Immunological Studies of Ribosomal Mutants in the Fungus 

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In the fungus Podospora anserina, two mutants with ribosomes apparently lacking one ribosomal protein were investigated using immunological techniques to determine whether these proteins were indeed missing. Antibodies directed against the wild type ribosomal proteins apparently absent in the mutants were raised from two-dimensional gel spots containing these proteins. These sera were used to detect cross-reacting proteins in the mutants. Immunological results have revealed in both mutants the presence of strongly altered ribosomal proteins which co-migrate with other wild type proteins on two-dimensional gel. The molecular weight of mutant proteins differs at least 25% from that of homologous wild type proteins. Furthermore, we observed that the growth of the mutants and the activity of their ribosomes are very reduced in both single mutants containing the altered proteins.

Bacterial mutants with altered ribosomes have proved to be of considerable utility to study ribosome structure and functions. In eukaryotes, such approaches are increasing. In the fungus Podospora anserina several mutants with altered ribosomal proteins were isolated. The first such mutant to be characterized was a cycloheximide-resistant strain (1). Indeed, the mutation leading to cycloheximide resistance has been localized in the structural gene for protein L21 of the 60 S subunit. We have also obtained mutant forms of the ribosomal proteins L16 and S15 in strains selected by reversion from sensitivity to ammonium acetate of the double mutant CyR1-1 CyR2-1 (2).

Proteins of the 40 S subunit were also found to be altered in some mutants (3). So, among emetine-resistant mutants, the strain EmR40 was found to exhibit an alteration of protein S14 and in the strain EmR69, resistance to emetine could be attributed to the alteration of proteins S13 and S17. If we consider the various mutant forms of ribosomal proteins in P. anserina and more generally in eukaryotes, the differences observed between the electrophoretic mobilities of mutant and corresponding wild type proteins are limited.

In this paper we report the characterization of two new mutant strains and the immunological studies that have allowed us to identify the altered ribosomal proteins on the two-dimensional polyacrylamide gels. The mutant proteins exhibit electrophoretic mobilities very different from those of the homologous wild type proteins. Indeed, the results presented for the two mutants show changes in the molecular weight of the proteins of at least 25%. In addition, we examined the effect of such mutations on the growth of the mutant strains and the activity of the ribosomes.

EXPERIMENTAL PROCEDURES

Organism—P. anserina is an Ascomycete closely related to Neurospora crassa. The biology and life cycle of P. anserina have already been described (4). The strains used for this study, CyR1-1 and CyR2-1, are cycloheximide-resistant mutants. The properties of these mutants have previously been reported (5).

Selection of Revertant Strains—Revertant strains were selected from the double mutant strain CyR1-1 CyR2-1 as described (2). The growth of the double mutant strain is inhibited by NH4 acetate (50 mM), and mutations which relieve this inhibition were screened as mutant sectors issued from the thallus.

Measurements of Growth—P. anserina is a filamentous fungus which forms a flat circular mycelium growing essentially at the periphery. The growth rate is then measured by the increase of the diameter of the thalli.

Two-dimensional Polyacrylamide Gel Electrophoresis of Ribosomal Proteins—Ribosomes were prepared as described (5). Ribosomal proteins were extracted by acetic acid in the presence of 33 mM MgCl2 (6). Two-dimensional gel electrophoresis was carried out as described by Kaltschmidt and Wittmann (7) with modifications introduced by Howard and Trout (8). In the first dimension (8 x 0.27 cm), basic proteins were separated at 4 °C for 15 h at 90 V. In the second dimension, the disc gels were overlaid on slab gels (12 x 8 x 0.27 cm) and were run for 24 h at 120 V. The gels were stained with 0.1% Coomassie brilliant blue (R-250) in 50% methanol, 7.5% acetic acid and destained with the same solution without Coomassie blue.

SDS-Gel Electrophoresis of Ribosomal Proteins—SDS-gel electrophoresis was performed according to the method of Laemmli (9). Slab gel was 0.1 cm thick.

Poly(U)-directed Polypeptide Synthesis—A detailed description of this system has been reported (5). Incubation was carried out for 20 min at 32 °C.

Immunization Procedure—Ribosomal proteins separated by two-dimensional electrophoresis were stained about half an hour with 0.1% Coomassie brilliant blue (R-250) in 50% methanol, 7.5% acetic acid. The ribosomal protein spots were excised from the gel, rinsed with distilled water for half an hour, and then dried with blotting paper. For each injection, 16 gel pieces containing the protein (total about 30 μg) were crushed in a Potter-Elvehjem homogenizer, mixed with 2 ml of 0.15 M NaCl, 10 mM potassium acetate buffer (pH 4.5), and emulsified with an equal volume of Freund's adjuvant. Antibodies were raised in an hybrid rabbit E.C.601 by injecting antigens subcutaneously over four or five sites. An injection was applied every 20 days during 2 months. The complete form of adjuvant was used in the two first injections and the incomplete form in the two last. Blood was collected before the first injection to get preimmune serum and rabbits were bled 8 days after the last booster injection. The blood was stored at 4 °C for 15 h to allow clot retraction. The clot was eliminated by centrifugation for 15 min at 3000 x g. The sera were dispensed in aliquots and stored at −20 °C.

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Electrophoretic Transfer Procedure—Electrophoretic transfer procedure was essentially achieved according to the method of Towbin et al. (10). Ribosomal proteins were separated by electrophoresis in the presence of SDS or urea as described above. After electrophoresis, SDS gels were equilibrated in 25 mM Tris (pH 8.3), 192 mM glycine, 0.1% SDS, 20% methanol and urea gels in 0.7% acetic acid, 20% methanol. After soaking for 10 min, the ribosomal proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose sheets (Millipore, HAWP 0.45 μm). A voltage of 24 V was applied for 3 h at 4°C in Pharmacia GD4 destained apparatus. The electrophoreses were those used to equilibrate the gels after electrophoresis. For urea gels, the nitrocellulose sheet faces the cathode and the polarity of the electrodes is reversed for SDS gels. Efficiency of the proteins transfer can be estimated by staining the nitrocellulose sheet. The ribosomal proteins were stained with Amido black (0.1% Amido black in 45% methanol, 7.5% acetic acid) and destained with 45% methanol, 7.5% acetic acid. Staining was achieved after 3 min.

Immunological Detection of Proteins on Nitrocellulose Sheet—After the transfer, the nitrocellulose sheets were soaked three times for 15 min in PBS and saturated for 4 h with 3% bovine serum albumin in PBS at room temperature. The sheets were washed for 30 min with PBS and incubated with the sera diluted in PBS containing 0.1% bovine serum albumin. Then they were rinsed five times with PBS, 0.1% Tween 20, and 0.3% lauryl Sarkosyl. They were subjected to autoradiography using Kodak X-Omat Plus B1 intensifying screen. Alignment of the films with the stained sheets was performed using marks left by radioactive ink. We can so identify on the autoradiogram the proteins which reacted with the serum.

RESULTS

The strain used in this study combines the two cycloheximide-resistant mutations CyR1-1 and CyR2-1. The properties of this double mutant strain have already been described (11). The mutation CyR1-1 leads to an alteration of the ribosomal protein L21 (1). No difference was observed in the ribosomal proteins pattern of the strain CyR2-1 compared to wild type. The double mutant CyR1-1 CyR2-1 is cold-sensitive and exhibits sensitivity to ammonium acetate at concentrations which do not affect the growth of wild type or the single mutant strains. This last characteristic was previously used to isolate ribosomal mutations which relieve sensitivity to ammonium acetate of CyR1-1 CyR2-1. We therefore obtained mutations in structural genes leading to alterations of proteins L16 and S15 (2). In the same way, another form of protein L21 more acidic than wild type protein was found in the mutant strain CyR1-10 (12).

Genetic Analysis of Revertants—Thirteen additional revertant strains (M13 to M25) have been isolated from CyR1-1 CyR2-1 on medium supplemented with 80 mM ammonium acetate. Sensitivity to ammonium acetate has been shown to be suppressed by mutations in either CyR1 and CyR2 genes or in external genes. To discriminate between these different possibilities, genetic analysis of the revertants was performed as already described (11). From such a genetic analysis, it could be concluded that six revertants (M13, M14, M17, M18, M22, and M25) arose from mutations in the gene CyR1 and two others (M15 and M16) from mutations in gene CyR2. The suppression of sensitivity to ammonium acetate in the other strains (M19, M20, M22, M24) resulted from mutations in external genes. A similar result was obtained when we analyzed cold-resistant revertants from cold-sensitive strain CyR1-1 CyR2-1 (11).

Electrophoretic Analysis of Revertants—The ribosomal proteins of the 13 revertants were compared with those of the parental strain CyR1-1 CyR2-1 by two-dimensional polyacrylamide gel electrophoresis. Results are summarized in Table I. In the six revertants which have the suppressor mutation in gene CyR1, protein L21 is affected. In mutants M13, M18, M22, and M25, a form identical with that of wild type was observed; for the two others, M14 and M17, the mobility of protein L21 is indistinguishable from that already described in the strain CyR1-10 (not shown). The ribosomal proteins of the mutants M15 and M16 carrying the reverse mutation in the gene CyR2 showed no difference. Out of the five last revertants (M19, M20, M21, M23, and M24), only M21 and M23 exhibit patterns different from that of the parental strain CyR1-1 CyR2-1 (Fig. 1). Indeed, the spot corresponding to the wild type protein L16 of 60 S subunit was absent from the gel on which total ribosomal proteins from M21 were run. No other difference was apparent; in particular no extra spot was present on the gel. Likewise in mutant M23, the protein S15 of 40 S subunit is apparently missing. However L12, a faint spot in wild type and in parental strain is much more stained. Therefore, the ribosomes of the mutants M21 and M23 are either devoid of these ribosomal proteins, or they possess variants of these proteins which co-migrate with other proteins on two-dimensional polyacrylamide gel. These two possibilities were investigated using an immunological approach.

Immunological Studies of Mutants M21 and M23—Two antiserum, one against wild type ribosomal protein L16 and the other against wild type S15, were used to test the presence of cross-reacting material in the ribosomes of strains M21 and M23. To achieve immunological reactions, the proteins separated on polyacrylamide gel were transferred to nitrocellulose sheets and were incubated with diluted sera. After washing away unbound antibodies, the sheets were soaked with 125I-protein A, which binds to immunoglobulins (13). The antigen-antibody-protein A complexes were revealed by autoradiography. So the detection of very small amounts of antigen (about 1 ng) is possible (14).

Specificity of the sera could be inferred from immunochromical studies performed with wild type proteins separated by two-dimensional electrophoresis. Autoradiograms illustrating these experiments are shown in Fig. 2, C and E. Only one spot can be seen on the autoradiogram in both cases when wild type ribosomal proteins were reacted either with antil16 serum or with anti-S15 serum. We could establish that the position of spots revealed by autoradiography corresponded to that of proteins L16 and S15 located by staining.

Table I

<table>
<thead>
<tr>
<th>Protein L21 is that observed in cycloheximide-resistant mutant CyR1-1.</th>
<th>Electro...</th>
<th>Consequences on the ribosomal proteins pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localisation of suppressor mutation</td>
<td>Form L21 identical with that of wild type</td>
<td></td>
</tr>
<tr>
<td>Revertant strains</td>
<td>CyR1</td>
<td>CyR2</td>
</tr>
<tr>
<td>M13</td>
<td>Form L21 indistinguishable with that of the strain CyR1-10</td>
<td></td>
</tr>
<tr>
<td>M18</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>M22</td>
<td>Protein L16 apparently lacking</td>
<td></td>
</tr>
<tr>
<td>M24</td>
<td>Protein S15 apparently lacking</td>
<td></td>
</tr>
<tr>
<td>M19</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>M21</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>M23</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>M20</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>M25</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>
Immunological Studies of Ribosomal Mutants in Eukaryotic Organism

Fig. 1. Two-dimensional electrophoresis of ribosomal proteins from mutant and wild type strains. Proteins (about 400 μg) were electrophoresed in the first dimension at pH 8.7 for 15 h in tubes of 2.7-mm diameter and in the second dimension at pH 4.5 for 24 h and stained with Coomassie brilliant blue (R-250) (8). The upper frames show parts of the distributions of ribosomal proteins from mutants M21 (A) and M23 (B). Position of apparently missing proteins is indicated by a circle; the arrows point out the position of mutant proteins determined by immunological techniques (see below). C, whole distribution of ribosomal proteins of wild type is given for comparison. The faintly stained spot corresponding to the protein L12 not visible on photography is marked by a star.

Fig. 2. Immun autoradiographic detection of mutant and wild type proteins L16 and S15 after two-dimensional gel electrophoresis. Total ribosomal proteins (20 μg) were run on two-dimensional gel according to Ref. 8, then transferred to nitrocellulose sheet. Treatment with serum and protein A was then performed as described under "Experimental Procedures." A shows the nitrocellulose sheet on which wild type proteins were transferred. Proteins were stained with 0.1% Amido black before the treatment, and proteins L12, L16, L22, and S15 are indicated by an arrow. B-F show autoradiograms obtained from ribosomal proteins treated with the sera. Wild type proteins were treated, respectively, with preimmune serum (B), with anti-L16 serum diluted 1:50 (C), and with anti-S15 serum diluted 1:25 (E). D shows autoradiogram obtained with the ribosomal proteins of M21 treated with anti-L16 serum diluted 1:50, and F shows autoradiogram obtained with the ribosomal proteins of M23 treated with anti-S15 serum diluted 1:25.

As neither L22 nor L12 react with the antisera, mutant proteins in M21 and M23 could co-migrate with these proteins on two-dimensional gels. Such a co-migration would explain the higher intensity of staining of L12 spot observed when M23 ribosomal proteins were analyzed (Fig. 1).
To ascertain the position of mutant proteins, ribosomal proteins from both mutants and wild type were run on two-dimensional gels, and the spots corresponding to protein L22 in M21, to L12 in M23, and to proteins L12, L16, L22, and S15 in wild type were excised. The proteins were extracted from the gel pieces, run on SDS gels, transferred on nitrocellulose sheet, and treated with the antisera. The results are shown in Fig. 3. As mentioned above, proteins L16 and S15 react with the respective serum and no reaction was observed with wild type proteins L22 and L12. Nevertheless, the spots corresponding to L22 in M21 and to L12 in M23 show positive reaction, respectively, with anti-L16 and anti-S15 sera. This result confirms the co-migration on two-dimensional gels of mutant L16 with protein L22 and mutant S15 with protein L12.

Electrophoresis on SDS Gels of Ribosomal Proteins—The electrophoretic mobilities on SDS gels of mutant and corresponding wild type proteins suggest the existence of large differences in molecular weights. To estimate these differences, the proteins were recovered from two-dimensional gels and their electrophoretic mobilities on SDS gels were compared with those of marker proteins (Fig. 4). We thus determined a value of 18,050 ± 300 for mutant protein L16 against a value of 24,000 ± 200 for wild type L16. The mutant protein S15 in M23 ribosomes (M, = 23,000 ± 200) is larger than the wild type protein (M, = 18,000 ± 300).

Effect on the Growth of Single Mutant Strains and on the Activity of Ribosomes—These differences in molecular weight are important, and in order to investigate the effects of such mutations on the activity of the ribosomes, we isolated among the offspring of the crosses M21 x wild type and M23 x wild type, single mutant strains carrying the gene encoding either the altered ribosomal protein L16 or S15. They were selected as cycloheximide-sensitive strains which, in back-cross with Cyrl-1 Cy2r-1, suppress the ammonium acetate-sensitive phenotype. In both crosses, we selected about 10 single mutant strains. The ribosomal proteins were separated on two-dimensional gels. According to the cross, mutant protein L16 or S15 was present in all these single mutant strains. Therefore, it can be inferred that in each revertant M21 and M23 the suppression of sensitivity to ammonium acetate and the alteration, respectively, of L16 and S15 are probably the consequences of a single mutational event. The growth of the single mutant strains containing the altered protein L16 or S15 was compared to that of wild type (Table II). The alteration of L16 leads to a reduction of about 30% of the growth rate at 26°C; at 11°C, the growth is extremely slow. Additionally, spore germination of such a mutant is delayed but the germination rate is not affected. For the strains which contain the altered S15, the linear growth is not significantly reduced.

**Table II**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Linear growth at 27°C</th>
<th>Poly(U)-dependent polyphenylalanine synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm/day</td>
<td>cpm/unit A560 ribosome</td>
</tr>
<tr>
<td>Wild type</td>
<td>12.5</td>
<td>3653</td>
</tr>
<tr>
<td>Mutant with altered S15</td>
<td>12, 3*</td>
<td>2280</td>
</tr>
<tr>
<td>Mutant with altered L16</td>
<td>7, 9</td>
<td>739</td>
</tr>
</tbody>
</table>

*The mycelium is less dense than this of wild type.*
modified, but the mycelium is less dense than wild type mycelium. All these observations led us to examine whether these mutations could affect the rate of polypeptide synthesis by mutant ribosomes. Table II gives the results obtained in poly(U)-dependent system. It is evident that the two mutations leading to alterations of L16 and S15 reduce the activity of the ribosomes; in particular, mutant ribosomes containing the altered L16 showed the lowest activity (20% remaining activity). So there is a good correlation between the effect of the mutations observed in vivo and in vitro.

We have attempted to isolate the double mutant strain carrying the genes encoding the mutant proteins L16 and S15 in the cross between the two single mutants. In the offspring of this cross, many spores do not germinate and among the viable offspring we could not identify a strain containing the two altered proteins. Therefore, it can be supposed that the double mutant strain would be lethal.

In previous reports (2, 3) we have already identified mutations in the structural genes coding for L16 and S15. The obtained mutant proteins exhibit electrophoretic mobilities different from those described here. The allelism of the different mutations affecting either protein L16 or S15 was then tested. For that purpose, the two strains containing different mutant forms of L16 were crossed. The ribosomes were isolated from 15 offsprings and the ribosomal proteins were run on two-dimensional gel. No recombinant containing wild type protein L16 was found. The same result was observed when offsprings of a cross between two strains containing different mutant forms of S15 were analyzed. Therefore, the mutations described here which alter the molecular weight of L16 and S15 would lie in the structural genes encoding these proteins.

DISCUSSION

In prokaryotes and especially in Escherichia coli, mutations leading to large alterations of ribosomal proteins are known (for review see Refs. 15 and 16). On the contrary, the alterations observed in ribosomal mutants of P. anserina and more generally in eukaryotes (17-19) are limited. The mutant proteins differ from the corresponding wild type in small variations in the net charge of the protein and their molecular weights are in most cases identical. The only known exception was described in Schizosaccharomyces pombe by Coddington and Fluri (20).

In this paper we reported the isolation and characterization of two mutants of P. anserina containing strongly altered ribosomal proteins. Indeed in the mutant strain M23, the 40 S subunit contains a mutant protein S15 which is about 5,000 or 28% larger than wild type S15. In mutant M21, the molecular weight of the altered L16 is 18,050, 25% smaller than that of wild type protein. The altered forms of L16 and S15 which co-migrate with other proteins on two-dimensional gel could be identified by immunochemical techniques. Specific sera against either wild type L16 or S15 were obtained by immunization of rabbits from gel spots containing the protein. About 120 μg of protein were necessary to get immune sera. Identification of reacting material in ribosomes was performed using radioimmunodetection after electrophoretic separation and blotting of ribosomal proteins (10).

According to genetic results, the mutations responsible for the alteration of the proteins were localized in the structural genes coding for L16 and S15. In the mutant M21, the mutation in the gene coding for L16 gives rise to shorter protein than wild type L16. A premature termination could occur during the translation of the mutant protein before reaching the normal termination codon. The mutation could be either a substitution or a frameshift mutation leading to a nonsense codon. In the mutant M23, the mutation in the structural gene for S15 would likely suppress the normal termination codon allowing readthrough to take place and leading the translation to terminate at a termination codon about 45 codons downstream from the normal one. One possible explication would be a point mutation in the termination codon or a frameshift mutation located just before the termination codon. The knowledge of nucleotide sequences of wild type and mutant genes would shed some light on these problems.

In spite of the large difference of molecular weight, the mutant proteins are normally integrated into the ribosomes. However, such mutations lead to prejudicial effects on the growth of the mutant strains and on the activity of the mutant ribosomes. Such results could explain why only slight alterations of ribosomal proteins are in most cases observed in the ribosomal mutants selected in eukaryotes. More severe alterations would produce either lethal or slow growing strains which cannot be identified in the generally used screenings.

REFERENCES

Immunological studies of ribosomal mutants in the fungus Podospora anserina.
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