Erythromycin Resistance Peptides Selected from Random Peptide Libraries*

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Translation of a 5-codon mini-gene encoded in Escherichia coli 23 S rRNA was previously shown to renders cells resistant to erythromycin (Tenson, T., DeBlasio, A., and Mankin, A. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5641–5646). Erythromycin resistance was mediated by a specific interaction of the 23 S rRNA-encoded pentapeptide with the ribosome. In the present study, peptides conferring erythromycin resistance were selected from in vivo expressed random peptide libraries to study structural features important for peptide activity. Screening of a 21-codon mini-gene library (the general structure ATG (NNN)20 TAA) demonstrated that only short peptides (3–6 amino acids long) conferred erythromycin resistance. Sequence comparison of erythromycin resistance peptides isolated from the 5-codon library (ATG (NNN)4 TAA) revealed a strong preference for leucine or isoleucine as a third amino acid and a hydrophobic amino acid at the C terminus of the peptide. When tested against other antibiotics, erythromycin resistance peptides rendered cells resistant to other macrolides, oleandomycin and spiramycin, but not to chloramphenicol or clindamycin. Defining the consensus amino acid sequence of erythromycin resistance peptides provided insights into a possible mode of peptide action and the nature of the peptide binding site on the ribosome.

It was assumed for a long time that the ribosome is indiffer-ent to the sequence of the polypeptide it is synthesizing. New evidence, however, indicates that nascent or newly synthesized polypeptides can affect functions of the ribosome in cis. In a number of cases, the newly translated peptide exerts its effect on translation while still being located within the ribosome. For example, short nascent peptides regulate stalling of the ribosome on mRNA, which is required for inducing the expression of chloramphenicol resistance (cat and cmlA) and erythromycin resistance (erm) genes (1, 2). Ribosome stalling depends on the amino acid sequence of the nascent peptide rather than on the nucleotide sequence of mRNA and occurs when the nascent peptide is only several amino acids long and should be located within the ribosome. Other examples include: translational bypass of the coding gap in bacteriophage T4 gene 60 mRNA (ribosome “hopping”), which depends on the amino acid se-

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1 The abbreviations used are: E-peptide, erythromycin resistance pentapeptide; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MIC, minimal inhibitory concentration.
EXPERIMENTAL PROCEDURES

Strains and Materials—E. coli JM109 strain (10) (endA1, recA1, gyrA96, thi, hsdR17 (rK-, mK+), relA1, supE44, Δ(lac-proAB), [F', traD36, proA, lacP28, rpsL15]) was used for most of the cloning experiments. For the library construction, the ligation mixtures were originally transformed into ultracompetent E. coli cells XL2-Blue MRF' (ΔmcrA) or XL2-Blue MRF' (ΔmcrAΔmsr msrR raf173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac I', proA, lacP28, rpsL15) (Stratagene). Synthetic oligonucleotides were from DNeasy. Enzymes were from Promega and New England Biolabs. Chemicals and antibiotics were from Fisher or Sigma.

Library Construction—pPOT1 vector described previously (8) was used for the construction of random peptide libraries. The original vector, which contains a Ptac promoter-Ttrp terminator expression cassette with a single Nhel cloning site, was modified by introducing sites for restriction nucleases EcoRI and AluI between the Ptac promoter and trp terminator. The resulting vector, pPOT1AE (see Fig. 1), was cut with EcoRI and AluI, and the linear plasmid was gel-purified.

Random peptide mini-gene DNA was prepared from synthetic oligonucleotides dGGCTTAAGGAGGTCACATATG(N)60TAACTAGCTGAA- ATTCCG or dGGCTTAAGGAGGTCACATATG(N)46TAACTAGCTGAA- ATTCCG. The oligonucleotides were PCR-amplified from a pair of primers, d(CGGAATTCAGCTAGTTA) and d(GGCTTAAGGAGGTCA- ATTCCG). The PCR products were cut with HincII and EcoRI and ligated with linearized pPOT1AE vector.

Plasmid libraries were transformed into E. coli XL2-Blue MRF' ultracompetent cells (Stratagene). An aliquot of transformed cells from each library was plated onto agar plates to estimate the number of clones in each library, whereas the rest of the cells were grown in 100 ml of LB medium containing 100 μg/ml ampicillin. When culture densities reached A550 = 0.8, cells were harvested, and plasmid libraries were isolated. The 5- and 21-codon libraries contained 5 × 105 and 1 × 106 clones, respectively.

Selection of Erythromycin Resistance Peptides—E. coli JM109 competent cells were transformed with the random mini-gene plasmid libraries and plated onto LB agar plates containing 100 μg/ml ampicillin, 150 μg/ml erythromycin, and 1 mM IPTG. Plates were incubated overnight at 37 °C. Colonies that appeared on plates were streaked on plates containing 100 μg/ml ampicillin and 150 μg/ml erythromycin or 100 μg/ml ampicillin, 150 μg/ml erythromycin, and 1 mM IPTG. Colonies growing in the presence but not in the absence of IPTG were taken for further analysis. Plasmids were isolated from all selected clones and retransformed into fresh competent cells, and phenotypes of the secondary transformants were checked by replica plating onto ampicillin/erythromycin or ampicillin/erythromycin/IPTG plates. Peptide mini-genes from the plasmids conferring retransformable IPTG-dependent erythromycin resistance were sequenced.

Selection of clones resistant to a higher concentration of erythromycin was performed in essentially the same way except that the selective plate contained 1 mg/ml instead of 150 μg/ml erythromycin.

Comparing Erythromycin Resistance of Cells Expressing Different E-peptides—Overnight cultures of cells expressing different E-peptides were grown in LB medium containing 100 μg/ml ampicillin. Cultures were diluted with LB medium containing 100 μg/ml ampicillin and 2 mM IPTG to the final density of A550 = 0.005. 3 ml of each culture was placed into two 15-ml tubes, and tubes were incubated at 37 °C with constant shaking. After a 1 h incubation, 10 μl of erythromycin solution (30 mg/ml) was added to one of the two tubes in each parallel trial, and cells were grown until the optical density of the control cultures reached A550 = 1. At this time, optical densities of all cultures were measured. Absorbance of cultures grown in the presence of erythromycin was divided by the absorbance of cultures grown in the absence of erythromycin, and the results were plotted.

Testing Antibiotic Resistance of E-peptide-expressing Cells—Overnight cultures were grown from cells transformed either with the empty pPOT1AE vector, a plasmid isolated from a randomly picked unselected clone expressing pentapeptide MDVEQ or a plasmid from Ery′ clones expressing E-peptide MSLKV. Cultures grown in LB medium containing 50 μg/ml ampicillin and 1 mM IPTG to A550 = 0.988. Erythromycin, oleandomycin, spiramycin, chloramphenicol, or clindamycin was then added to concentrations of 100, 1000, 2000, 1, and 50 μg/ml, respectively. Cultures were grown until optical density of the control culture, grown only in the presence of ampicillin and IPTG, reached A550 = 1. At this time, optical densities of all cultures were measured and normalized relative to the control culture.

FIG. 1. A 21-codon mini-gene library in pPOT1AE vector. pPOT1AE is identical to the pPOT1 vector described previously (8) except that AluI and EcoRI cloning sites were introduced into a single cloning Nhel site of pPOT1. IPTG-inducible Ptac promoter is shown as a black bar, and the lac operator (Olae) and trp terminator (Trtp) are shown as hatched bars. The transcription start site is indicated by an arrow. AluI and EcoRI sites used for cloning of the mini-gene library are shown. The Shine-Dalgarno sequence (S-D.), initiator AUG codon, and terminator UAA codon of the mini-gene are underlined. Positions of β-lactamase gene (Ap′) and lac I′ genes in the plasmid are shown by open arrows.

FIG. 2. Size distribution and amino acid sequences of erythromycin resistance peptides isolated from the 21-codon library. A, distribution of peptide sizes (number of amino acids) encoded in mini-genes in randomly picked unselected clones (open bars) and erythromycin-resistant clones (shaded bars). The y axis represents the number of sequenced clones that encoded peptides of a particular size. B, amino acid sequences of peptides encoded in mini-genes in erythromycin-resistant clones. Peptide sequences are aligned relative to the C-terminal amino acid.
RESULTS

Construction of Random Mini-gene Libraries—Two random mini-gene plasmid libraries were constructed for isolation of peptides whose expression renders cells resistant to erythromycin. Random mini-genes were generated by PCR amplification of oligonucleotides containing initiator and terminator codons separated by 12 (for 5-codon library) or 60 (for 21-codon library) nucleotides from both libraries showed no significant bias in nucleotide composition in the randomized segment of the mini-gene. The PCR-amplified mini-gene library was introduced unidirectionally in the pPOT1AE vector (8), where transcription of the mini-gene was ensured by translation of the mini-gene. The PCR-amplified mini-gene library was introduced unidirectionally in the pPOT1AE vector (8), where transcription of the mini-gene was controlled by a strong IPTG-inducible Pτac promoter. Sequencing mini-genes from a number of randomly picked unselected clones from both libraries showed no significant bias in nucleotide composition in the randomized segment of the mini-gene.

The 5-codon library contained ~500,000 clones. Since the total number of various pentapeptides (with fixed methionine in the first position) is 20^5 = 160,000, it was assumed that most possible pentapeptides were encoded in the 5-codon library. The 21-codon library had ~10^6 clones. Naturally, only a relatively small segment of the sequence space corresponding to all possible peptides encoded in 21-codon-long open reading frames were represented in this library.

Isolation of Erythromycin-resistant Clones from 21-codon Random Mini-gene Library—Due to an occasional presence of stop codons in a random open reading frame, the 21-codon library can encode peptides ranging in size from 1 to 21 amino acids. This library was used primarily to determine the predominant size of erythromycin resistance peptides. Clones that became erythromycin-resistant due to expression of peptide mini-genes were selected by plating the 21-codon library on agar medium containing ampicillin, erythromycin, and IPTG. For most clones that appeared on the plate, the Eryr phenotype was retransformable with the plasmid and depended on the presence of IPTG in the medium, indicating that expression of the peptide mini-gene was necessary for the resistance. Plasmids from 12 Eryr clones were sequenced alongside of plasmids isolated from several unselected, randomly picked clones. Whereas mini-genes in unselected clones showed a broad distribution of sizes of the encoded peptides (open bars in Fig. 2), the mini-genes in 12 isolated Eryr clones encoded only short peptides in a very narrow range of sizes, from 3 to 6 amino acids (filled bars in Fig. 2). Thus, it appears that only short peptides can confer resistance to erythromycin.

Erythromycin Resistance Peptides from 5-codon Mini-gene Library—The experiment with the 21-codon library showed that expression of predominantly short peptides can render cells erythromycin resistant. Furthermore, the first described erythromycin resistance peptide (E-peptide) is encoded in a 5-codon-long open reading frame in the E. coli 23 S rRNA (8). Therefore, the next selection and all subsequent experiments were done with a plasmid library where random mini-genes contained only 5 codons. By analogy with the rRNA-encoded E-peptide, the erythromycin resistance pentapeptides selected from the library are referred to as E-peptides.

More than 100 Eryr clones were selected from the 5-codon library on ampicillin/IPTG plates containing 150 µg/ml erythromycin. The relation of the Eryr phenotype to the expression of plasmid-encoded peptides was confirmed by IPTG-dependence of erythromycin resistance and by its co-transference with the plasmid. Peptide mini-genes from ~50 Eryr clones were sequenced. Only 1 of these clones had an in-frame stop codon in the mini-gene that coded for a tetrapeptide MILV; pentapeptides were encoded in all the rest of the clones. Sequences of E-peptides expressed in Eryr clones showed significant devia-

FIG. 3. Nucleotide sequences of mini-genes and amino acid sequences of the encoded peptides expressed in erythromycin-resistant clones isolated on plates with 150 µg/ml erythromycin (A), 1 mg/ml erythromycin (B), and randomly picked unselected clones (C) from the 5-codon library. Conserved Leu or Ile in the third position and hydrophobic amino acids in the C-terminal position of the peptide sequence are underlined. Asterisks in the peptide sequences correspond to stop codons in peptide mini-genes.

2 S. Douthwaite, personal communication.
Resistance in different antibiotics—

To investigate whether E-peptide expression affects sensitivity to antibiotics other than erythromycin, cells transformed with the plasmid coding for the E-peptide MSLKV were grown in the presence of several antibiotics known to interact with the large ribosomal subunit (Fig. 5). Expression of the E-peptide increased cell resistance not only to erythromycin but also to two other macrolide antibiotics, spiramycin and oleandomycin, whereas sensitivity to chloramphenicol and clindamycin was not affected.

**DISCUSSION**

In the present study we asked the question, Which properties of a peptide make possible its functional interaction with the ribosome resulting in resistance to erythromycin? To answer this question, we used random mini-gene libraries for isolation of a variety of erythromycin resistance peptides. The use of mini-gene expression libraries has a number of advantages compared with the other combinatorial methods exploiting libraries of synthetic peptides (15) or phage display libraries (16). First, it is much easier to synthesize a random DNA sequence of the peptide gene than a random amino acid sequence of the peptide itself, leading to better representation of a random peptide sequence space in a mini-gene library. Second, phenotypic selection permits not only screening of hundreds of thousands of peptide sequences in a single experiment but also amplification of the “signal” (the selected sequences) by growing cells that passed the selection. Third, in contrast to phage display libraries where a random amino acid sequence is expressed as a segment of a larger protein, the mini-gene library peptides are expressed in their free form, which can be critical for assessing functionality of the peptide. Because of these advantages, random mini-gene libraries can be used for isolation of different functional peptides including enzyme cofactors, inhibitors, etc.

In our experiments, a number of clones expressing erythromycin resistance peptides were isolated from 21- and 5-codon libraries. Comparison of peptide sequences allowed us to draw the first conclusions about the sequence and size requirements for peptide activity. Thus, screening of the 21-codon library primarily revealed the preferred size of erythromycin resistance peptides. Each of the random codons in the library mini-gene can be either 1 out of a possible 61 sense codons or 1 of the 3 stop codons. The probability that, out of 20 random codons, none will be a terminator codon is \((61/64)^{20} = 0.38\); therefore, about two-thirds of the clones in the 21-codon library are expected to have in-frame stop codons. Indeed, as expected, a broad distribution of sizes of the encoded peptides were found..
in unselected, randomly picked clones. In contrast, the majority of peptides expressed in Ery\textsuperscript{r} clones fell within an amazingly narrow size range; 11 out of 12 peptides were 4, 5, or 6 amino acids long. Though it is possible that more extensive screening could reveal some functional peptides larger than hexapeptides, this experiment showed a clear tendency of erythromycin resistance peptides to be 4–6 amino acids long. In agreement with this finding, the originally described rRNA-encoded E-peptide was 5 amino acids long (8).

Previously it had been demonstrated that any mutation eliminating the stop codon of the rRNA-encoded E-peptide abolished erythromycin resistance (8). This showed that a mere presence of the E-peptide sequence at the N terminus of a longer polypeptide could not render ribosomes resistant to erythromycin. The results of screening a 21-codon library not only confirmed this observation but also indicated that the E-peptide sequence is not functional when present at the C terminus of a longer oligopeptide (otherwise we could isolate clones coding for long peptides where a critical sequence would be located close to the C terminus). Thus, we can conclude that an erythromycin resistance peptide cannot be part of a longer protein and that the size of the peptide is essential for its activity. The strict size limitation may mean that the peptide binding site cannot accommodate a longer polypeptide.

If analysis of clones isolated from the 21-codon library revealed peptide size preference, then screening the 5-codon library provided clues to the sequence features that are important for E-peptide activity. Comparison of pentapeptide sequences found in Ery\textsuperscript{r} clones selected at 150 \(\mu\)g/ml erythromycin showed a strong tendency of E-peptides to have Leu or Ile in the third position and a hydrophobic amino acid in the C-terminal position. Not only did these sequence signatures appear in the majority of isolated E-peptides (Fig. 3A), but there is also a correlation between the degree of peptide activity and the presence of Leu or Ile in the third position and a hydrophobic amino acid at the C terminus (Fig. 4). Peptides expressed in clones growing at a very high concentration of erythromycin (1 mg/ml) show even stronger selectivity at positions 3 and 5; most of such peptides (with only one exception) have Leu or Ile in the third position, and all peptides but one have a hydrophobic amino acid, most commonly Val, at the C terminus (Fig. 3B). In addition, peptides expressed in the highly resistant cells frequently have hydrophobic amino acids at the second and fourth positions: 14 out of 16 clones resistant to 1 mg/ml erythromycin express peptides with a hydrophobic amino acid in the second position, and in 8 of these peptides, a hydrophobic amino acid is present also at the fourth position. As a result, most of the peptides isolated from highly resistant clones are very hydrophobic, suggesting that the peptide binding site is also of a hydrophobic nature and presumably not exposed to the solvent.

The ribosome appears to be the primary target of action of E-peptides since translation of the E-peptide mRNA \textit{in vitro} rendered ribosomes resistant to erythromycin (8). At the same time, synthetic E-peptide did not affect sensitivity of the cell-free translation system to erythromycin. This led to a hypothesis that E-peptide enters the ribosome co-translationally and acts in \textit{cis}, affecting properties only of that ribosome on which it has been translated. The simplest way in which E-peptide can render the ribosome resistant to erythromycin is by direct blocking of the drug binding site on the ribosome. This hypothesis is in good agreement with the known mode of erythromycin action and the \textit{cis} nature of the E-peptide effect. Erythromycin interacts with a vacant ribosome in the vicinity of the peptidyltransferase center and inhibits protein synthesis by sterically hindering growth of the nascent peptide (17). \textit{In vitro}, the antibiotic does not inhibit formation of the first peptide bond, but it can inhibit the peptidyltransferase reaction when the donor substrate becomes 2 or more amino acids long (7, 18); nascent peptide chains longer than 2–5 amino acids (depending on the nature of polymerized amino acids) prevent erythromycin binding (19–21). Therefore, in the cell, erythromycin can bind only to the vacant ribosome that has already released a newly synthesized protein but before several amino acids of a newly initiated protein are polymerized. If translated E-peptide does not leave the ribosome and remains tightly bound, the erythromycin binding site will be blocked, and the ribosome will be immune to erythromycin. A newly initiated nascent peptide may possibly go “around” the bound E-peptide or, alternatively, displace it.

A model of E-peptide-ribosome interaction is shown in Fig. 6. The binding site of E-peptide is located most probably in the large ribosomal subunit, in or immediately near the nascent peptide channel, and overlaps with the erythromycin binding site (shown in shading in Fig. 6). Erythromycin starts to inhibit protein synthesis at a step when the third amino acid is added to the growing nascent peptide (22); therefore, if the C-terminal peptide residue is positioned in the ribosomal P-site, then the third residue from the C terminus would be located very close to the hypothetical erythromycin binding site. The bulky hydrophobic side chain of leucine or isoleucine may interfere with interaction of erythromycin with its binding site in the vicinity of the peptidyltransferase center. The peptide may enter its binding site co-translationally from the side of the peptidyltransferase center; this would explain a \textit{cis}-mode of E-peptide action. E-peptide binding is probably stabilized by the interaction of the essential amino acids with the ribosome components, rRNA or proteins. Three amino acid positions in the peptide appear to be primarily important. Besides Leu or Ile in the third position and a hydrophobic residue at the C terminus, the N-terminal formylmethionine may be also critical for peptide binding. Though importance of fMet is difficult to assess since by default it is present in all library-coded E-peptides, the

\begin{figure}
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\caption{Proposed model of E-peptide action. Erythromycin binding site (ERY) is shown in gray, and the binding sites of chloramphenicol (CAM) and clindamycin (CLD) are shown as open triangles. The third position of the peptide, commonly represented by Leu (as shown in the figure) or Ile, is assumed to overlap with the erythromycin binding site. The conserved amino acids (N-terminal formyl methionine, the third Leu or Ile, and C-terminal hydrophobic amino acid commonly represented by Val, as shown in the figure) may form specific contacts with rRNA or ribosomal proteins.}
\end{figure}
fact that E-peptide cannot be part of a longer protein suggests that the position or formylation of the N-terminal methionine is crucial for peptide activity.

Expression of E-peptide rendered cells resistant to other macrolide antibiotics: oleandomycin, which is similar to erythromycin and has a 14-atom lactone ring, and spiramycin, a macrolide with a 16-atom ring. At the same time, E-peptide did not affect cell sensitivity to structurally different chloramphenicol and clindamycin. All drugs tested compete for binding to the ribosome (23); however, the binding sites of chloramphenicol and clindamycin do not precisely coincide with the binding site of macrolides, as demonstrated by RNA footprinting and the difference in the mode of action of these drugs (17, 24, 25). Thus, the site of E-peptide action probably overlaps specifically with the binding site of macrolides but not with that of other antibiotics interacting with the ribosome in the vicinity of the peptidyltransferase center.

In the proposed model, E-peptide is assumed to interact with the large ribosomal subunit in the vicinity of the peptidyltransferase center (Fig. 6). A similar site of action was proposed for the cis-acting peptides regulating expression of erm, cat, and cmlA antibiotic-resistant genes (2, 26). These peptides, acting in a form of peptidyl tRNA, cause ribosome stalling on mRNA in the presence of low, noninhibitory concentrations of erythromycin (27) or chloramphenicol (28). It is conceivable that erythromycin resistance E-peptides and regulatory cis-acting peptides may utilize a basically similar mechanism where tight binding of a peptide to the ribosome in the vicinity of the peptidyltransferase center causes erythromycin resistance in the case of E-peptide or ribosome stalling in the case of regulatory peptides of erm, cat, and cmlA genes. The lack of apparent similarity between the consensus sequence of E-peptide and sequences of other cis-acting peptides may be related to the fact that stalling peptides become active only in the presence of low concentrations of chloramphenicol or erythromycin. Application of a random library approach, which proved useful in the E-peptide studies, may provide insights into functionally important features of other cis-acting peptides and may eventually lead to a better understanding of how the ribosome “talks” to the protein it is synthesizing.

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