Inhibition of *Escherichia coli* RecA coprotease activities by DinI

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In *Escherichia coli*, the SOS response is induced upon DNA damage and results in the enhanced expression of a set of genes involved in DNA repair and other functions. The initial step, self-cleavage of the LexA repressor, is promoted by the RecA protein which is activated upon binding to single-stranded DNA. In this work, induction of the SOS response by the addition of mitomycin C was found to be prevented by overexpression of the *dinI* gene. *dinI* is an SOS gene which maps at 24.6 min of the *E.coli* chromosome and encodes a small protein of 81 amino acids. Immunoblotting analysis with anti-LexA antibodies revealed that LexA did not undergo cleavage in *dinI*-overexpressed cells after UV irradiation. In addition, the RecA-dependent conversion of UmuD to UmuD' (the active form for mutagenesis) was also inhibited in *dinI*-overexpressed cells. Conversely, a *dinI*-deficient mutant showed a slightly faster and more extensive processing of UmuD and hence higher mutability than the wild-type. Finally, we demonstrated, by using an *in vitro* reaction with purified proteins, that DinI directly inhibits the ability of RecA to mediate self-cleavage of UmuD.

Keywords: inducible evolution/LexA/SOS mutagenesis/
SOS response/UmuD

Introduction

Most living organisms have some genetic network system(s) that enables them to cope with the consequences of DNA damage (Friedberg *et al.*, 1995 and references therein). In *Escherichia coli* cells, >20 unlinked genes are induced, which function in DNA repair, recombination, mutagenesis, inhibition of cell division and other activities. This phenomenon is often referred to as the SOS response. The intracellular signal for SOS induction is believed to be single-stranded (ss) DNA that results from the interruption of DNA replication at the damaged sites (Sassanfar and Roberts, 1990; Higashitani *et al.*, 1995). Upon binding to ssDNA, RecA undergoes a conformational change to an activated form that is able to mediate cleavage of LexA, the general repressor for the SOS

genes, thereby enhancing expression of many genes, including the *recA* and *lexA* genes. If the cell carries a prophage such as λ , the phage repressor is cleaved similarly, depending on the activity of activated RecA protein (Roberts and Devoret, 1983). Since LexA and λ phage repressors were later found to have an ability to autodigest at the same site *in vitro* under alkaline pH conditions, the term coprotease was introduced to denote that activated RecA protein promotes the otherwise latent activities of such repressors (Little, 1991). SOS-induced mutagenesis at DNA lesions requires the function of the *umuDC* operon (Kato and Shinoura, 1977; Kitagawa *et al.*, 1985; Perry *et al.*, 1985); one of the products, UmuD, is converted to an active form UmuD', in a RecA-dependent fashion (Buckhardt *et al.*, 1988; Nohmi *et al.*, 1988; Shinagawa *et al.*, 1988). The target proteins for the activated RecA coprotease activity (LexA, λ phage repressor, UmuD and others) retain common amino acid residues critical for autodigestion (Slilaty and Little, 1987; Battista *et al.*, 1990). In addition to such a key role in SOS induction and mutagenesis, RecA plays an indispensable role in homologous recombination and recombinational repair, as a so-called recombinase. Upon binding to ssDNA, and then to another DNA (single-stranded or double-stranded), it mediates pairing between homologous DNAs and strand exchange (reviewed by Kowalczykowski *et al.*, 1994).

The process for induction of the SOS response is now well understood at the molecular level, yet much less is known about how SOS-induced cells return to a steady state and how the SOS response is turned off. It has been believed that, as DNA damage is repaired and consequently the SOS-inducing signal to activate RecA disappears, the intact LexA re-accumulates to regain repression of the SOS genes (Little and Mount, 1982). This model, however, has not been verified by any concrete experimental evidence as far as we know. A prolonged duration of the SOS response seems harmful for bacterial cells in their efforts to maintain genomic stability, because the SOS response includes multiple pathways for mutagenesis. The *umuDC*-dependent pathway induces mutations at DNA lesions, and its activity is regulated in a way coordinated to the SOS response (Battista *et al.*, 1990). However, another *dinB/P*-dependent pathway, which does not require the activity of activated RecA protein once induced, enhances mutagenesis most probably at non-damaged sites (Kim *et al.*, 1997). As a consequence, one may very well expect that there might be a factor, for example a feedback regulator, that functions in turning off the SOS response.

Recently, we have reported that the *dinI* gene, an SOS gene that maps at 24.6 min of the *E.coli* chromosome, acts as a multicopy suppressor for the cold-sensitive (cs) phenotype of the *dinD68* mutant (Yasuda *et al.*, 1996). *dinD68* mutant cells form long filaments without any

DNA-damaging treatment at temperatures $<20^{\circ}\text{C}$ (Kudo *et al.*, 1977). The *dinD68* cs phenotype is suppressed by ΔrecA or *lexA* (Ind^{-}) mutations (Ohmori *et al.*, 1995b) or by a multicopy plasmid carrying the intact *lexA* gene (Yasuda *et al.*, 1996), implying that the cs phenotype depends on induction of the SOS response. While studying the mechanism by which *dinI*-carrying plasmids suppressed the *dinD68* cs phenotype, we observed that induction of the SOS response was prevented in cells carrying such plasmids. Here, we present evidence which indicates that DinI has an activity in attenuating RecA coprotease activities, and probably functions as a modulator of SOS-induced mutagenesis.

Results

In vivo evidence for the SOS inducibility of *DinI*

The DNA sequence of *dinI* indicates that its coding region consists of 81 codons. A plasmid carrying the *dinI* gene with its cognate regulatory region produced a small protein of the expected size in an *in vitro* transcription-translation coupled system (Yasuda *et al.*, 1996). The sequence 5'-ACCTGTATAAATAACCAGTA-3', which differs by only five nucleotides from the consensus SOS-box sequence (5'-TACTGTATATATACAGTA-3'), is located in the *dinI* promoter region in such a way as to overlap with the putative -10 signal sequence necessary for transcription initiation. Lewis *et al.* (1994) showed that LexA binds *in vitro* to a DNA fragment containing the *dinI* regulatory region, but not to the same fragment with an alteration of the first T residue to C within the above sequence upstream of *dinI*. Before analyzing the *dinI* gene function further, we therefore wished to obtain firm evidence indicating that DinI is indeed induced during the SOS response.

We constructed a plasmid (named pYP74) to overproduce DinI, by placing the *dinI* coding region downstream of a potent *Ptac* promoter in the vector plasmid pYP73. DinI was overproduced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) in the culture medium to MC1061 cells carrying pYP74, and then it was purified to near homogeneity by multiple chromatographic steps (Figure 1), as described in detail in Materials and methods. We determined the amino acid sequence of the purified DinI protein from the N-terminus to the 25th residue, which matched perfectly with that predicted from the DNA sequence. We subsequently raised polyclonal antibodies against DinI in a rabbit, and used the sera to detect the DinI protein expressed *in vivo* from its natural sequence by immunoblotting analysis. As shown in Figure 2, an ~6 kDa protein band, which had the same mobility as the purified DinI (lane 8), appeared in MC1061 cells after addition of mitomycin C (MMC, 1 $\mu\text{g/ml}$) (lane 2). The 6 kDa band was not detected in an MC1061 derivative carrying *lexA1* (Ind^{-}) (lane 3), $\Delta\text{recA } 518::\text{kan}$ (lane 4), *dinI1::kan* (lane 6) or $\Delta\text{dinI2::kan}$ (lane 7) after MMC treatment. In contrast, the band was observed without MMC addition in a *lexA51* (Def) derivative of MC1061 (lane 5). These results verify that *dinI* is indeed induced by DNA damage and that its expression is regulated by both *lexA* and *recA*, like other SOS genes.

Effect of overexpression of *dinI* on SOS induction

To study *dinI* gene function, we compared the UV sensitivity of MC1061 and its *dinI1::kan* mutant derivative SY117,

Fig. 1. Purification of DinI. An aliquot of each fraction obtained during the purification process was subjected to SDS-16.5% PAGE with the Tricine buffer system. Proteins were visualized by Coomassie Blue staining. Lane 1, crude extract; lane 2, fraction precipitated with 90% saturated ammonium sulfate; lane 3, Q-Sepharose fraction; lane 4, Sephacryl S-100 fraction; lane 5, molecular weight markers.

Fig. 2. Expression of the *dinI* gene. Cell lysates prepared from MC1061 and its derivatives were analyzed as described in Materials and methods. The DinI antiserum used here is specific but cross-reacts with another protein in all of the lysates, generating a band above the DinI band. This extra band serves as an internal control ensuring that equal amounts of proteins were applied to the gel. Lanes 1 and 2, MC1061; lane 3, KN2012; lane 4, MS6; lane 5, SY184; lane 6, SY117; lane 7, SY183; lane 8, purified DinI. The relevant genotype is indicated above the lane number. MMC (1 $\mu\text{g/ml}$) was added to the cultures, except for those for lanes 1 and 5.

but found no discernible difference between them (Figure 3A). In contrast, the *dinI* gene placed on a multicopy plasmid (pTZ19ULC, a derivative of pTZ19U with a reduced copy number) conferred a profound UV sensitivity on MC1061, which was more severe than that conferred by the intact *lexA* gene on the same vector plasmid (Figure 3B). The plasmids used in this experiment, pMspB and pMspC, which originally were isolated for their abilities to suppress the cs phenotype of the *dinD68* mutant, carry a *Bam*HI chromosomal segment containing *dinI* or *lexA*, respectively (Yasuda *et al.*, 1996). The UV sensitization caused by the *lexA* gene on the multicopy plasmid may be explained by the fact that excess amounts of LexA repressor prevent induction of the SOS response (Hori *et al.*, 1981a).

We therefore tested whether plasmid-borne *dinI* could

Fig. 3. Effect of *dinI* mutation or overexpression on UV sensitivity. (A) MC1061 (○) and its derivatives carrying *dinI1::kan* (Δ) or *uvrD98::Tn10* (□). (B) MC1061 carrying pMspB (*dinI*+, ▲), pMspC (*lexA*+, ■) or no plasmid (●). (C) GW2730 (*lexA71::Tn5*) carrying pYP74 (*dinI*+, ▼) or pYP73 (vector, ▽). IPTG (0.1 mM) was added to both cultures.

Fig. 4. Inhibition of *sulA-lacZ* expression by the *dinI*⁺ plasmid. SY2, an MC1061 derivative carrying the *sulA-lacZ* fusion, was used for these studies. The bacteria carrying (A) pMspB (*dinI*⁺, Δ) or its vector pTZ19ULC (▲), (B) pYP74 (*dinI*⁺, □, ■) or its vector pYP73 (○, ●) were grown in L-broth containing ampicillin (50 μg/ml). IPTG (0.1 mM) was also added to some of the cultures in (B) (■, ●). When the density reached $\sim 1 \times 10^8$ cells/ml, a sample was taken (time 0). MMC at the final concentration of 0.2 μg/ml was added to the rest, from which samples were taken at various times and assayed for β-galactosidase activity (Miller, 1972).

also prevent SOS induction. For this purpose, we employed the MC1061 derivative, SY2, with a *sulA-lacZ* fusion under SOS control, which allowed us to measure induction of the SOS response by β-galactosidase activity. As shown in Figure 4A, pMspB carrying *dinI* suppressed induction of *sulA* after addition of MMC (0.2 μg/ml), while a

slight expression of *sulA* was observed after prolonged incubation. When DinI was overproduced further by adding IPTG (0.1 mM) to cells carrying pYP74, no induction of *sulA* was observed (Figure 4B). We estimated the cellular amount of DinI protein by semi-quantitative immunoblotting analysis with purified DinI as standard. MC1061 cells contained DinI at <500 copies per cell without SOS induction and at ~2300 copies per cell when the SOS response was induced by MMC. MC1061 cells carrying pMspB contained DinI at ~4000 copies per cell without MMC and at ~8500 copies per cell with MMC addition, while MC1061 cells carrying pYP74 contained DinI at $\sim 10^5$ copies per cell when induced by IPTG addition. DinI overproduction seems to have little or no adverse effect on bacterial cell growth, as cells carrying pYP74 grew normally in the presence or absence of IPTG. Furthermore, when we measured β-galactosidase activity from a *dinD-lacZ* fusion carried on a λ phage genome, λC43 (Ohmori *et al.*, 1995b), we obtained essentially similar results to those with the *sulA-lacZ* fusion (data not shown). These results indicate that multicopy *dinI* inhibits induction of the SOS response, thus accounting for why plasmid-borne *dinI* suppresses the *dinD68* cs phenotype.

The inhibition of SOS induction by DinI overproduction might be explained in three ways: (i) the overproduced DinI protein might function as an alternative repressor for SOS-regulated genes; (ii) it might reduce the intracellular level of RecA that is normally required for SOS induction; or (iii) it might inhibit LexA self-cleavage that is mediated by activated RecA. The first possibility could be eliminated since the multicopy *dinI* plasmid did not affect constitutive expression of *sulA-lacZ* in a *lexA51* (Def) derivative of SY2, SY184 (Table I). The second possibility also seemed to be less likely; when we measured the intracellular level of RecA by immunoanalysis with anti-RecA antibodies, we detected, at most, a 50% reduction in the amounts of RecA in cells containing pMspB, compared with cells containing the vector plasmid (data not shown). To test the third possibility, we followed LexA degradation after UV irradiation by immunoblotting with anti-LexA antibodies, under conditions where re-synthesis of LexA was inhibited by the presence of chloramphenicol. The result shown in Figure 5 indicates that, while LexA is completely degraded within 10 min of UV irradiation in control cells, the level of the intact LexA protein remained almost

Table I. Effect of *dinI* overexpression in *lexA51*(Def) cells

Plasmid	IPTG	MMC	<i>sulA-lacZ</i> expression (β -gal activity, Miller units)
pYP73 (vector)	–	–	2454
	+	–	2417
	–	+	2123
	+	+	2126
pYP74 (<i>dinI</i> ⁺)	–	–	1967
	+	–	2200
	–	+	2087
	+	+	1855

Fig. 5. Inhibition of LexA degradation by *dinI* overexpression. MC1061 cells carrying pYP73 (vector) or pYP74 (*dinI*⁺) were grown in EYM9 containing ampicillin (50 μ g/ml) and IPTG (0.1 mM) at 37°C. When the density reached $\sim 2 \times 10^8$ cells/ml, the cultures were supplemented with chloramphenicol (100 μ g/ml) and incubated further for 10 min. Then, aliquots were taken from both cultures (time 0), and the remainder was UV irradiated (8 J/m²). Additional aliquots were taken from the UV-irradiated samples at 5, 10 and 20 min, and subjected to SDS–PAGE followed by immunoblotting with anti-LexA antibodies. In lane M, purified LexA protein was loaded, and a faster moving band corresponds to a product of self-cleavage. The LexA antiserum used here cross-reacts with other proteins in the cell lysates, generating two fast moving bands in lanes 1–8.

Table II. Effect of *dinI* overexpression on λ prophage induction

Phage	Plasmid	IPTG	Titer
λ cI ⁺	pYP73 (vector)	–	5.9×10^9
		+	6.0×10^9
	pYP74 (<i>dinI</i> ⁺)	–	2.2×10^9
		+	8.5×10^5
λ cI857	pYP73 (vector)	–	6.6×10^8
		+	5.0×10^8
	pYP74 (<i>dinI</i> ⁺)	–	6.1×10^8
		+	3.4×10^8

unchanged in the *dinI*-overexpressed cells even 20 min after UV irradiation.

We then tested whether *dinI* overexpression could suppress degradation of the λ cI repressor, which is also a process promoted by RecA coprotease activity. As shown in Table II, plasmid-borne *dinI* inhibited the induction of λ cI⁺ prophage by addition of MMC, but did not affect heat induction of the λ cI857 (Ts) prophage which is independent of the coprotease activity. This result implies that DinI overproduction inhibits activated RecA-mediated cleavage of the cI repressor, but not any further steps of the phage development.

Fig. 6. Inhibition of UmuD processing by *dinI* overexpression. The experiments were carried out with (A) SY184 (*lexA51*) or with (B) DE667 (*lexA51 recA730*), both carrying pYP88 (*umuD*⁺). (A) SY184 carrying pYP73 (vector) or pYP74 (*dinI*⁺) were grown in L-broth containing ampicillin (50 μ g/ml), chloramphenicol (100 μ g/ml) and IPTG (0.1 mM). At a cell density of $\sim 1.5 \times 10^8$ cells/ml, MMC (0.2 μ g/ml) was added. Aliquots were taken at the indicated times, and subjected to SDS–PAGE, followed by immunoblotting with anti-UmuD antibodies. In lane M, purified UmuD and *in vitro* processed UmuD' were loaded. (B) The DE667 cells carrying pYP73 or pYP74 were grown in L-broth containing ampicillin (50 μ g/ml) and chloramphenicol (100 μ g/ml) with or without IPTG (0.1 mM). At a cell density of $\sim 5 \times 10^8$ cells/ml, the cells were harvested, lysed and subjected to SDS–PAGE, followed by immunoblotting with anti-UmuD antibodies. Equal amounts of cell extract were applied in lanes 1–4, and ~ 3 -fold more extract was applied in lane 5. Purified UmuD and UmuD' were loaded in lanes M1 and M2, respectively.

Effect of DinI overexpression on UmuD processing

Another well-studied reaction that is dependent on RecA coprotease activity is the conversion of UmuD to UmuD', which is an essential step for SOS-induced mutagenesis at DNA damaged sites. To examine whether UmuD processing is also affected by DinI overexpression, we used SY184 carrying the *lexA51* (Def) mutation, in which UmuD is constitutively produced and its processing can be initiated upon activation of RecA by DNA-damaging treatment. Furthermore, we transformed SY184 with a multicopy plasmid (pYP88) carrying *umuD* to increase the intracellular level of UmuD so as to make UmuD detection easier. As shown in Figure 6A, in the control cells, UmuD was completely converted to UmuD' at 120 min after MMC (lane 4). In contrast, little or no UmuD' was detected 120 min after MMC addition in the *dinI*-overexpressed cells (lane 8).

We repeated a similar experiment in a *lexA51* (Def) *recA730* mutant strain, DE667, carrying the *umuD*⁺ plasmid pYP88. In this strain, UmuD' is the predominant form in the absence of any DNA-damaging treatment, because RecA730 is constitutively activated (Figure 6B, lanes 1–3). Even in such backgrounds, overproduction of DinI reduces UmuD processing by $>50\%$ (lanes 4 and 5). It should be noted that the total amounts of UmuD and UmuD' in lane 4 seem to be much lower than those

Table III. Effect of *dinI* overexpression on P1 transduction

Plasmid	IPTG	Transduction frequency
pYP73 (vector)	–	3.1×10^{-7}
	+	3.3×10^{-7}
pYP74 (<i>dinI</i> ⁺)	–	3.5×10^{-7}
	+	2.1×10^{-9}

in lanes 1–3. This might be because UmuD is more unstable than UmuD' (Frank *et al.*, 1996). The result shown in Figure 6B implies that the overproduced DinI protein might inhibit the coprotease activity of constitutively activated RecA730 protein as well as that of the wild-type RecA protein. Another important implication from the result shown in Figure 6B is that an excess amount of DinI protein appears to be necessary to inhibit RecA730 coprotease activity. Without IPTG induction of the plasmid-borne DinI protein, the DE667 strain should contain significant amounts of DinI constitutively expressed from the chromosomal *dinI* gene as a consequence of the *lexA51* (Def) mutation; however, the amount does not appear to be sufficient to prevent RecA730-mediated processing of UmuD (lanes 1–3).

Effect of DinI overproduction on DNA recombination

The UV sensitization caused by multicopy plasmids carrying *dinI* may be explained by inhibition of SOS induction. However, pYP74 conferred a moderate UV sensitivity on an SOS constitutive strain GW2730 (*lexA71::Tn5*) in the presence of IPTG (Figure 3C), suggesting that the overproduced DinI protein inhibits other function(s) related to DNA repair, e.g. recombination repair. Since RecA also plays a crucial role in recombination, we examined the effect of *dinI* overexpression on RecA-mediated homologous DNA recombination. As indicated in Table III, the P1 transduction frequency of the *zic11::Tn10* marker was severely reduced in MC1061 cells carrying pYP74 (*dinI*⁺) in the presence of IPTG to <1% of the frequencies in the same cells in the absence of IPTG. This level is fairly similar to that of a *recA*-deficient derivative of MC1061. The results described above indicate that *dinI* overexpression results in inhibition of both of the two major activities of RecA, i.e. coprotease and recombinase.

Higher mutability in a *dinI* null mutant

To examine the defect of a *dinI* null mutant in more detail, we followed processing of plasmid (pYP88)-encoded UmuD after addition of MMC in a *lexA51* (Def) strain, DE192, and its Δ *dinI2::kan* derivative, SY221. The result shown in Figure 7 indicates that the conversion of UmuD to UmuD' proceeded slightly faster in SY221 than in DE192. Furthermore, since the samples contain equal amounts of the total proteins, the fact that the UmuD' band in SY221 is much darker at any time point after 60 min indicates that SY221 has a higher concentration of UmuD' than DE192. This should also be related to the difference in stability between UmuD and UmuD' (Frank *et al.*, 1996), as discussed above for the result shown in Figure 6B. Such an inhibitory effect on UmuD processing by the single-copy *dinI* gene was observed reproducibly,

Fig. 7. Effect of the *dinI* mutation on UmuD processing. DE192 (*lexA51*) and SY221 (*lexA51* Δ *dinI2::kan*) cells, both carrying pYP88 (*umuD*⁺), were grown in L-broth containing chloramphenicol (100 μ g/ml) at 37°C. At a cell density of $\sim 1.5 \times 10^8$ cells/ml, MMC (0.2 μ g/ml) was added to the cultures (time 0). Aliquots were taken at the indicated times, and subjected to SDS-PAGE, followed by immunoblotting with anti-UmuD antibodies.

Fig. 8. Increase in induced mutation frequency in a *dinI* mutant. The frequency of rifampicin-resistant mutations induced by UV irradiation was examined with DE192 (*lexA51*), SY221 (*lexA51* Δ *dinI2::kan*) and SY229 (*lexA51* *umuC::Tn5*).

although it is much more subtle than that by the multicopy gene as seen in Figure 6. Given that faster and more extensive UmuD processing occurred in SY221 than in DE192, we expected that SY221 would show a higher frequency of *umuDC*-dependent mutation than DE192. We therefore compared the frequencies of UV-induced mutation to rifampicin resistance in SY221 and DE192. As shown in Figure 8, SY221 indeed exhibited an ~3- to 5-fold increase in the mutation frequency. These results with the *dinI* null mutant indicate that the single copy of *dinI* is effective in modulating the activated RecA coprotease activity, at least, for UmuD processing.

Evidence for a direct effect of DinI on the RecA coprotease activity

To distinguish whether DinI exerts its effect either directly by interacting with RecA itself or with a RecA-ssDNA complex, or indirectly through other factor(s), we examined the effect of purified DinI on *in vitro* UmuD processing. First, RecA (3 μ g, 0.079 nmol) and M13mp7 ssDNA (60 ng, 0.2 nmol in nucleotide) were mixed in the presence of ATP γ S (1 mM), 10 mM MgCl₂ and other salts, and pre-incubated for 10 min at 37°C to allow for complex formation. When UmuD (3 μ g, 0.2 nmol) was added to this mixture and incubated further for an additional 90 min, >90% of UmuD was converted to UmuD' (lane 5). To study the effects of DinI on this processing,

Fig. 9. Effect of purified DinI on UmuD processing *in vitro*. (A) The *in vitro* reactions were performed as described in Materials and methods. The products were analyzed by SDS–16.5% PAGE with the Tricine buffer system, and visualized by Coomassie Blue staining. In lanes M1, M2 and M3, purified UmuD, RecA and DinI proteins were applied. The reaction in lane 1 lacked ssDNA, and the reactions in lanes 2–5 contained all the requirements for *in vitro* UmuD processing and the indicated amounts of DinI. (B) The density of the UmuD and UmuD' bands in lanes 2–5 were measured, and the relative amounts of UmuD or UmuD' compared with the sum of the two were calculated and plotted against the amount of DinI added to the reaction.

different amounts of DinI were added to the pre-incubated RecA–ssDNA–ATP γ S mixture and then, after a further 10 min incubation, UmuD was added to the reaction mixture. After 90 min incubation, the reaction products were subjected to analysis by SDS–PAGE. As shown in Figure 9, the UmuD processing was inhibited more severely as larger amounts of DinI were added to the reaction mixture; 28.2, 52.8 and 73.5% inhibition was observed in the presence of 0.67, 1.67 and 2.68 nmol of DinI, respectively (lanes 6–8). Thus, a vast molar excess of DinI over RecA or UmuD was required to produce an inhibitory effect *in vitro*. As a control experiment, we added similar amounts of bovine serum albumin instead of DinI, and found no effect (data not shown). Moreover, almost identical results were obtained when RecA, DinI and UmuD were added in a different order in the reaction mixture; e.g. DinI was first incubated with M13mp7 ssDNA and then RecA was added to the mixture, followed

by the addition of UmuD. Since the RecA–ssDNA–ATP γ S complex, once formed, is believed to be very stable, the above results for the *in vitro* reaction imply that DinI directly inhibits the coprotease activity of RecA bound to ssDNA, rather than interfering with the formation of the ternary complex.

Discussion

Our results presented in this study indicate that overproduction of DinI leads to inhibition of RecA's coprotease and recombinase activities. Furthermore, we showed in the *in vitro* experiment with purified proteins (Figure 9) that DinI inhibited the RecA coprotease activity toward UmuD self-cleavage even when it was added after the stable RecA–ssDNA–ATP γ S complex had been formed. This result implies that DinI is capable of interacting with RecA in the ternary complex, although DinI may also interact with free RecA molecules as well. The interaction between DinI and RecA may not be so strong, as a vast excess of DinI is necessary to produce efficient inhibition of the RecA coprotease activities *in vivo* (Figure 6B) and also *in vitro* (Figure 9), where the presence of a 10-fold molar excess of DinI over RecA resulted in ~28% inhibition of UmuD processing.

Formation of the RecA–ssDNA–ATP ternary complex is a common prerequisite for RecA's coprotease and recombinase activities. *In vitro* studies on the two RecA activities showed that an excess amount of ssDNA or the presence of dsDNA inhibited LexA processing (Takahashi and Schnarr, 1989; Rehrauer *et al.*, 1996), and that an uncleavable LexA mutant protein blocked the DNA strand exchange reaction (Harmon *et al.*, 1996). These results led to the conclusion that the two RecA reactions are mutually exclusive, involving common sites on the ternary complex. This conclusion seems to be consistent with the results of electron microscopic reconstruction studies which suggest that the binding site for LexA repressor lies within the deep helical groove of the RecA nucleofilament (Yu and Egelman, 1993). The site is also believed to be the secondary DNA-binding site that is essential for DNA strand transfer (Story *et al.*, 1992). It is conceivable that the site for binding to LexA within the RecA ternary complex would serve as the binding site for UmuD and phage repressors as well, even though different residues in RecA might critically affect the interaction between RecA and each of the target proteins (Dutreix *et al.*, 1989). If the coprotease and recombinase reactions involve common sites within the RecA–ssDNA–ATP ternary complex, both activities may be inhibited by a factor that interacts with the ternary complex near the common sites, as well as by those that interfere with ternary complex formation.

We examined whether DinI could inhibit LexA processing *in vitro* under similar conditions to that for the UmuD reaction, but thus far we have not been able to detect any inhibitory effect of DinI on *in vitro* LexA processing, in spite of our repeated attempts. It seems plausible that the experimental conditions that we have tested are sub-optimal for DinI to inhibit the RecA-dependent processing of LexA. Since LexA processing was prevented *in vivo* when DinI was overproduced (Figure 5), we expect that DinI should inhibit the RecA-

dependent cleavage of LexA *in vitro* under conditions that mimic the *in vivo* conditions more faithfully (see also below for further discussion). Obviously, further *in vitro* experiments are necessary to prove or disprove the above model for the action of DinI.

ImpC, a homolog of DinI

The primary sequence of DinI shows similarity to that of several other proteins (Yasuda *et al.*, 1996), among which the similarity to ImpC (82 amino acids) is most suggestive of their *raison d'être*. In a LexA-controlled operon on the *Salmonella typhimurium* plasmid TP110, the *impC* gene is followed by *impA* and *impB* (Lodwick *et al.*, 1990), which are homologous in both structure and function to *umuD* and *umuC* on the *E. coli* chromosome, respectively. No gene similar to *impC* is present in other homologous operons such as *mucAB* and *samAB* carried by the *S. typhimurium* plasmids pKM101 and pSLT, respectively (Perry *et al.*, 1985; Nohmi *et al.*, 1991). It has been described, although no experimental data were presented, that *impC* was not absolutely required for the *imp*-promoting mutagenic activity, and that its overexpression interfered with SOS induction (Lodwick *et al.*, 1990). In fact, we observed that a multicopy plasmid carrying the *impC* gene inhibited induction of the SOS response in an experiment using the *sulA-lacZ* fusion similar to that in Figure 4 (data not shown). Thus, structurally related DinI and ImpC proteins appear to have a similar function, i.e. a negative regulatory role.

The effect on UmuD processing of the *dinI* gene when expressed chromosomally could be detected; a slightly faster and more extensive processing of UmuD was reproducibly observed in a *dinI* null mutant when compared with the wild-type (Figure 7). Furthermore, this faster processing of UmuD to UmuD' was associated with an increase in the frequency of SOS-induced rifampicin resistance mutations (Figure 8). If *dinI* functions as a feedback regulator for the SOS response, it would be expected that, during the recovery phase of the SOS response, wild-type cells might restore the normal level of intact LexA proteins faster than the *dinI* mutant cells; however, when we examined the level of intact LexA after UV irradiation by immunoblotting, we could detect no discernible difference between the two strains (data not shown). The effect of DinI on LexA processing was only observed when it was overexpressed. Thus, LexA processing appears to be less susceptible than UmuD processing to inhibition by DinI. Such a difference in sensitivity to inhibition by DinI between UmuD and LexA processing may be attributed to a large difference in their reactivities; UmuD is cleaved much less efficiently than LexA *in vivo* (Shinagawa *et al.*, 1988; Woodgate and Ennis, 1991) and *in vitro* (Buckhardt *et al.*, 1988). The inhibitory effect on UmuD processing can be amplified further since UmuD' readily forms mutagenically inactive heterodimers with intact UmuD in preference to an active UmuD' homodimer (Battista *et al.*, 1990). Furthermore, this heterodimer formation leads to the rapid proteolysis of mutagenically active UmuD' by the ClpXP protease (Frank *et al.*, 1996). Thus, we conclude that the function of *dinI* (and probably also of *impC*) is more suited for a fine-tuning role in the mutagenic process by preventing extensive processing of UmuD (and ImpA), than as a

feedback regulator of the overall SOS response by inhibiting LexA processing. This interpretation should account for why *impC* comprises an operon together with *impA* and *impB*, and is expressed in a coordinated way.

Other genes with anti-SOS function

psiB is a gene conserved among conjugative plasmids such as F, which was shown to be responsible for a plasmid-coded anti-SOS function (Bagdasarian *et al.*, 1986; Dutreix *et al.*, 1988; Golub *et al.*, 1988). It not only prevents induction of *sfiA* (= *sulA*) and prophage λ , but also mutagenesis and intrachromosomal recombination (Bailone *et al.*, 1988). The degradation of LexA after UV irradiation was found to be inhibited in the cells carrying a *psiB*⁺ plasmid (Célérier *et al.*, 1988). Thus, the function of *psiB* is very similar to that of *dinI*, yet no sequence similarity is found between PsiB (144 amino acids) and DinI. *psiB* is located near *oriT*, the origin of conjugational transfer, and it undergoes zygotic induction (Bagdasarian *et al.*, 1992). *psiB* seems not to be under SOS control, and its role is believed to be to prevent the recipient cells from inducing the SOS response during the transfer of the ssDNA. If this is indeed the case, PsiB is expected to inhibit LexA processing more efficiently than DinI, either by preventing formation of the RecA-ssDNA-ATP ternary complex or by interacting with the ternary complex in a similar way to that described for DinI.

isfA is a mutation recently localized at 85 min on the *E. coli* chromosome, which was found by virtue of its inhibitory effect on various SOS functions (Bebenek and Pierzykowska, 1995). The mutation was shown to suppress UmuD processing by the RecA coprotease activity (Bebenek and Pierzykowska, 1996). The authors postulated that the *isfA* mutation led to constitutive expression of the gene (*isfA*), which might be expressed at the end of the DNA repair with the function of turning off the induced SOS system. However, since neither the *isfA* gene nor its product have been identified yet, no further information on the question of how *isfA* expression is regulated and of how the gene product acts to inhibit SOS induction is available at the present time.

Antagonistic functions in the SOS response

It has been generally accepted that the SOS response is induced mainly, although not exclusively, for the repair of DNA damage, while the functions of some other SOS-inducible genes still remain unclear. Here, we wish to emphasize another aspect of the SOS response, focusing on SOS-induced mutagenesis. The SOS mutagenic functions include the *dinB/P*-dependent pathway that induces mutations most probably at non-damaged sites, albeit at a low frequency unless overexpressed (Kim *et al.*, 1997). It is very difficult to assign any role for repair to this pathway that seems to be more ubiquitous than the *umuDC*-dependent pathway (Ohmori *et al.*, 1995a; Kulaeva *et al.*, 1996). The role of the *umuDC*-dependent pathway, which is more potent in mutagenesis than the *dinB/P*-dependent one, has been considered as a last resort for survival of cells with damaged DNA, by keeping replication proceeding past a DNA lesion at the cost of generating mutations. However, the effect of a mutation in *umuDC* on cellular survival after UV irradiation appears very weak when we consider that the activity is regulated at multiple levels

Table IV. Bacterial strains used

Strain	Relevant genotype	Source or reference
MC1061 ^a		laboratory collection
KN1808	<i>uvrD98::Tn10</i>	laboratory collection
KN2012	MC1061 <i>lexA1</i> (Ind ⁻) <i>zjb564::Tn10</i>	Ohmori <i>et al.</i> (1995)
MS6	MC1061 Δ <i>recA518::kan</i>	Ohmori <i>et al.</i> (1995)
SY2	MC1061 <i>sulA::lacZ'</i> YA::kan	Ohmori <i>et al.</i> (1995)
SY101	MC1061 <i>uvrD98::Tn10</i>	P1(KN1808)×MC1061
SY117	MC1061 <i>dinI1::kan</i>	Yasuda <i>et al.</i> (1996)
SY181	MC1061 (λ C43 <i>dinD::lacZ</i>) ⁺	Ohmori <i>et al.</i> (1995)
SY183	MC1061 Δ <i>dinI2::kan</i>	this work
KH07	DE667 <i>zjb564::Tn10</i>	P1(KN2012)×DE667
SY184	SY2 <i>lexA51</i> (Def) <i>zjb564::Tn10</i>	P1(KH07)×SY2
JC8947	<i>umuC::Tn5</i>	NIG collection
DE667 ^b	<i>lexA51</i> (Def) <i>recA730</i>	R.Woodgate, via T.Nohmi
DE192 ^c	<i>lexA51</i> (Def)	R.Woodgate
SY221	<i>lexA51</i> (Def) Δ <i>dinI2::kan</i>	P1(SY183)×DE192
SY229	<i>lexA51</i> (Def) <i>umuC::Tn5</i>	P1(JC8947)×DE192
GW2730 ^d	<i>lexA71::Tn5</i> <i>recA441</i>	NIG collection
KN2003	W3110 <i>pyrE41</i> <i>zic11::Tn10</i>	laboratory collection

^aThe genetic markers are F⁻ Δ *lacX74* *rpsL* *araD139* Δ (*ara leu*)7697 *galU* *galK* *hsr*⁻ *hsm*⁺.

^bOther markers are F⁻ *thi-1* *sulA211* Δ (*lac-gpt*)5 Δ (*uvrB-chlA*) *mtl-1* *rpsL31*.

^cAs DE667 but *uvr*⁺.

^dOther markers are F⁻ Δ (*lac*)169 *rpsL31* *his-4* *thr-1* *leu-6* *argE3* *ilv* *galK2* *sulA11*.

by elaborate systems (Buckhardt *et al.*, 1988; Nohmi *et al.*, 1988; Shinagawa *et al.*, 1988; Battista *et al.*, 1990; Frank *et al.*, 1996; this work). Moreover, *umuDC*-dependent translesion synthesis is not the only way to continue replication in the presence of DNA damage; recombinational repair that, similarly to activation of the *umuDC* pathway, requires RecA–ssDNA–ATP ternary complex formation, should also be able to keep replication continuing in the presence of DNA damage in a different way and more efficiently (Friedberg *et al.*, 1995). We therefore infer that the role of SOS mutagenesis including both the *umuDC* and *dinB/P* pathways is to generate, by chance, a mutant in the population exposed to a DNA-damaging agent or other stresses such as starvation (Taddei *et al.*, 1995), such that it is better adapted to the stressed environments (Echols, 1981). On the other hand, since most mutations generated by any means should be detrimental for growth in the short-term, it becomes important for an individual cell to keep the mutation frequency low, especially by tightly controlling the more potent *umuDC*-dependent mutagenic pathway. In conclusion, we believe that the SOS response comprises many functions that act in various antagonistic ways, but their respective functions may have their own meaning on different time scales, and under different environmental conditions.

Materials and methods

Strains and media

The bacterial strains used are all derivatives of *E. coli* K12 and are listed in Table IV. Among them, SY183 (MC1061 Δ *dinI2::kan*) was constructed by replacing the 0.6 kb *PvuII*–*HpaI* segment with the kanamycin resistance gene from pUC4K (Pharmacia-LKB), using the gene disruption method that we developed previously (Ohmori *et al.*, 1995b). The plasmids used are listed in Table V. An expression vector, pYP73, was constructed from pKK223-3 (Pharmacia-LKB) by inserting the *lacI*^q gene at the unique *SphI* site. The *dinI* coding region was inserted between the *EcoRI* and *PstI* sites of the multiple cloning sites of pYP73 to construct pYP74. pYP88 was constructed by inserting an ~1.0 kb *BglIII* fragment from pGW2101 that contained the *umuD*⁺ gene into the *BamHI* site of pACYC184 (Chan and Cohen, 1978).

Table V. Plasmids used

Plasmids	Vector or precursor	Relevant feature	Source or reference
pTZ19ULC	pTZ19U	<i>amp</i> ⁺	Yasuda <i>et al.</i> (1996)
pMspB	pTZ19ULC	<i>dinI</i> ⁺	Yasuda <i>et al.</i> (1996)
pMspC	pTZ19ULC	<i>lexA</i> ⁺	Yasuda <i>et al.</i> (1996)
pKK223-3		<i>amp</i> ⁺	Pharmacia-LKB
pYP73	pKK223-3	<i>amp</i> ⁺ <i>lacI</i> ^q	this work
pYP74	pYP73	<i>dinI</i> ⁺	this work
pGW2101	pBR322 Δ <i>rop</i>	<i>umuD</i> ⁺ <i>C</i> ⁺	Nohmi <i>et al.</i> (1988)
pACYC184		<i>cat</i> ⁺ <i>tet</i> ⁺	Chan and Cohen (1978)
pYP88	pACYC184	<i>cat</i> ⁺ <i>umuD</i> ⁺	this work

L-broth [1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl (pH7.4)] was used for cultivation of bacteria throughout this study, unless otherwise indicated. L-agar contained 1.5% agar in L-broth. EYM9 medium was M9 medium (Miller, 1972) supplemented with thiamine (10 µg/ml), glucose (2 mg/ml), MgSO₄ (1 mM), MgCl₂ (0.1 mM), thymine (20 µg/ml) and each of the amino acids required (10 µg/ml). When necessary, ampicillin (50 µg/ml), kanamycin (20 µg/ml), tetracycline (20 µg/ml), chloramphenicol (100 µg/ml) or rifampicin (50 µg/ml) were added.

Proteins and antibodies

LexA was purified as described (Hori *et al.*, 1981b). Antibodies against the purified LexA protein were raised in a rabbit. In some early experiments, the anti-LexA antibodies provided by A.Higashitani (NIG, Mishima) were used. Purified UmuD is a gift of H.Iwasaki (Osaka University). Rabbit antibodies against RecA and UmuD were provided by A.Shinohara (Osaka University) and R.Woodgate (NIH, Bethesda, MD), respectively.

Purification of DinI

MC1061 carrying pYP74 was grown in 10 l of L-broth at 37°C. When the density reached ~1×10⁸ cells/ml, IPTG was added at the final concentration of 0.1 mM and, after further shaking for an additional 5 h, the cells were harvested. The cell paste (23.6 g wet weight) was resuspended in 130 ml of buffer A [10 mM Tris–HCl (pH 7.5)/0.1 M NaCl/1 mM EDTA/10 mM 2-mercaptoethanol] containing lysozyme (5 mg/ml) and phenylmethylsulfonyl fluoride (PMSF, 1 mM). After cell disruption by ultrasonication, the cell suspension was centrifuged at 14 000 g for 30 min and at 77 000 g for 30 min, and the supernatants were supplemented with streptomycin sulfate (3%, w/v) to remove

nucleic acids. The crude extract thus obtained was fractionated with ammonium sulfate (40–90% saturation). The precipitate was resuspended in buffer B [20 mM Tris–HCl (pH 7.5)], treated with Hi-Trap Desalting and applied to HiPrep 16/10 Q-Sepharose FF (obtained from Pharmacia-LKB) that was pre-equilibrated with buffer B. DinI protein was eluted at ~0.3 M NaCl in a 0–0.5 M linear gradient of NaCl in buffer B. Pooled fractions containing DinI were then applied to HiPrep 16/60 Sephacryl S100 HR (obtained from Pharmacia-LKB) pre-equilibrated with buffer C [20 mM Tris–HCl (pH 7.5)/0.4 M NaCl] (Figure 1). The DinI protein thus purified showed a single band on SDS–PAGE (Figure 1).

Mutation assay

Overnight cultures of DE192 or its derivative carrying a *dinI* or a *umuC* mutation were diluted into fresh L-broth, and grown to 2×10^8 cells/ml by vigorous shaking at 37°C. The bacterial cells were collected by centrifugation, and resuspended in one-fifth of the original volume of 0.1 M MgSO₄. The cell suspensions were irradiated with UV at various doses. Aliquots were diluted 20-fold into fresh L-broth and cultured overnight in the dark at 37°C. After appropriate dilution with saline (0.84% sterilized NaCl solution), the cells were plated on L-agar with or without rifampicin (50 µg/ml) in duplicate. After incubating the plates overnight at 37°C, the colony numbers were counted.

Recombination assay

The frequency of recombination mediated by P1 phage transduction was measured as follows. MC1061 carrying pYP73 (vector) or pYP74 (*dinI*⁺) was cultivated in 20 ml of L-broth containing ampicillin (50 µg/ml) in the presence or absence of IPTG (0.1 mM). When the density reached $\sim 5 \times 10^8$ cells/ml, the cells were collected and resuspended in the same medium but also containing 2.5 mM CaCl₂. P1 phages (1×10^9 p.f.u.) that were prepared from KN2003 carrying the *zic11::Tn10* marker were added to the cultures. After mild shaking for 30 min at 37°C, the cells were collected by centrifugation, washed with 5 ml of saline and then resuspended in 1 ml of saline. After appropriate dilution, the cells were plated in duplicate on L-agar plates containing ampicillin (50 µg/ml); some of the plates also contained tetracycline (20 µg/ml) and/or IPTG (0.1 mM). After incubating the plates overnight at 37°C, the colony numbers were counted.

Immunoblotting analysis

Aliquots of the bacterial cultures were mixed with one-third volume of chilled 20% trichloroacetic acid solution, and kept on ice for 30 min. The precipitated proteins were washed twice with chilled acetone and resuspended in 50 mM Tris–HCl (pH 7.5)/1% SDS. After boiling, the protein concentrations in the samples were measured with Micro BCA Protein Assay Reagent (Pierce), and diluted so that each contained an equal concentration of total protein. Aliquots of the samples were either applied to SDS–12.5% PAGE gels with the standard buffer system (Laemmli, 1970) for LexA and RecA or to SDS–16.5% PAGE gels with the Tricine buffer system (Schägger and von Jagow, 1987) for UmuD and DinI. Separated proteins were electrotransferred to an Immobilon P membrane (Millipore) or Hybond ECL Western (Amersham), and subsequently probed with rabbit antibodies raised against DinI, LexA, RecA or UmuD. The target proteins were visualized by using either the ECL Western blotting analysis system (Amersham) or the Western-Light Plus™ Chemiluminescent Detection system (Tropix). For quantification of the densities of the bands, the membranes were exposed to Kodak X-Omat film and the developed films were subjected to densitometric analysis using the software Sigma Gel (Jandel Corporation).

In vitro assay for the UmuD processing

The *in vitro* reactions were carried out essentially following the method described by Burckhart *et al.* (1988). The reaction mixtures (45 µl) contained the basal medium [40 mM Tris–HCl (pH 8.0), 10 mM MgCl₂, 30 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM ATPγS], 60 ng of M13mp7 single-stranded DNA, 3 µg of RecA, 3 µg of UmuD and DinI. M13mp7 ssDNA and RecA were first added to the basal medium, and pre-incubated at 37°C for 10 min. The mixture was supplemented with different amounts of DinI, and further incubated for an additional 10 min. After adding UmuD (3 µg) to the mixtures and incubating for a further 90 min, the reactions were terminated by adding the sample buffer for SDS–PAGE (Laemmli, 1970). Products were analyzed by SDS–16.5% PAGE with the Tricine buffer system and staining with Coomassie Brilliant Blue R-250.

Other methods

β-Galactosidase was assayed as described (Miller, 1972). Genetic methods followed the standard protocols (Miller, 1972), and most of the

procedures for DNA manipulations followed those described (Sambrook *et al.*, 1989).

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