

Cellular localization of Type I restriction–modification enzymes is family dependent[☆]

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Abstract

Cellular localization of Type I restriction–modification enzymes *EcoKI*, *EcoAI*, and *EcoR124I*—the most frequently studied representatives of IA, IB, and IC families—was analyzed by immunoblotting of subcellular fractions isolated from *Escherichia coli* strains harboring the corresponding *hsd* genes. *EcoR124I* shows characteristics similar to those of *EcoKI*. The complex enzymes are associated with the cytoplasmic membrane via DNA interaction as documented by the release of the Hsd subunits from the membrane into the soluble fraction following benzonase treatment. HsdR subunits of the membrane-bound enzymes *EcoKI* and *EcoR124I* are accessible, though to a different extent, at the external surface of cytoplasmic membrane as shown by trypsinization of intact spheroplasts. *EcoAI* strongly differs from *EcoKI* and *EcoR124I*, since neither benzonase nor trypsin affects its association with the cytoplasmic membrane. Possible reasons for such a different organization are discussed in relation of the control of the restriction–modification activities in vivo.

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Type I restriction–modification (R–M) systems consist of complex oligomeric enzymes that exhibit restriction endonuclease (REase [1]) or DNA methyltransferase (MTase) activities dependent upon the methylation status of the target sequence on a DNA substrate [2,3]. All Type I R–M systems seem to originate from a common ancestor [4,5]. Evolution has led to enzymes with significantly different specificities, which have been classified into four distinct families: IA (e.g., *EcoKI*), IB (e.g., *EcoAI*), IC (e.g., *EcoR124I*), and ID (e.g., StySBLI), based on genetic complementation, cross-hybridization of genes, and antibody cross-reactivity [6,7]. Families IA, IB, and ID are encoded by allelic genes within the immigration control region of the bacterial genome [8]. *EcoR124I*, the well-characterized

member of IC family, is of plasmid origin [9]. However, a chromosomally encoded member of this family (*EcoprrI*) has been mapped at a location distinct from the immigration control region [5,10]. Despite similarity in the genetic and transcriptional organization, the various families produce dissimilar Type I enzymes with little homology of gene sequence.

The three enzyme subunits HsdR, HsdM, and HsdS, encoded by the closely linked genes *hsdR*, *hsdM*, and *hsdS*, assemble into a large complex with a stoichiometry of R₂M₂S₁ which has both REase and MTase activities, or into a smaller complex M₂S₁ with only MTase activity. The HsdS subunit confers DNA sequence specificity to both complexes [11]. The affinities of the HsdR subunits to bind to the MTase trimer vary between families. While *EcoKI* forms a stable pentamer [12], *EcoR124I* has a tendency to lose one HsdR polypeptide [13] and *EcoAI* readily loses both HsdR subunits [14]. Although the gene order varies between different families, the genes are in all cases expressed

[☆] Abbreviations: R–M, restriction–modification; REase, restriction endonuclease; MTase, methyltransferase.

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from two promoters: P_{Res} (for transcription of *hsdR*) and P_{Mod} (for transcription of *hsdM* and *hsdS* genes). In spite of this genetic organization no support exists for transcriptional regulation of the Type I R–M enzyme [15,16]. However, following transfer of the *hsd* genes to a new host, the enzyme must have a control mechanism that prevents restriction activity preceding modification. It has recently been shown that this temporal control is achieved using post-translational mechanisms either through control of the subunit assembly pathway or via proteolysis of the assembled enzyme (for reviews, see [5,17]).

Despite their small size and lack of obvious cell compartments, bacteria have a complex and dynamic intracellular organization. Proteins are localized to specific subcellular regions, many are known to be membrane associated, i.e., directly or indirectly (through an anchor protein) recognize some components of the cell envelope. Protein function can depend on cellular position, so the analysis of the intracellular location of a protein can be crucial for understanding its activity [18]. As far as Type I R–M enzymes are concerned, an indirect indication of their surface localization was published as early as 1966 [19]. Recently, we have presented direct evidence that *EcoKI* is associated with the inner membrane (probably through interaction with chromosomal DNA), with the HsdR subunit of the complex enzyme accessible to membrane impermeant reagent (trypsin) on the outer surface of the cytoplasmic membrane [20]. Our results have been supported by the finding that the membrane-associated fraction of *EcoKI* is responsible for residual restriction activity of the enzyme under condition of restriction alleviation [21]. To check if such membrane topology is common also for other Type I R–M enzymes, we have analyzed cellular localization of *EcoAI* and *EcoR124I*, representatives of IB and IC families, respectively.

Materials and methods

Bacterial strains, plasmids, and microbiological techniques. Table 1 lists the *Escherichia coli* strains and plasmids used in this study. The appropriate strains were transformed with the plasmids coding for endonuclease, methyltransferase, and individual Hsd subunits either of the Type IC enzyme *EcoR124I* or of the Type IB enzyme *EcoAI*. Cells were grown in Luria–Bertani (LB) medium with addition of antibiotics (ampicillin, 100 mg/ml; chloramphenicol, 50 mg/ml) where required. Transformation and manipulation of nucleic acids were performed using the methods described in [22].

Plasmid pAC15M was prepared by insertion of the *EcoRI* fragment from pJS4M with the *EcoR124I hsdM* gene into pACYC184.

Protein purification. The *EcoR124I* MTase and HsdR subunit were purified as described in [13,23]. The *EcoR124I* REase was reconstituted in vitro by mixing the purified HsdR subunit and MTase at a molar ratio of 2:1. The *EcoAI* REase was reconstituted in vitro by mixing the purified subunits (kindly provided by Dr. Janscak [24]) at a molar ratio of 2HsdR:2HsdM:1HsdS.

Preparation of subcellular fractions and total cell extract. Late logarithmic phase cells were converted to spheroplasts by lysozyme–EDTA treatment according to the method of Minsky et al. [25]; conversion was monitored by phase-contrast microscopy. Spheroplasts were collected by centrifugation at 13,000g for 20 min and subjected to osmotic lysis (in some experiments disrupted also by sonication). Lysates were separated by centrifugation at 50,000g for 1 h into the cytoplasmic (supernatant) and membrane (pellet) fractions. Membrane proteins from the pellet were solubilized in SDS sample buffer by gentle oscillation in an ultrasonic bath and boiled for 5 min. To prepare total cell proteins, aliquots of bacteria were harvested, resuspended in SDS sample buffer, and boiled for 5 min.

Analysis of proteins. Corresponding amounts of solubilized proteins were separated by SDS–PAGE [26] and transferred to nitrocellulose membrane in CAPS buffer, pH 11, using a semi-dry blotter (Sigma). Hsd polypeptides were identified by rabbit polyclonal antibodies anti-*EcoR124I* and anti-*EcoAI* according to standard Western blotting protocol using Super Signal West Pico Chemiluminescent Substrate (Pierce).

Accessibility of Hsd subunits in intact spheroplasts to trypsin. Spheroplasts were suspended in the isotonic buffer and incubated with trypsin (from bovine pancreas, Fluka) at 30 °C for 2 h as described previously [20]. Proteolysis was stopped by the addition of soybean trypsin inhibitor (Fluka) followed by osmotic lysis of spheroplasts and their separation to membrane and cytoplasmic fractions. Controls without either trypsin or trypsin inhibitor were included.

Table 1
Bacterial strains and plasmids

	Characteristics	Reference
<i>E. coli</i> strains		
BL21(DE3)	$F^- dcm, ompT hsdS (r_{\bar{B}} m_{\bar{B}}) gal \lambda$ (DE3)	[38]
JM109(DE3)	$F^+ tra\Delta36, lacI, \Delta(lacZ)M15, proAB recA 1, endA 1, gyrA 96 (Na^R), hsdR 17, mcrA, relA 1, supE, sbcBC, thi-1, \Delta(lac proAB) \lambda$ (DE3)	[39]
Plasmids		
pCP1005	<i>hsdS</i> _{124I} <i>hsdM hsdR</i> on pUR51, Ap ^R	[40]
pJS4M	A derivative of pUC119 and pET3A carrying the <i>hsdM</i> and <i>hsdS</i> genes of the <i>EcoR124I</i> R–M system under control of the P_{T7g10} promoter. Ap ^R	[33]
pLP25R	pTZ19R carrying the <i>hsdR</i> gene expressed from its natural promoter, Ap ^R	[41]
pAC15M	The vector pACYC184 carrying the <i>hsdM</i> gene of <i>EcoR124I</i> under the P_{T7g10} promoter, Cm ^R	This work
pJS491	A derivative of pTZ19R carrying the <i>hsdS</i> gene of <i>EcoR124I</i> under control of the $T7_{g10}$ promoter, Ap ^R	[33]
pFFP30	<i>hsd</i> region of <i>EcoAI</i> on <i>HindIII</i> fragment cloned into pBR322, Ap ^R	[42]
pACRM _A	The vector pACYC184 carrying the <i>hsdM</i> and <i>HsdR</i> genes of <i>EcoAI</i> under control of the $T7_{g10}$ promoter, Cm ^R	[14]
pACM _A	The vector pACYC184 carrying the <i>hsdM</i> gene of <i>EcoAI</i> under control of the $T7_{g10}$ promoter, Cm ^R	[14]

Benzonase treatment. Spheroplasts were subjected to osmotic lysis. Benzonase (Merck) was added to the suspension together with $MgCl_2$ (up to 10 mM) and incubated on ice for 20 min. Lysed spheroplasts were separated into cytoplasmic and membrane fractions as above. The amount of REase and MTase subunits that remained associated with cytoplasmic membrane after benzonase treatment was checked.

Results and discussion

Localization of the Type IC R–M enzyme *EcoR124I*

To localize *EcoR124I* in the bacterial cell the presence of Hsd subunits in subcellular fractions was checked by immunoblotting method. Membrane and cytoplasmic fractions were prepared after osmotic lysis of spheroplasts of JM109(DE3) strain carrying plasmids coding for endonuclease (pCP1005), methyltransferase (pJS4M) as well as for individual subunits HsdR (pLP25R) and HsdM (pAC15M). As demonstrated in Fig. 1, individual HsdR and HsdM subunits are soluble cytoplasmic proteins. Complex REase and MTase are associated with cytoplasmic membrane (Fig. 2). In addition to membrane-bound enzyme, subunits of the REase and MTase were also found in the cytoplasmic fraction; these could represent a balance between membrane-associated assembled enzyme and free subunits or intermediates which are known to play a regulative role [3,12]. HsdR and HsdM became membrane-associated only when co-produced with the HsdS subunit—as an assembled REase or MTase. If the integrity of the membrane was disrupted by sonication of spheroplasts, the membrane-bound *EcoR124I* subunits were released into the soluble fraction (data not shown).

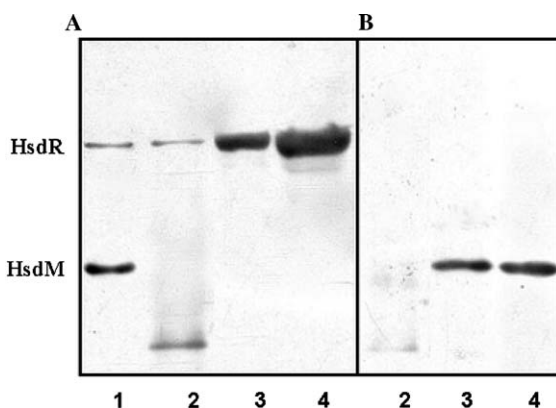


Fig. 1. Immunoblotting of membrane and cytoplasmic proteins from *E. coli* JM109(DE3) carrying plasmids coding for individual *EcoR124I* Hsd subunits: pLP25R/HsdR (A), pAC15M/HsdM (B). Lane 1, standard (purified *EcoR124I*); lanes 2, membrane fractions; lanes 3, cytoplasmic fractions; and lanes 4, total cell extract. The polyclonal antibody used in these experiments fails to detect HsdS. Only under high concentration of antibody a corresponding signal appears, however, an unidentified protein (present also in strains deleted for *hdsS*) interferes with the position of HsdS.

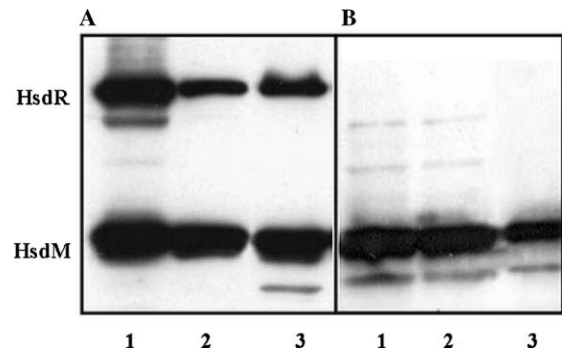


Fig. 2. Immunoblotting of subcellular fractions from *E. coli* JM109(DE3) harboring plasmid pCP1005 with the REase operon (A) or plasmid pJS4M with the MTase operon (B). Lanes 1, total cell proteins; lanes 2, cytoplasmic fractions; and lanes 3, membrane fractions.

Benzonase—an Mg^{2+} - or Mn^{2+} -dependent endonuclease—efficiently hydrolyzes both single- and double-stranded DNA to short oligonucleotides [27]. We have found for the *EcoKI* system [20] that the presence of benzonase during osmotic lysis of spheroplasts resulted in the release of REase and MTase components from the membrane fraction. Analogous experiments with *EcoR124I* led to the same results confirming the involvement of DNA in the interaction of these R–M enzymes with the cytoplasmic membrane (Fig. 3).

Treatment of intact spheroplasts of JM109(DE3) [pCP1005] with trypsin, which, under the given experimental conditions, attacks only proteins on the outer face of cytoplasmic membrane [25,28] results in proteolysis of the HsdR subunit of the REase complex, while MTase components remain protected (Fig. 4). If proteolysis was allowed to continue after lysis of spheroplasts (control

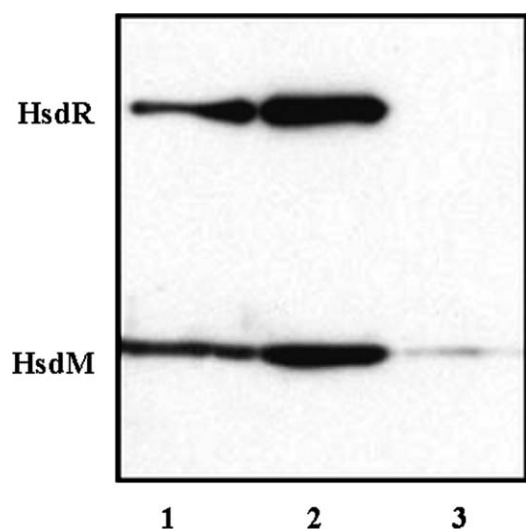


Fig. 3. The effect of benzonase on the association of *EcoR124I* with cytoplasmic membrane. Immunoblot of membrane proteins from spheroplasts *E. coli* JM109(DE3)[pCP1005] treated with benzonase in the course of lysis. Lane 1, standard (purified *EcoR124I*); lane 2, control without benzonase; and lane 3, benzonase 1 U/ml.

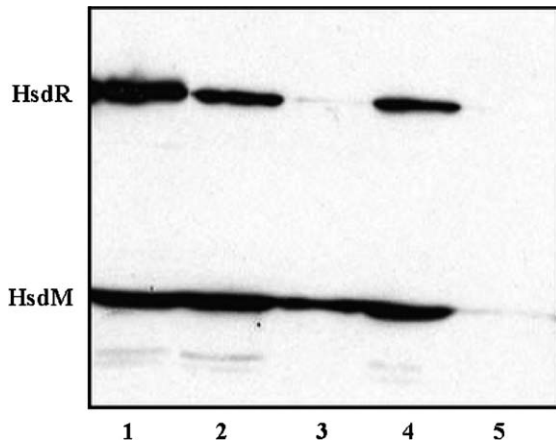


Fig. 4. Trypsin accessibility of HsdR subunit of *EcoR124I* REase in intact spheroplasts of *E. coli* JM109(DE3)[pCP1005]. Immunoblotting of membrane fractions. Lane 1, total cell proteins; lane 2, control without trypsin; lane 3, trypsin 0.2 mg/ml, 120 min, 30 °C; proteolysis stopped by trypsin inhibitor before osmotic lysis of spheroplasts; lane 4, inhibitor added together with trypsin at the beginning of incubation; and lane 5, control without inhibitor.

without trypsin inhibitor) all subunits were completely digested. Up to this point the cellular localization of *EcoR124I* is analogous to that we found for *EcoKI* [20]. Assembled enzyme is associated with the cytoplasmic membrane, with the HsdR subunit exposed on the periplasmic side of the inner membrane. The only difference is the size of proteolytic products; in *EcoKI* two distinct polypeptides were detected on Western blots [20], while the HsdR subunit of *EcoR124I* is proteolyzed to much smaller products that are not retained in the gel (Fig. 4, lane 3).

We have previously observed [29] the phenomena of a transient state of *EcoR124I* restriction deficiency in *E. coli* strains carrying pCP1005 plasmid after treatment with the non-mutagenic surfactant dimethyl sulfoxide (DMSO), which is known to affect the composition and organization of membrane components [30]. The restriction phenotype of *EcoKI* was not changed by such treatment. Hubacek et al. [29] suggested that different structures (receptors) could be involved in the localization of the two enzymes. Our results indicate that the HsdR subunit of *EcoR124I* is more exposed on the outer side of the cytoplasmic membrane (Fig. 4) than the HsdR of *EcoKI* [20]. Such topology could explain the different degrees of trypsin accessibility of the HsdR subunits in intact spheroplasts as well as different sensitivities of these two systems to DMSO. These results correspond with the data for post-translational regulation of restriction activity by ClpXP protease. While ClpXP-dependent proteolysis of HsdR subunit was shown to be involved in the regulation of restriction activity following transmission of the *hsd* genes of the *EcoKI* R–M system [31], the HsdR subunit of *EcoR124I* is not sensitive to the cytoplasmic ClpXP protease [32].

Localization of the Type IB R–M enzyme *EcoAI*

The similarity in the localization of *EcoKI* and *EcoR124I*—representatives of two different Type I families led us to analyze the localization of a representative of the third family. Therefore, an analogous set of experiments has been performed with the Type IB R–M enzyme *EcoAI*. Subunits of REase from the strain BL21(DE3)[pFFP30] were again found in both membrane and cytoplasmic fractions. Individually produced HsdR and HsdM subunits, from strains BL21(DE3)[pACRM_A] and BL21(DE3)[pACM_A], were soluble cytoplasmic polypeptides (data not shown). The association of REase with the cytoplasmic membrane and solubility of “free” R and M polypeptides seems to be a common feature of all the three Type I families. HsdS varies in solubility depending upon the family; HsdS of *EcoKI* and *EcoR124I* are insoluble [12,33], while HsdS of *EcoAI* is soluble as demonstrated by Janscak and Bickle [14].

Remarkable differences have been found, however, in the response of *EcoAI* to treatment with trypsin as well as with benzonase. In contrast to *EcoKI* and *EcoR124I*, treatment of intact spheroplasts of BL21(DE3)[pFFP30] with trypsin did not produce proteolysis of the HsdR subunit of the membrane-bound *EcoAI* REase (Fig. 5). The HsdR subunit itself is not structurally resistant to trypsin, as proved by complete proteolysis of Hsd subunits in the control without trypsin inhibitor. Therefore, the HsdR subunit of *EcoAI* must be anchored within the spheroplast membrane and/or somehow protected (by other proteins) so that it is not accessible to the protease at the periplasmic surface of the inner membrane.

Even more surprising is the resistance of the membrane association of *EcoAI* REase to benzonase as

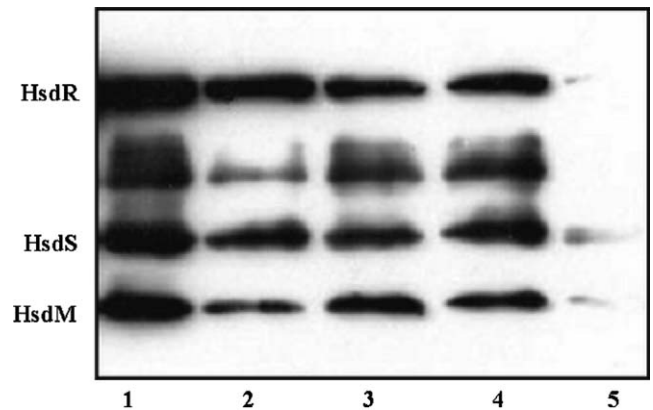


Fig. 5. Hsd subunits of *EcoAI* in membrane fractions isolated from trypsin treated spheroplasts BL21(DE3)[pFFP30]. Lane 1, total cell proteins; lane 2, control without trypsin; lane 3, trypsin 0.2 mg/ml, 120 min, 30 °C; lane 4, trypsin 0.4 mg/ml, 120 min, 30 °C; and lane 5, as lane 3, without inhibitor. The unidentified protein detected under HsdR by our *EcoAI* polyclonal antibody is present also in host strain without expression of *EcoAI* subunits.

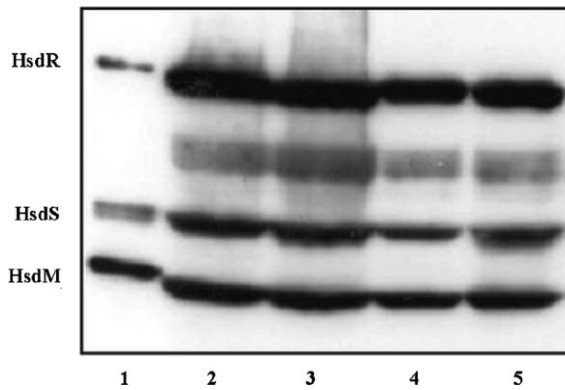


Fig. 6. The effect of benzonase on *EcoAI*-membrane association. Immunoblot of membrane proteins of BL21(DE3)[pFFP30]. Lane 1, standard (purified *EcoAI*); lane 2, total cell proteins; lane 3, control without benzonase; lane 4, benzonase 1 U/1 ml of lysed spheroplast; and lane 5, benzonase 30 U/1 ml of lysed spheroplast.

shown on immunoblots of the membrane fractions from BL21(DE3)[pFFP30] (Fig. 6). In experiments with the *EcoKI* [20] and *EcoR124I* (this paper, Fig. 3) systems, benzonase released REase and MTase from membrane into the soluble fraction. Under the same conditions, even at a much higher concentration of benzonase (Fig. 6), *EcoAI* remains associated with the cytoplasmic membrane. The association of chromosomal DNA and replicative plasmids with the bacterial membrane has been well documented [34–36]. The DNA-mediated association of *EcoKI* and *EcoR124I* with the cytoplasmic membrane may ensure an access of these enzymes to their natural substrate—hemimethylated DNA. When Type I enzymes act on unmethylated substrates, they function mainly as REase [1]. Since *EcoAI* is able to methylate unmethylated DNA as efficiently as the hemimethylated one, the stable *EcoAI* MTase complex may be the preferred enzyme that acts on host DNA and thus DNA is not a pre-requisite for membrane association of the REase.

Family-dependent differences in membrane topology of the Type I R–M enzymes are summarized into a working model (Fig. 7). It is not possible, on the basis of the given experiments, to draw a definite conclusion about the topology of such complex enzymes. However, the results reported here clearly document that Type I R–M enzymes interact with the cytoplasmic membrane and that the nature of these interactions is what differs from family to family. Such dynamic interactions offer a scope for a temporal control of restriction activity in the bacterial cell. One such control mechanism involves the ClpXP proteolysis of HsdR subunits of Type IA and IB systems. A fraction of *EcoKI* enzyme, resistant to ClpXP, was shown to be membrane-associated. This fraction is thought to be targeted against invading DNA [20,21]. Thus, the membrane association can provide a degree of the targeting of the REase that is required for efficient protection of the host against invading phage

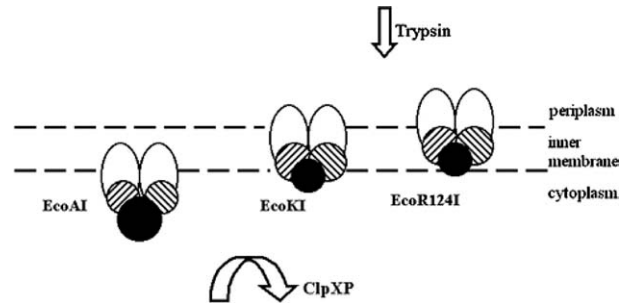


Fig. 7. The model showing possible cell localization of different families of Type I R–M systems. The restriction enzymes (complex of 2 HsdR, white; 2 HsdM, hatched; and 1 HsdS, black) vary in the extent of embedding into the cytoplasmic membrane shown by trypsin accessibility. The fraction of membrane-associated enzymes is protected against cytoplasmic protease ClpXP.

DNA. Type IC *EcoR124I* R–M enzyme is not subjected to control by ClpXP proteolysis [37] and has evolved a temporal control based on a weak final stage in the subunit assembly pathway [13,17]. In this control mechanism, interaction of R–M enzyme with the cytoplasmic membrane could play a role in keeping a balance between membrane-attached enzyme and soluble (cytoplasmic) intermediates.

It appears that the cellular localization of the oligomeric multifunctional R–M enzymes, particularly their dynamic interactions with the cytoplasmic membrane, plays an important role in switching between restriction and modification activities and also in discriminating between resident and foreign DNA.

Acknowledgments

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