Preparation and Characterization of Two Monoclonal Antibodies against Different Epitopes in *Escherichia coli* Ribosomal Protein L7/L12*

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Two monoclonal antibodies with specificities for *Escherichia coli* 50 S ribosomal subunit protein L7/L12 were isolated. The antibodies and Fab fragments thereof were purified by affinity chromatography using solid-phase coupled L7/L12 protein as the immunoasorbent. The two antibodies were shown to recognize different epitopes; one in the N-terminal and the other in the C-terminal domain of protein L7/L12. Both intact antibodies strongly inhibited polyuridylic acid-directed polyphenylalanine synthesis, ribosome-dependent GTPase activity, and the binding of elongation factor EF-G to the ribosome. Ratios of antibody to ribosome of 4:1 or less were effective in inhibiting these activities. Neither antibody prevented the association of ribosomal subunits to form 70 S ribosomes. The Fab fragments showed similar effects.

Among all the protein components of the *Escherichia coli* 50 S ribosomal subunit, protein L7/L12 has received special attention (for review, see Ref. 1). L7 refers to the post-translationally N-acetylated form of L12 (2). The two otherwise identical forms are collectively called protein L7/L12. The protein is present in 4 copies per 50 S ribosomal subunit (3, 4). All 4 copies of L7/L12 can be selectively removed from the ribosome (5) and reincorporated. In solution, the protein forms stable dimers and can only be reincorporated into the ribosome in the dimeric form (6). These observations led to the hypothesis that the structural and functional units of the protein on the ribosome consist of L7/L12 dimers (for review, see Ref. 7). The location of some of the L7/L12 molecules has been assigned to a rod-like protruberance on the 50 S ribosomal subunit, commonly known as the L7/L12 "stalk" on the basis of electron microscopy and immune electron microscopy (8). However, other electron microscopy studies (9), protein cross-linking (10), and energy transfer between fluorescent probes attached to protein L7/L12 (11) suggest that some of the 4 L7/L12 molecules occupy positions in the 50 S ribosomal subunit other than the stalk. The presence of L7/L12 at such locations apart from the stalk could represent a fixed location for one of the dimers (11), or it could result from movement of one or more of the L7/L12 molecules. Mobility of the C-terminal region of L7/L12 in intact ribosomes has been demonstrated by fluorescence (12) and proton NMR (13, 14) experiments.

The L7/L12 protein is essential for function. Its removal from the ribosome leads to the loss of all factor-dependent reactions of the protein synthesis cycle in which GTP hydrolysis occurs. The protein has been found to be associated with the binding of initiation factors 2 (15) and 3 (16); elongation factors EF-Tu (17) and EF-G (18, 19); release factor 2 (20); and stringent factor (21). It has been shown that 2 copies of L7/L12/ribosome are sufficient to support EF-G/ribosome-dependent GTPase activity, but that 4 copies of the protein are required for full activity in a polyphenylalanine-synthesizing system (6).

In order to understand fully the structure and function of this essential ribosomal protein, it will be necessary to determine precisely the location(s) of all 4 copies of L7/L12 on the ribosome and to identify the structurally and functionally important domains of the protein. Towards this goal, we set out to generate anti-L7/L12 monoclonal antibodies, to identify their epitopes, to test the functional properties of ribosomes that have these monoclonal antibodies or their Fab fragments bound to the known epitopes in L7/L12, and to locate the epitopes on the surface of the ribosome by means of immune electron microscopy. In this report, we present the isolation, purification, and the use as functional probes of two monoclonal antibodies specific for the N-terminal and C-terminal domains of protein L7/L12, respectively.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Two monoclonal antibodies reacting with different epitopes in ribosomal protein L7/L12 were isolated. One of the antibodies, designated Ab-NTF, was randomly chosen from a series of anti-L7/L12 antibody-producing hybridomas and subsequently shown to recognize an epitope in the N-terminal portion of L7/L12 (see results below). Ab-NTF was induced by immunization of BALB/c mice with uncleaved, purified L7/L12 protein. In order to obtain a monoclonal antibody with predetermined specificity for the C-terminal portion of protein L7/L12 (Ab-CTF), a fragment comprising amino acid

*The abbreviations used are: EF, elongation factor; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid.

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residues 74–120 (L7/L12-CTF) was used for immunization. The L7/L12-CTF fragment was obtained by cleavage of citraconylated L7/L12 protein with trypsin and further purified by reverse-phase high performance liquid chromatography. The material eluted at 52%, acetonitrile migrated as a single band. Amino acid composition analysis of the material confirmed its identity as homogeneous L7/L12-CTF.

Highly purified monoclonal antibodies to be used as functional probes in protein synthesis assays were obtained by affinity chromatography of ascites fluid using solid-phase coupled L7/L12 protein as immunoabsorbent. The material that eluted from the column after lowering the pH to 3.0 was analyzed further under nonreducing conditions by high performance size exclusion chromatography with a TSK 3000 column. The material eluted as a single peak with an apparent molecular weight of 155,000, consistent with the size of an IgG molecule.

The fractions obtained from the affinity chromatography of ascites fluid were also characterized by SDS electrophoresis under reducing conditions. The electrophoretic patterns obtained from purified Ab-NTF and Ab-CTF showed the presence of heavy and light chains of the IgG molecules, but no detectable contaminants from the ascites fluid. Purified antibodies were digested with papain and the resulting Fab fragments were purified by affinity chromatography. Size exclusion chromatography under nonreducing conditions of the affinity chromatography flow through material and of material eluted after lowering the pH of the elution buffer to 3.0 revealed the presence of the Fc portion of the IgG molecule and the Fab fragments. Electrophoretic analysis of the two peak fractions under reducing conditions also identified the Fc fragment and the heavy and light chain portions of the Fab fragments. Both the Ab-NTF and Ab-CTF were found to belong to the IgG, k subclass.

The reactivity of the isolated antibodies with 30 S and 50 S ribosomal subunit proteins was assessed by immunoblot analysis. Fig. 1A shows the stained pattern obtained after SDS electrophoresis and electroblotting onto nitrocellulose of 50 S ribosomal protein L7/L12 (lane 1), total protein from 30 S (lane 2) and 50 S (lane 3) ribosomal subunits. Immunoblot analysis of these protein patterns with Ab-NTF (Fig. 1C) reveals that both antibodies react with fragment 1-73 and not 74-120. Furthermore, it does not react with fragment 29-120. Either cleavage at position 28-29 destroys the epitope because it spans 1-28 and 29-73, or it lies within residues 1-28. Panel B summarizes the results.

Fig. 1. Reactivity of Ab-NTF and Ab-CTF with 30 S and 50 S ribosomal proteins. A, ribosomal proteins were separated by 17.5% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose by electroblotting and stained with Amido Black. 1, L7/L12; 2, total protein from the 30 S ribosomal subunit; 3, total protein from the 50 S ribosomal subunit. B, reaction of Ab-NTF as determined by immunoblot analysis of the blot shown in A. 1, L7/L12 protein; 2, total 30 S protein; 3, total 50 S protein. C, same analysis as in B using Ab-CTF.
hysterin in the polyphenylalanine synthesis assay (Fig. 4B). Preincubation of 50 S ribosomes with a 5–8-fold molar excess of Fab fragments over ribosomal subunits resulted in a degree of inhibition comparable to that produced by a 2–4-fold molar excess of intact antibody over ribosomal subunits.

Poly(U)-directed polyphenylalanine synthesis is dependent on the association of the ribosomal subunits to form 70 S ribosomes in the reaction mixture. Inhibition of subunit association or, alternatively, dissociation of 70 S ribosomes caused by the antibodies would result in inhibition of polyphenylalanine synthesis. An effect of Ab-NTF on subunit association would be consistent with the lower reactivity of 70 S versus 50 S shown in Fig. 2. To test this possibility, antibodies were preincubated with 50 S ribosomal subunits followed by addition of a 3-fold excess of 70 S ribosomal subunits over 50 S subunits. The mixtures were then analyzed by sucrose gradient centrifugation. The presence of neither Ab-NTF nor Ab-CTF prevented the formation of 70 S ribosomes: the sucrose gradient profiles were identical to those obtained from control ribosomes in the absence of antibody. In addition, the antibodies, despite their bivalent nature, did not cause the formation of any detectable 50 S ribosomal subunit dimers.

To investigate further the inhibitory effect of the antibodies in the polyphenylalanine synthesis system, the ribosome-dependent GTPase activity, also a requirement for the protein synthesis-elongation cycle, was assayed. Fig. 5 shows that this activity was inhibited by Ab-NTF and Ab-CTF, while the presence of nonimmune mouse IgG had little effect. The antibodies, when preincubated with L7/L12 showed no significant inhibition (results not shown). Subsequent analysis of the binding of elongation factor EF-G to 70 S ribosomes revealed that both Ab-NTF and Ab-CTF prevented the factor-ribosome complex formation in the presence of GTP and fusidic acid (Fig. 6). This was also found when a noncleavable analog of GTP (in the absence of fusidic acid) was used in the assay (result not shown).

DISCUSSION

Two antibodies against mutually exclusive epitopes of protein L7/L12 have been prepared and used as probes of ribosome function. The epitope of one antibody (Ab-CTF) is located within residues 74–120, the C-terminal portion of the molecule. That of the other (Ab-NTF) is located within residues 1–73. Attempts to define the epitopes more precisely have been inconclusive, but suggest that the Ab-NTF epitope is located within the N-terminal one-third of the antibody fails to react with fragment 29-120. The Ab-NTF was obtained by immunization with the intact protein and the Ab-CTF by immunization with the 74–120 residue fragment. The latter result shows the feasibility of generating monoclonal antibody probes of predetermined specificity for ribosomal proteins.

The monoclonal antibodies have been tested for their effects on ribosome function. The results refine and extend those obtained previously with polyclonal antibodies of undetermined epitope specificity (22). Polyclonal antibodies to L7/L12 were found to inhibit polyphenylalanine synthesis (23), EF-G-dependent GTP hydrolysis (24), and EF-G binding to the ribosome (25). Our results agree with these earlier findings. Both NTF and CTF antibodies prevent association of elongation factor EF-G with the ribosome. Consequently, both EF-G-dependent GTP hydrolysis and polyphenylalanine synthesis are inhibited. A 2–4-fold excess of antibodies over ribosomes led to strong inhibition of the functions assayed, while in earlier studies, using polyclonal antibodies, a 20–100-fold molar excess of antibody over ribosomes was required to
Monoclonal Antibodies against Ribosomal Protein L7/L12

Fig. 4. Effect of anti-L7/L12 monoclonal antibodies and Fab fragments on poly(U)-directed polyphenylalanine synthesis. A, Ab-NTF and Ab-CTF were preincubated with 50 S ribosomal subunits at the molar ratios of antibody to ribosomes indicated. The preincubated ribosomal subunits were then tested for their polyphenylalanine synthesis activity as indicated in the legend of Table I. B, the Fab fragments of Ab-NTF and Ab-CTF were tested as in A. ○, Ab-CTF; ●, Ab-NTF.

Table I
Effect of antibodies against L7/L12 and nonimmune immunoglobulin on polyphenylalanine synthesis

| Additions | Polystyrene synthesis | cpm | % inhibition |
|-----------|------------------------|-----|--------------|
| 1. 70 S   |                        |     |              |
| + Ab-NTF  | 32,800                 | 3,760 | 89           |
| + Ab-CTF  | 3,300                  |
| 2. 70 S   |                        |     |              |
| + Nonimmune IgG | 40,900 | 41,000 | 0           |
| 3. 50 S + 30 S | + Ab-NTF | 5,160 | 1,450 | 72 |
|            | + Ab-NTF + L7/L12 | 2,390 | 3,870 | 25 |
| 4. 50 S + 30 S | + Ab-CTF | 4,800 | 920 | 81 |
|            | + Ab-CTF + L7/L12 | 4,580 | 4,280 | 11 |

achieve the same result (22-25). This difference is most likely the result of the monoclonal nature and the affinity purification of our antibody preparations.

Proteins L7/L12 bind to the 50 S particle through interactions of the N-terminal region with protein L10 (for review, see Ref. 7). There is convincing evidence that the copies of L7/L12 are arranged in parallel with the C termini at the distal portion of the L7/L12 stalk (1, 12). The results obtained in this study demonstrate the functional importance of the C-terminal portion of protein L7/L12, and support a similar conclusion from studies with 50 S ribosomes that were reconstituted with the N-terminal portion of L7/L12 (26).

Cross-linking (18, 27, this laboratory), affinity labeling (28), and immune electron microscopy (29) indicate that EF-G binds to the body of the ribosome near the base of the stalk. Immune electron microscopy shows that one Ab-CTF binds at the tip of the stalk. The mechanism by which this antibody inhibits EF-G binding is not clear at this time. The result suggests that the C termini may not always occupy a fixed location distal from the body of the ribosomal subunit at the tip of the stalk.

Preliminary studies on the effect of Ab-NTF show that incubation of 50 S subunits with these antibodies leads to the appearance of particles without stalks. On the other hand, 2 copies of L7/L12 remain on the ribosome as shown by radioactive labeling. The appearance of stalks has previously been studied as a function of the ratio of L7/L12 to 50 S core particles in reconstitution experiments and the results suggest that only one dimer is required for the appearance of a stalk (9). The possible specificity of the antibody induced release

2 H. Olson and D. Glitz, unpublished results.
3 A. Sommer, D. Tewari, and R. R. Traut, unpublished results.
with respect to the multiple copies of L7/L12 and the locations of all the copies of L7/L12 are being investigated.

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**Monoclonal Antibodies against Ribosomal Protein L7/L12**

**Preparation and Characterization of Monoclonal Antibodies Against Different Epitopes in L7 and L12 Ribosomal Proteins**

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**EXPERIMENTAL PROCEDURES**

**Thiophosphate**

**Antibodies against Ribosomal Protein L7/L12**

Purified monoclonal antibodies against Ribosomal Proteins L7/L12 were produced using the hybridoma technique. The antibody producing hybridoma cell line was established from a mouse immunized with purified L7/L12 protein. The antibodies were affinity-purified using Protein A-Sepharose and characterized for specificity and affinity.

**Immunological studies and light microscopy**

L7/L12 epitopes were identified using specific monoclonal antibodies against L7/L12. The antibodies were used in immunofluorescence and immunoperoxidase techniques to study the distribution and expression of L7/L12 in various tissues.

**Immunohistochemical studies**

Immunohistochemical studies of L7/L12 expression were performed using monoclonal antibodies against L7/L12. The antibodies were used in immunohistochemical staining techniques to study the localization of L7/L12 in different cell types.

**Preparation of L7/L12 peptides**

Peptides corresponding to the amino acid sequences of L7/L12 were synthesized and used as immunogens to generate antibodies against L7/L12. The antibodies were characterized for specificity and affinity.

**Conclusions**

The monoclonal antibodies against L7/L12 were characterized for specificity and affinity. The antibodies were used in immunohistochemical and immunofluorescence techniques to study the expression and localization of L7/L12 in different cell types. The results indicate that L7/L12 is expressed in various cell types and is involved in different biological processes.