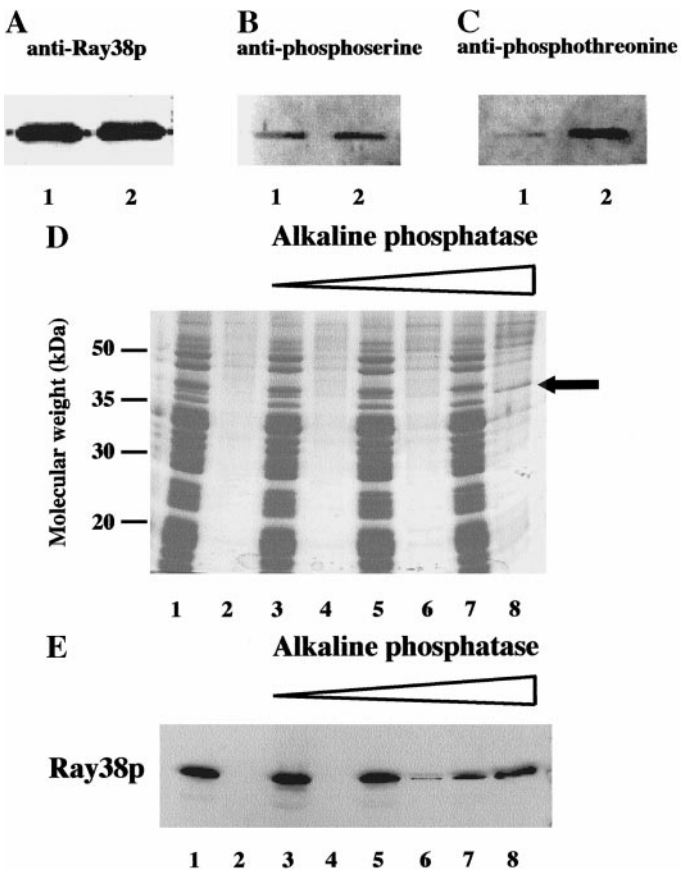

FIG. 5. Ray38p is a serine/threonine phosphoprotein and the released Ray38p is highly phosphorylated at threonine residues. (A–C) Ray38p was purified by anti-Ray38p antiserum as described under Materials and Methods. Lane 1; Ray38p purified from S-100 of 0.5 M KCl ribosome-wash. Lane 2; Ray38p purified from S-100 fraction of wild-type cells grown in the presence of 50 μg/ml CYH for 1.5 h. Purified Ray38p was subjected to 15% SDS–PAGE and blotted to a membrane. Western blots were performed with anti-Ray38p antiserum (A), anti-phosphoserine antibody (B), and anti-phosphothreonine antibody (C). The amount of loaded Ray38p was the same in these three experiments. (D) Dose-dependent effect of alkaline phosphatase on the associating Ray38p to ribosomes. Ribosomes were incubated for 30 min at 30°C in the absence (lanes 1 and 2) or presence of 1 unit (lanes 3 and 4), 5 units (lanes 5 and 6), or 20 units (lanes 7 and 8) of alkaline phosphatase, respectively. After the treatment of alkaline phosphatase, ribosomes were reprecipitated by ultracentrifugation. Ribosome pellets (lanes 1, 3, 5, and 7) and supernatant (lanes 2, 4, 6, and 8) were separated by 15% SDS–PAGE. The position of Ray38p was indicated by an arrow. (E) Western blot analysis of the gel in D using anti-Ray38p antiserum.

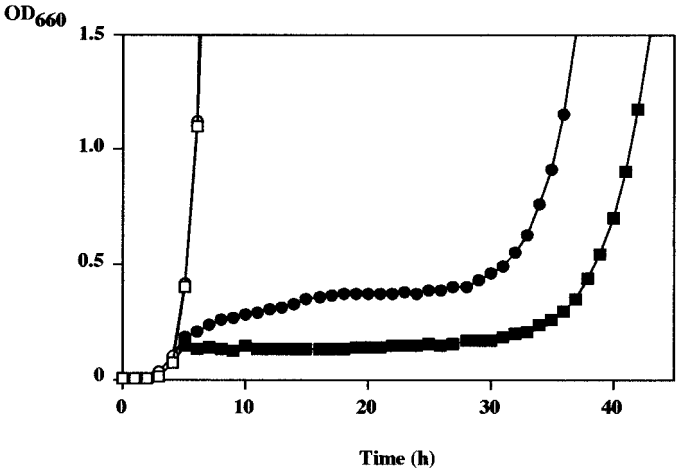

FIG. 6. Effects of the disruption of *RAY38* on the cell growth. Growth of cells was monitored by an automatically recording incubator TN1506 (Advantec). CYH was added to each medium at the time when OD_{660} was 0.1. Strain: rectangles, CMT100; circles, ΔRAY38. Open symbols indicate absence of CYH and closed symbols indicate presence of 1 mg/ml.

ulates the FRAP/mTOR pathway which activates S6 kinase that phosphorylates 40S ribosomal protein S6 (16, 17). Those protein inhibitors also stimulate the p38 mitogen-activated protein kinase and JNK/SAPK pathway, which involves a phosphorylation cascade (18–20). Although the molecular mechanism is not clear, it appears that anisomycin or CYH generates a signal that activates a kind of phosphorylation cascade in *C. maltosa* that results in hyper-phosphorylation of Ray38p and its liberation from ribosomes. The involvement of a hypothetical phosphorylation cascade is consistent with the fact that the release of Ray38p from ribosomes is temperature-dependent and not dependent on *de novo* protein synthesis.

We presumed that a similar signal was also involved with the induction of *L41-Qs*, which, however, occurred without the release of Ray38p from ribosomes under the histidine-starved condition. In *S. cerevisiae*, histidine starvation induces the production of Gcn4p, which is a transcriptional activator that binds to specific sites in many promoters of amino acid synthetic genes (21). A similar nucleotide sequence to the Gcn4p-binding sites is present in the promoter of *L41-Q2a*, and depletion of this sequence greatly reduced the production of Q-type L41 (5). Therefore, there might be two signaling pathways for the production of Q-type L41, one initiated from CYH, anisomycin or a nutritional shift-down, and the other from the histidine starvation that ultimately enhances the production of Gcn4p-like protein. Recent isolation of a gene encoding a Gcn4p homologue of *C. maltosa* in our laboratory will greatly facilitate the understanding of the latter pathway.

What is the function of Ray38p? The *ray38* null mutant was viable, and hence *RAY38* is not essential. After the addition of CYH, this null mutant did not immediately stop growing, and it recovered faster from the growth arrest than did the parental CMT100 strain. This indicates that Ray38p itself is not required for the induction of Q-type L41. The CYH-resistant ΔL41-Ps strain, which expresses constitutively *L41-Qs*, did not show the release of Ray38p in the presence of CYH. Therefore, the extra phosphorylation and the release of Ray38p are not required for the induction of *L41-Qs*, but Ray38p seems to facilitate the conversion of the L41 ribosomal protein from P-type to Q-type. Ray38p might be involved in strict recession of ribosomal function under the translation-inhibitory conditions, and its release from the ribosomes might allow leaky translation that is necessary for the inducible synthesis of Q-type L41.

Ray38p has significant homology to a *S. cerevisiae* protein, Stm1p (14). The gene *STM1* was isolated as a multicopy suppressor for temperature-sensitive mutations of *TOM1*, *HTR1/MPT5*, and *POP2*, which are involved in the regulation of mitotic progression (*TOM1* and *HTR1*) or transcription (*POP2*), respectively (14, 22). Irrespective of these genetic data, the function of *STM1* has not been clear, because of the incomplete suppression of the mutations described above and viability of the null stm1 mutant. The product Stm1p was a relatively abundant protein (35,000 copies/cell) and previously identified as G4p2, a protein that has specific affinity to quadruplex nucleic acid and that was also purified as a purine motif triple-helical DNA-binding protein (23, 24). Although these target structures are supposed to occur in the telomeres of chromosomal ends or in guanine tracts in 26S rRNA, it is not known whether Stm1p functions through binding to these sites. Ray38p has a nuclear localization-like domain like Stm1p dose, but the RGG motif is shown only in Ray38p. Thus, it is possible that the two have different functions. We believe, however, that our findings regarding Ray38p could facilitate understanding of the function of Stm1p. Study of the ribosome-associated protein Ray38p is important for understanding the functions of ribosomes. The function of Ray38p is now under investigation in our laboratory.

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