

Phosphorylation of Type IA restriction-modification complex enzyme EcoKI on the HsdR subunit

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Abstract

Phosphorylation of Type I restriction-modification (R-M) enzymes EcoKI, EcoAI, and EcoR124I – representatives of IA, IB, and IC families, respectively – was analysed *in vivo* by immunoblotting of endogenous phosphoproteins isolated from *Escherichia coli* strains harbouring the corresponding *hsd* genes, and *in vitro* by a phosphorylation assay using protein kinase present in subcellular fractions of *E. coli*. From all three R-M enzymes, the HsdR subunit of EcoKI system was the only subunit that was phosphorylated. Further, evidence is presented that HsdR is phosphorylated *in vivo* only when coproduced with HsdM and HsdS subunits – as part of assembled EcoKI restriction endonuclease, while the individually produced HsdR subunit is not phosphorylated. *In vitro* phosphorylation of the HsdR subunit of purified EcoKI endonuclease occurs on Thr, and is strictly dependent on the addition of a catalytic amount of cytoplasmic fraction isolated from *E. coli*. So far this is the first case of phosphorylation of a Type I R-M enzyme reported.

Introduction

Type I restriction-modification (R-M) systems provide the host bacteria with immunity to infection by foreign DNA. Recent years have witnessed a renaissance of interest in Type I R-M enzymes. The progressing genome-sequencing projects have revealed that these enzymes are widely distributed in a broad range of microorganisms in nature (Roberts *et al.*, 2005).

The Type I R-M systems are divided into five families (Type IA, e.g. EcoKI; Type IB, e.g. EcoAI; Type IC, e.g. EcoR124I; ID, e.g. StySBLI and Type IE, e.g. KpnBI). This classification is based on sequence similarity, functional complementation, and antibody cross-reactivity. Type I R-M systems are multisubunit, multifunctional complexes composed of three different subunits, HsdS (the DNA-binding subunit), HsdM (the methylation subunit), and HsdR (the restriction subunit), which are present in an R₂M₂S₁ stoichiometry (Murray, 2000; Roberts *et al.*, 2005). This heteromeric complex can act as a DNA methyltransferase (MTase), or a DNA-dependent ATPase, DNA translocase, and a restriction endonuclease (REase). HsdS is responsible for the recognition of a specific DNA sequence. HsdM and HsdS alone are sufficient to assemble an independent MTase with a stoichiometry of M₂S₁.

The three enzyme subunits are encoded by the closely linked genes *hsdR*, *hsdM*, and *hsdS*, which are expressed

from two promoters: P_{Res} (for transcription of *hsdR*) and P_{Mod} (for transcription of *hsdM* and *hsdS*). Besides this genetic organization, no evidence is available on the transcriptional regulation of the Type I R-M enzyme (Prakash-Cheng & Ryu, 1993). However, after the transfer of the *hsd* genes to a new host, the enzyme must have a control mechanism that prevents restriction activity preceding modification to avoid damage to the host unmodified chromosome. It has been shown that this temporal control can occur posttranslationally either *via* the subunit assembly pathway (Firman *et al.*, 2000), or *via* ClpXP proteolysis of the assembled enzyme (Makovets *et al.*, 1998; Doronina & Murray, 2001). These controls protect resident bacterial chromosome not only after the transfer of the *hsd* genes to a new host but also under conditions that lead to DNA damage. Consequent repair of DNA can generate unmodified restriction sites, which are manifested by decreased restriction of incoming unmodified phage DNA – a phenomenon first described as restriction alleviation by EcoKI (Bertani & Weigle, 1953; Blakely & Murray, 2006). Another mechanism playing an important role in the control of restriction and modification activities is based on differences in membrane localization of the Type I enzymes (Holubova *et al.*, 2004).

One cannot exclude combination and/or cooperation of these control mechanisms or even existence of other, not yet revealed, regulatory tools. Protein phosphorylation

represents one of the fundamental regulatory mechanisms in eukaryotes. Recently, analysis of bacterial genomes revealed the widespread presence of eukaryotic-type Ser/Thr protein kinase and protein phosphatase genes in many bacteria (Kennelly, 2002). Using two-dimensional gel electrophoresis, putative isoforms of Hsd subunits were revealed, most likely reflecting posttranslational modifications, especially phosphorylation (Nguyen *et al.*, 2002). Therefore it was decided to investigate possible phosphorylation of the three representatives of IA, IB, and IC families of Type I R-M enzymes – EcoKI, EcoAI, and EcoR124I. In this paper, it is shown that only the EcoKI HsdR subunit can be phosphorylated when expressed as part of the complex endonuclease.

Materials and methods

Bacterial strains, plasmids, and microbiological techniques

Table 1 lists the *Escherichia coli* strains and plasmids used in this study. The strains were transformed with the plasmids coding for endonuclease, methyltransferase, and individual HsdR subunits of EcoKI, EcoAI, and EcoR124I. Cells were grown in Luria–Bertani (LB) medium with antibiotics where required. Transformation and manipulation of nucleic acids were performed using the methods described in Sambrook *et al.* (1989). *In vivo* restriction and modification assays were carried out as described previously (Hubacek *et al.*, 1998).

Protein purification

The EcoKI REase was purified as described by Weiserova *et al.* (1993). The EcoR124I MTase and HsdR subunit were

purified according to Janscak *et al.* (1998). The EcoR124I REase was reconstituted *in vitro* by mixing the purified HsdR subunit and MTase at a molar ratio of 6 : 1. The EcoAI REase was reconstituted *in vitro* by mixing the purified subunits (kindly provided by Dr Janscak) at a molar ratio of 8HsdR : 2HsdM : 1HsdS.

Cell fractionation

Late-logarithmic-phase cells were harvested from 30 mL of culture, washed with STE buffer (10 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, pH 8), resuspended in STE supplemented by 10 mM NaF and 3 mM PMSE, and lysed by sonication. Centrifugation of cell lysates at 50 000 g for 1 h yielded separation of cytoplasmic (supernatant) and membrane (pellet) fractions. The washed pellet was resuspended in STE buffer. Aliquots of subcellular fractions containing 30 µg of proteins were used for the protein kinase assay.

Immunoprecipitation

Late-logarithmic-phase cells were harvested from 30 mL of culture, washed with immunoprecipitation buffer (IP) (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet 40, 1 mM EDTA), resuspended in IP buffer supplemented with 0.1 mM NaF, 1 mM DTT and 0.1 mM PMSE, and lysed by sonication. Aliquots of cell lysates (500 µg protein) were mixed with 5 mg of pre-equilibrated Protein A-Sepharose, and IP buffer was added to a final volume of 1 mL. After a 1-h incubation at 4 °C, Protein A-Sepharose was removed by centrifugation. Monoclonal antibody raised against phosphoserine or phosphothreonine was added to the precleared lysate, and the mixture was incubated for 1 h at 4 °C.

Table 1. Bacterial strains and plasmids

	Characteristics	Reference
<i>E. coli</i> strains		
C122	Prototroph, Δ <i>hsd</i>	British Culture Collection Strain No. 122
BL21 (DE3)	F ⁻ <i>dcm</i> , <i>ompT hsdS</i> (<i>r_B</i> <i>m_B</i>) <i>gal</i> λ (DE3)	Studier & Moffatt (1986)
Plasmids		
pVMC3	A derivative of pBg3 and pVM14 carrying the <i>hsdR</i> , <i>hsdM</i> , <i>hsdS</i> genes of the EcoKI R-M system, Ap ^R	Weiserova <i>et al.</i> (1993)
pACWR93	A derivative of pACYC184 carrying the <i>hsdR</i> gene of the EcoKI R-M system, Cm ^R	Hubacek <i>et al.</i> (1998)
pVM Δ C23	A derivative of pMS _K C23 carrying the <i>hsdM</i> and <i>hsdS</i> genes of the EcoKI R-M system, Ap ^R	Weiserova <i>et al.</i> (1994)
pCP1005	A derivative of pUR51 carrying the <i>hsdR</i> , <i>hsdM</i> , <i>hsdS</i> genes of the EcoR124I R-M system, Ap ^R	Firman <i>et al.</i> (1985)
pLP25R	pTZ19R carrying the <i>hsdR</i> gene of the EcoR124I R-M system, Ap ^R	Zinkevich <i>et al.</i> (1997)
pJS4M	A derivative of pUC119 and pET3A carrying the <i>hsdM</i> and <i>hsdS</i> genes of the EcoR124I R-M system under control of the P _{T7g10} promoter. Ap ^R	Patel <i>et al.</i> (1992)
pFFP30	<i>hsd</i> region of EcoAI on HindIII fragment cloned into pBR322, Ap ^R	Fuller-Pace <i>et al.</i> (1985)
pJP22	A derivative of pFFP30 and pET15b carrying the <i>hsdR</i> gene of the EcoAI R-M system under control of the P _{T7g10} promoter. Ap ^R	Janscak & Bickle (1998)
pJP21	A derivative of pFFP30 and pET32a carrying the <i>hsdM</i> and <i>hsdS</i> genes of the EcoAI R-M system under control of the P _{T7g10} promoter. Ap ^R	Janscak & Bickle (1998)

Afterwards, Protein A-Sepharose (5 mg) was added and the incubation was continued for 1 h at room temperature. The immunoreactive complexes were separated by centrifugation and washed five times with IP buffer. The sediment was suspended in sodium dodecylsulfate (SDS) sample buffer with 4% β -mercaptoethanol and boiled for 5 min. The Protein A-Sepharose was removed by centrifugation and the samples were analysed by Western blot.

Electrophoresis and Western blots

Aliquots of total cell extracts before and after immunoprecipitation were separated by SDS-polyacrylamide gel electrophoresis (PAGE; 10% polyacrylamide) (Laemmli, 1970) using purified enzymes as standard markers. Separated proteins were transferred onto a PVDF membrane in CAPS buffer, pH 11, using a semi-dry blotter (Sigma). Individual subunits of EcoKI, EcoAI, or EcoR124I were identified using the corresponding polyclonal antibodies raised in a rabbit against individual purified enzymes. The immune complexes were detected by chemiluminescence using the Super Signal West Pico Chemiluminescent Substrate protocol (Pierce).

In vitro phosphorylation of R-M enzymes by subcellular fractions of *E. coli*

Purified R-M enzymes were used as substrates, and subcellular fractions served as source(s) of kinase activity; no subcellular fraction was added when autophosphorylation was tested. The standard mixture for protein kinase assay (25 μ L) contained 50 mM Tris-Cl, pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 30 μ g of proteins of a subcellular fraction, 2 μ g of purified R-M enzyme, 10 mM NaF, 10 mM MnCl₂, and 10 μ M [γ -³²P] ATP (2 μ Ci). The reaction was started by the addition of ATP and terminated after 20 min of incubation at room temperature by adding 5 \times SDS sample buffer; the reaction mixtures were analysed by SDS-PAGE. After Coomassie Blue staining, the dried gels were scanned in a Fuji BAS 5000 PhosphorImager.

Phosphoamino acid analysis

For the analysis of phosphoamino acids, the *in vitro* phosphorylated R-M enzymes were subjected to SDS-PAGE (10% gel) and transferred onto a PVDF membrane using a Semi-Dry Blotter (Sigma). Radioactive protein bands detected by autoradiography were excised and hydrolysed in 6 M HCl at 110 °C for 2 h (Kamps & Sefton, 1989). The acid-resistant phospho-amino acids thus liberated were separated by two-dimensional thin-layer chromatography (TLC) (Grimm & Nordeen, 1999). Standard phospho-amino acids were run simultaneously, and their positions were detected

by staining with ninhydrin. Phosphorylated amino acids were detected by PhosphorImager.

Results and discussion

Phosphorylation *in vivo*

The attempts to detect Hsd subunits by monoclonal antibodies against phospho-threonine and phospho-serine directly in the crude extracts failed, most probably due to a very low concentration of Type I enzymes in the cell extracts (data not shown). Only 100–200 molecules of chromosomally encoded EcoKI REase were estimated in an *E. coli* cell (Dryden *et al.*, 1997). In the present experiments, all REases were expressed from plasmids but the genes were under control of their natural promoters to keep the balance of the concentrations of the enzymes as natural as possible. In the case of the *hsd* genes expressed under the T7 promoter, the appropriate strain BL21(DE3) was grown under noninduced conditions. It was shown previously that the background level of T7 RNA polymerase is sufficient for *in vivo* analysis of R-M functions (Janscak *et al.*, 1998), which were tested before each experiment.

Therefore, in this study, immunoprecipitation was used, followed by SDS-PAGE and immunoblot analysis as a powerful technique for detection of rare proteins that otherwise would be difficult to detect. First, phosphorylation of EcoKI REase subunits was investigated by immunoprecipitation analysis using monoclonal antibodies against phosphothreonine and phosphoserine. Cell-free extract prepared from late-logarithmic-phase cells C122[PVMC3] (Fig. 1a, lane 1) was mixed with antibodies and Protein A-Sepharose as described in 'Materials and methods'. The immunoreactive complexes were separated by SDS-PAGE, followed by immunoassay analysis using rabbit polyclonal antibodies against EcoKI. The HsdR subunit was clearly detected among phosphoproteins when immunoprecipitated with antiphosphothreonine antibody (Fig. 1b, lane 2). On the other hand, no immunological cross-reactivity was observed in the experiment with antiphosphoserine antibody (Fig. 1b, lane 3). HsdM and HsdS subunits were not phosphorylated on either serine or threonine (Fig. 1b, lanes 2, 3).

To exclude any false-positive signals, control precipitation was performed without the presence of any monoclonal antiphospho-antibodies (Fig. 1b, lane 4). Immunoprecipitation of cell-free extract from C122, where no Hsd subunits were expressed, yielded no signals as well (Fig. 1b, lane 5).

To answer the question regarding whether active EcoKI is necessary for phosphorylation of HsdR, the phosphorylation of HsdR in the strain where only the HsdR subunit was expressed and no EcoKI complex enzyme could be formed was tested (Fig. 1a, lane 2). For this reason,

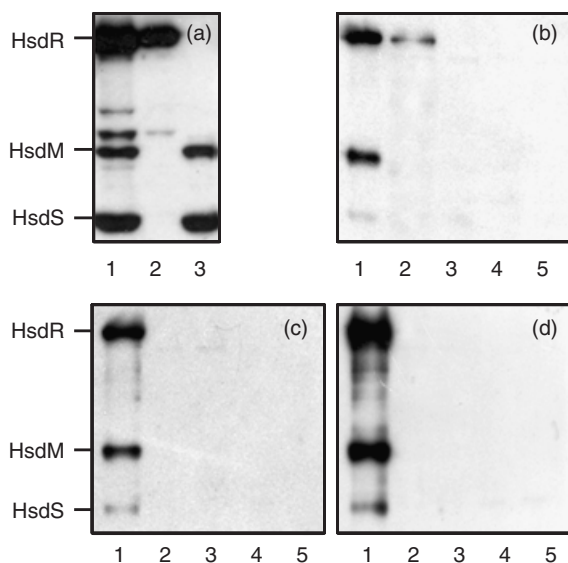


Fig. 1. *In vivo* phosphorylation of EcoKI subunits. (a) Control immunodetection of Hsd subunits expressed in cell-free extracts of *Escherichia coli* C122 carrying plasmids coding for REase (pVMC3) (lane 1), HsdR subunit (pACWR93) (lane 2), and MTase (pVMΔC23) (lane 3) before immunoprecipitation. Phosphoproteins were immunoprecipitated from cell-free extracts of *E. coli* C122 clones expressing complex EcoKI REase (b), individual HsdR subunit (c), and MTase (d). The immunoprecipitates were separated by SDS-PAGE, blotted onto a PVDF membrane, and Hsd subunits were visualized by immunodetection with anti-EcoKI. (b–d) Lanes 1, standard (purified EcoKI); lanes 2, immunoprecipitation with monoclonal antibody against phosphothreonine; lanes 3, immunoprecipitation with monoclonal antibody against phosphoserine; lanes 4, control immunoprecipitation without antiphosphothreonine or antiphosphoserine; lanes 5, cell lysate of C122 strain expressing none of the Hsd subunits immunoprecipitated with antiphosphothreonine.

immunoprecipitation was performed of a cell-free extract from C122[pACWR93] with antiphosphothreonine (Fig. 1c, lane 2) or antiphosphoserine (Fig. 1c, lane 3), followed by immunoassay analysis using anti-EcoKI. Interestingly, no phosphorylation of HsdR was detected on either serine or threonine. This finding implies that a functional endonuclease is required for phosphorylation of HsdR.

In an analogous set of experiments, it was tested whether phosphorylation of HsdM or HsdS subunits occurs in the strain that harbours *hsdM* and *hsdS* genes (C122 [pVMΔC23]), where HsdM and HsdS form functional MTase (Fig. 1a, lane 3). As evident from Fig. 1d, lane 2 and 3, no phosphorylation of HsdM or HsdS was detected. It is therefore concluded that HsdM and HsdS are not phosphorylated regardless of being assembled in EcoKI REase or MTase complexes.

A parallel set of experiments was performed with the Type IB R-M enzyme EcoAI and the Type IC R-M enzyme EcoR124I. In contrast to EcoKI, no subunit of EcoAI and EcoR124I enzyme was phosphorylated on either serine or

threonine in the cells harbouring appropriate plasmids coding for endonucleases, methyltransferases, or for individual HsdR subunits (see Table 1). The production of Hsd subunits was confirmed in the analysed cell lysates by antiEcoAI and antiEcoR124I antibodies (data not shown).

Phosphorylation *in vitro*

To confirm the *in vivo* results, the purified REases were subjected to *in vitro* analysis. Phosphorylation test was carried out in the presence of [γ - 32 P]ATP, Mn^{2+} and Mg^{2+} as described in 'Materials and methods'.

To rule out a possibility that the phosphorylation of HsdR could result from an autophosphorylation, EcoKI REase was analysed for this activity in an *in vitro* kinase assay. No Hsd subunits of the purified EcoKI enzyme were phosphorylated in the absence of *E. coli* cell extracts (data not shown); hence, the autophosphorylation could be excluded.

Further, the study focused on the ability of inherent protein kinase(s) in cytoplasmic and membrane fractions of *E. coli* C122 to phosphorylate purified EcoKI enzyme (Fig. 2). Phosphorylation patterns were shown to differ clearly in dependence on the cell fraction used. When the cytoplasmic fraction was used as the source of kinase activity, the HsdR subunit of EcoKI was detected as a phosphorylated product (Fig. 2b, lane 1), while no phosphorylation of EcoKI enzyme occurred in the presence of membrane fraction (Fig. 2d, lane 1).

To identify the amino acid residue of HsdR serving as a target of the cytoplasmic kinase, the radioactive band was excised and analysed as described in 'Materials and methods'. Phospho-amino acid analysis showed that 32 P-labelled HsdR subunit of EcoKI was phosphorylated exclusively at threonine residue(s) (Fig. 3).

Although no phosphorylation was detected *in vivo*, *in vitro* phosphorylation experiments were performed with the Type IB R-M enzyme EcoAI and the Type IC R-M enzyme EcoR124I. Purified EcoAI or EcoR124I REases were added to the reaction mixture containing the cytoplasmic fraction of *E. coli* C122. In agreement with the *in vivo* immunodetection analysis, no subunit of either EcoAI or EcoR124I was phosphorylated (data not shown).

Conclusion

Protein phosphorylation is an important post translational modification that serves many key functions to regulate a protein's activity, localization, and protein–protein interactions (Kennelly, 2002). Transfer of the phosphoryl group even on a single target residue has a considerable impact on the protein structure and consequently on its function (Sala-Newby & Campbell, 1991). Both localization and protein–protein interactions (subunit assembly) are involved in the correct function of Type I multisubunit R-M enzymes

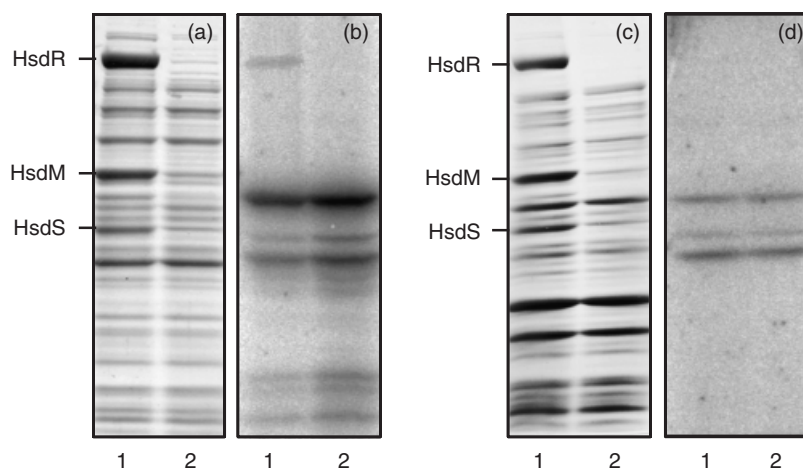


Fig. 2. Analysis of *in vitro* phosphorylation of purified EcoKI enzyme subunits by kinase activity of cytoplasmic (b) and membrane (d) fractions of *Escherichia coli* C122 (lanes 1); lanes 2, control without EcoKI. After *in vitro* phosphorylation reaction, proteins were separated by SDS-PAGE, the gels were stained with Coomassie Blue (a, c), and the radioactivity of the phosphoproteins was monitored by PhosphorImager (b, d).

(Firman *et al.*, 2000; Holubova *et al.*, 2004). Moreover, it has been shown that HsdR of EcoKI is proteolysed by ClpXP protease only when it is a part of an assembled translocation-proficient EcoKI complex, preferentially in cytoplasmic fraction, while in the membrane fraction HsdR is not degraded (Doronina & Murray, 2001). This is in keeping with the present results showing that HsdR of EcoKI is phosphorylated at a threonine residue by an as yet unidentified cytoplasmic kinase only as part of a complex REase and with the previous finding that the membrane-associated fraction of EcoKI is responsible for the residual restriction activity of the enzyme under a condition of restriction alleviation (Holubova *et al.*, 2000; Doronina & Murray, 2001). It may be speculated that HsdR of EcoKI requires phosphorylation/dephosphorylation for better susceptibility to ClpXP proteolysis. The cytoplasm-localized HsdR subunits might be phosphorylated in the domain, which in the case of the membrane-associated EcoKI complex, is embedded in the cytoplasmic membrane. Trypsinization of spheroplasts revealed that solely the HsdR subunit of the complex enzyme was digested by the protease, as was demonstrated by the disappearance of the band corresponding to the HsdR polypeptide that was replaced by two smaller polypeptides T1 and T2 (Holubova *et al.*, 2000). Using NETPHOS 2.0 Server (Blom *et al.*, 1999), 16 threonine residues were predicted as possible targets for phosphorylation: 11 present on fragment T1 and five on fragment T2.

In contrast to EcoKI, no subunit of EcoAI or EcoR124I was found to be phosphorylated. These results correspond to the data on posttranslational regulation of their restriction activity by the ClpXP protease. The HsdR subunit of EcoR124I is not sensitive to the cytoplasmic ClpXP protease at all (Kulik & Bickle, 1996) and efficient transmission of the EcoAI *hsd* genes was found to be less dependent on ClpXP than transmission of the *hsd* genes for EcoKI (Makovets *et al.*, 1998). This is probably due to the instability of the EcoAI REase complex in contrast to the MTase complex,

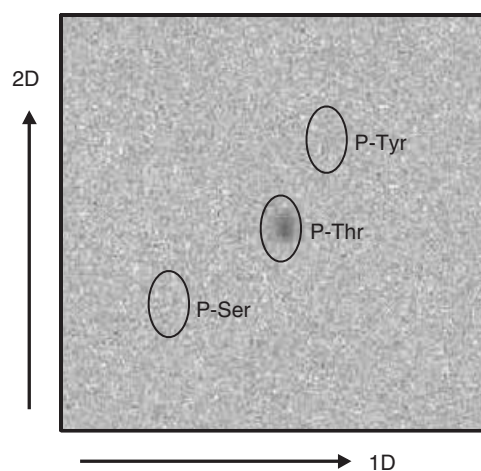


Fig. 3. 2D TLC analysis of phosphorylated amino acids of the HsdR EcoKI subunit. The acid-stable phosphoamino acids from ^{32}P -labelled HsdR were separated into two consecutive dimensions and radioactivity was monitored by PhosphorImager. The positions of the nonradioactive phosphoamino acid standards phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) identified by ninhydrin are circled.

which is stable (Janscak & Bickle, 1998) and efficiently methylates the unmethylated targets (Suri & Bickle, 1985) while EcoKI and EcoR124I prefer the hemimethylated DNA substrate. In addition, the EcoR124I enzyme forms a stable restriction-deficient $\text{R}_1\text{M}_2\text{S}_1$ complex (Janscak *et al.*, 1998). Apparently, subunits assembly pathway of the EcoR124I and EcoAI R-M enzymes play a significant role in the control mechanism governing restriction activity (Janscak & Bickle, 1998; Firman *et al.*, 2000).

It is supposed that phosphorylation–dephosphorylation of HsdR might be involved in the regulation of restriction activity of EcoKI either by ClpXP-dependent proteolysis, and/or in the control of its subcellular localization. The present results do not rule out the possible role of phosphorylation in the subunit assembly of a functional complex

REase by modulating the stability of HsdR subunit, as *in vivo* phosphorylation was detected in a restriction-proficient strain. This implies that phosphorylation of HsdR is a prerequisite for normal function of the EcoKI R-M system, while under the condition of restriction alleviation, dephosphorylation might be a signal for ClpXP proteolysis. Although the localization of the phosphorylated Thr residue(s) and the exact biological implication of its modification remain to be determined, our results open a new direction in the study of regulation of function of Type I R-M enzymes.

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