ALTERATION OF RIBOSOMAL PROTEIN S2 IN KASUGAMYCIN-RESISTANT MUTANT DERIVED FROM ESCHERICHIA COLI AB312

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Summary
A new type of kasugamycin-resistant mutant has been isolated from E. coli K12, strain AB312 (Hfr, lac, thr, leu, thi, strA, fus). In a cell-free protein-synthetic system, the resistance is localized in the ribosome but not in the supernatant fraction. On initiation complex formation, the resistance is associated with the washed ribosome but not with initiation factors. In reconstitution of the 30S ribosomal subunit, the resistance is due to the protein(s) but not to 16S RNA. In two-dimensional electrophoresis, protein S2 is deficient in the 30S ribosomal subunit of kasugamycin-resistant mutant. The results indicate that the kasugamycin-resistance is attributed to alteration of ribosomal protein S2.

Kasugamycin, an aminoglycosidic antibiotic, is a selective inhibitor of initiation in bacterial protein synthesis (1,2), and a useful tool for investigating the mechanism of regulation of cistron-specific initiation, because the antibiotic differentially inhibits initiation of translation of maturation and coat protein cistrons on f2 phage RNA (3,4,5). The mechanism of inhibition of protein synthesis seems to be different from that of streptomycin and other aminoglycosides, since kasugamycin does not induce codon misreading (6). On the resistance to kasugamycin, ksgA and ksgB have been reported (7,8). In these mutants, the resistance is attributed to alteration of 16S ribosomal RNA or permeability, but not to that of ribosomal protein (9).

In the present experiment we have isolated a new type of kasugamycin-resistant mutant, in which the resistance is associated with alteration of ribosomal protein S2.

Methods

Several kasugamycin-resistant mutants were isolated from *E. coli* AB312 (Hfr, *lac*, *thr*, *leu*, *thi*, *strA*, *fus*) by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (10). The incorporation of [^{14}C] valine with poly UG was performed by the same procedure as described previously, except that S30 and f2 RNA were replaced by 0.1M NH_4Cl-washed ribosomes 2 mg/ml, S100 1 mg/ml, and poly UG 100 μg/ml (3). The formation of 70S initiation complex followed the method previously described, using poly UG 100 μg/ml instead of f2 RNA (1). The 30S ribosomal subunit was reconstituted by the technique of Traub and Nomura (11). The method of Kaltschmidt and Wittmann (12) was applied to the two-dimensional polyacrylamide gel electrophoresis, using the apparatus of YEDA, Research and Development Co., Rehovot, Israel.

Results

The poly UG-directed incorporation of valine was markedly inhibited by kasugamycin with the 70S ribosomes obtained from kasugamycin-

Table 1. Localization of kasugamycin resistance in the ribosomes obtained from *E. coli* AB312 ksg^r: Incorporation of valine with poly UG.

S100	Ribosome	[^{14}C] Val incorporated cpm (% of control) Concentration of kasugamycin (μg/ml) 0	100
S	S	14225 (100)	130 (1)
S	R	15980 (100)	16230 (103)
R	S	15495 (100)	3442 (21)
R	R	17648 (100)	17135 (97)

The reaction mixtures contained: Tris-HCl, pH 7.5, 50 mM, KCl 30 mM, $Mg(AcO)_2$ 9 mM, 2-mercaptoethanol 6 mM, 0.1 M NH_4Cl-washed ribosomes 2 mg/ml, S100 1 mg protein/ml, poly UG 100 μg/ml, *E. coli* tRNA 0.15 mg/ml, [^{14}C] valine (165 μCi/μmole) 0.2 μCi/ml, amino acids except valine 0.025 mM, ATP 2 mM, PEP 5 mM, pyruvate kinase 20 μg/ml, and 0.1 mM of GTP, 0.2 ml in each tube. It was incubated at 37°C for 10 min. The TCA-insoluble radioactivity was determined with correction for the values obtained in parallel mixtures without messenger.

Table 2. Localization of kasugamycin resistance in the washed ribosomes derived from *E. coli* AB312 ksg^r: 70S initiation complex formation on poly UG.

Washed ribosome	Initiation factor	[^{14}C] fMet-tRNA bound cpm (% of control) Concentration of kasugamycin (μg/ml) 0	10	100
S	S	1822 (100)	906 (49)	0 (0)
S	R	1774 (100)	1117 (63)	213 (12)
R	S	1945 (100)	2228 (115)	1672 (86)
R	R	1858 (100)	1821 (98)	1301 (70)

The reaction mixtures contained: Tris-HCl, pH 7.5, 50 mM, NH_4Cl 60 mM, $Mg(AcO)_2$ 6 mM, 2-mercaptoethanol 10 mM, 1 M NH_4Cl-washed 70S ribosomes 2 mg/ml, initiation factor 0.6 mg/ml, [^{14}C]fMet-tRNA ([^{14}C]Met 222 mCi/mmole) 160 μg/ml, poly UG 100 μg/ml, and GTP 0.2 mM, 0.1 ml in each tube. It was incubated at 37°C for 15 min. The radioactivity, collected on Millipore filter, was assayed with correction for the values without messenger.

sensitive strain, but was not significantly affected with those from the resistant mutant in ribosome-S100 recombination systems. On the contrary, the S100 of sensitive and resistant strains showed the same sensitivity to the antibiotic. The results indicated that the kasugamycin resistance is attributed to alteration of 0.1 M NH_4Cl-washed 70S ribosomes, but not to that of S100 (Table 1).

Since kasugamycin affects initiation of protein synthesis (1,2), the sensitivity of initiation factors of different origins was then examined. The 70S initiation complex formation on poly UG was markedly blocked by kasugamycin with the 70S ribosomes of the sensitive strain, while it was hardly affected with those of the resistant mutant. No significant difference of the antibiotic effects was demonstrated with the initiation factors of both origins. The results showed that the kasugamycin resistance is localized in the 1 M NH_4Cl-washed ribosomes, but not in the initiation factors (Table 2).

In reconstitution of ribosomes, the kasugamycin resistance was associated with the 30S subunit but not with the 50S subunit (The

data are not shown). In the reconstitution experiment of the 30S subunit, the incorporation of valine with poly UG was more significantly affected by the antibiotic when the total proteins of the 30S ribosomal subunit of the sensitive strain were used than when those of the resistant mutant. No significant difference was observed with the origin of 16S RNA. The results indicated that the kasugamycin resistance is due to the change of the ribosomal protein but not to that of 16S RNA (Table 3).

The pattern of 30S ribosomal proteins of the sensitive and resistant cells were compared by the two-dimensional polyacrylamide gel electrophoresis. The spot of S2 was deficient and a new spot appeared near the S2 site in case of the resistant mutant, while all the other spots showed good correspondence in both strains. The results suggested that the kasugamycin resistance is attributed to alteration of ribosomal protein S2 (Fig. 1).

Discussion

Kasugamycin selectively inhibits initiation of bacterial protein

Table 3. Localization of kasugamycin resistance in the 30S ribosomal protein: Reconstitution of the 30S ribosomal subunit obtained from *E. coli* AB312 ksg^r.

Reconstituted 30S		[^{14}C]Val incorporated cpm (% of control)				
16S RNA	Total protein	Concentration of kasugamycin (μg/ml) 0	10	20	50	100
S	S	2043(100)	1369(67)	1001(49)	797(39)	695(34)
S	R	1489(100)	1429(94)	1117(75)	938(63)	878(59)
R	S	2016(100)	1350(67)	1169(58)	968(48)	847(42)
R	R	1578(100)	1515(96)	1294(82)	1168(74)	1010(64)

The reactions were performed as described in Table 1, except that the reconstituted 30S ribosomal subunit 0.66 mg/ml, 50S subunit 1.32 mg/ml and initiation factor 0.6 mg/ml were used instead of the ribosomes in the mixtures.

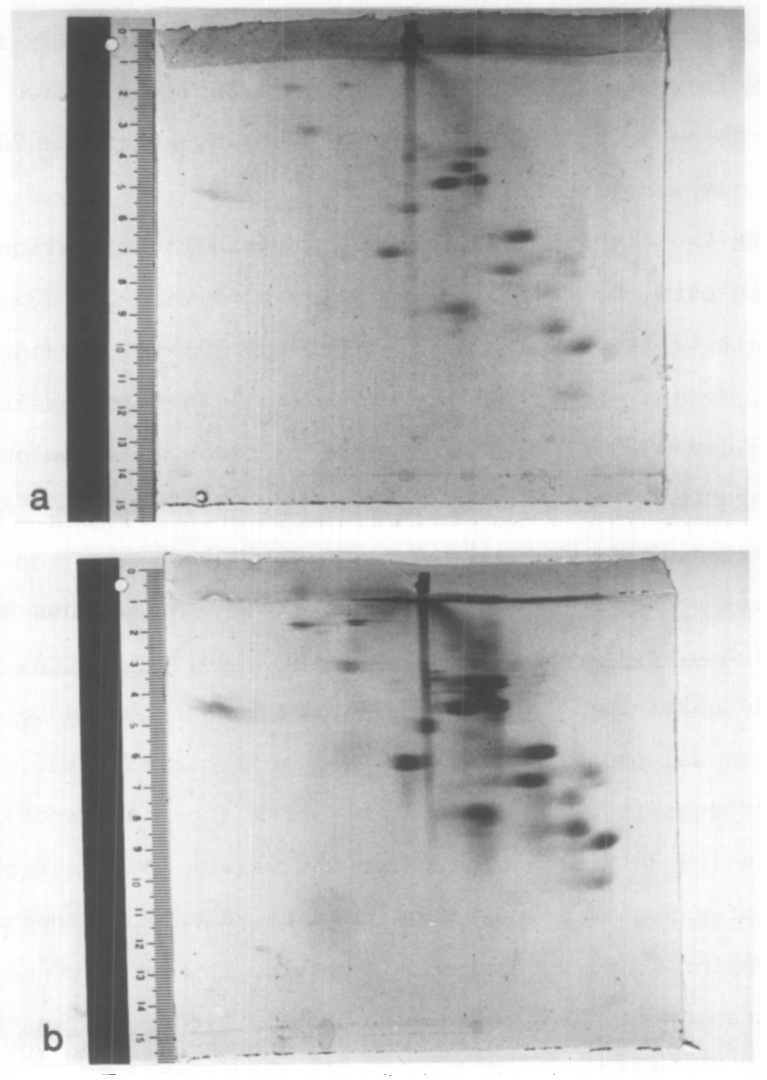

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis of 30S ribosomal proteins. (a) E. coli AB312 ksg^S (b) E. coli AB312 ksg^r

synthesis by blocking the 30S initiation complex formation on f2 RNA and poly AUG. The antibiotic also inhibits the 70S initiation complex formation with GTP or GDPCP, while streptomycin blocks the formation with GTP but not with GDPCP (1). Unlike streptomycin, kasugamycin does not significantly release fMet-tRNA from the 70S

initiation complex. The antibiotic does not significantly affect fMet-puromycin synthesis and the binding of Ala-tRNA, which is the second amino acid of f2 phage coat protein, after the 70S initiation complex formation (2).

Concerning the function of ribosomal protein in initiation of protein synthesis, Bollen et al. (13) reported that the 30S ribosomal subunit is missing S1 and S2 after the 30S initiation complex formation. Nomura et al. (14) described that S6 is necessary for IF2 and AUG-dependent fMet-tRNA binding to ribosomes. Lelong et al. (15) reported that antibody against S2, S5, S6, S12, S13, and S14 preferentially inhibits the binding of fMet-tRNA.

In the present study, a kasugamycin-resistant mutant has been isolated from E. coli AB312 (Hfr, lac, thr, leu, thi, strA, fus) (10), and demonstrated that the resistance is attributed to alteration of ribosomal protein S2. We have also isolated kasugamycin-resistant mutants from E. coli Q13, in which the resistance is localized in 16S RNA (9). The reason why we are able to isolate a new type of kasugamycin-resistant mutants, altering ribosomal protein, remains to be determined. However, it may be connected with the mutation of the parent strain (Hfr, lac, thr, leu, thi, strA, fus).

From the studies on the mode of action of kasugamycin (1-3) and the mechanism of resistance, we propose an assumption that ribosomal protein S2 may be one of initiation-specific proteins concerning the binding of fMet-tRNA to the 30S ribosomal subunit and the regulation of cistron-specific initiation.

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References

1. Okuyama, A., Machiyama,N., Kinoshita, T., and Tanaka, N. (1971) Biochem. Biophys. Res. Commun. 43, 196-199.
2. Okuyama, A., Watanabe, T., and Tanaka, N. (1972) J. Antibiotics 25, 212-218.
3. Okuyama, A., and Tanaka, N. (1972) Biochem. Biophys. Res. Commun. 49, 951-957.
4. Okuyama, A., and Tanaka, N. (1973) Biochem. Biophys. Res. Commun. 52, 1463-1469.
5. Kozak, M., and Nathans, D. (1972) J. Mol. Biol. 70, 41-55.
6. Tanaka, N., Yamaguchi, H., and Umezawa, H. (1966) J. Biochem. (Tokyo) 60, 429-434.
7. Sparling, P.F. (1968) Science 167, 56-57.
8. Sparling, P.F., Ikeya, Y., and Elliot, D. (1973) J. Bacteriol. 113, 704-710.
9. Helser, T.L., Davies, J.E., and Dahlberg, J.E. (1971) Nature New Biol. 232, 12-14.
10. Tanaka, N., Kawano, G., and Kinoshita, T. (1971) Biochem. Biophys. Res. Commun. 42, 564-567.
11. Traub, P., and Nomura, M. (1969) J. Mol. Biol. 40, 391-413.
12. Kaltschmidt, E., and Wittmann, H.G. (1970) Analyt. Biochem. 36, 401-412.
13. Bollen, A., Petre, J., and Grosjean, H. (1972) FEBS Lett. 24, 327-330.
14. Nomura, M., Mizushima, S., Ozaki, M., Traub,P., and Lowry, C.V. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 49-61.
15. Lelong, J.C., Gros, D., Gros, F., Bollen, A., Maschler, R., and Stöffler, G. (1974) Proc. Nat. Acad. Sci. 71, 248-252.