RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis

(DNA damage/error-prone repair/posttranslational processing/protease/gene fusion)

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ABSTRACT Induction of the Escherichia coli SOS system increases the ability of the cell to perform DNA repair and mutagenesis. Products of the recA and umuD,C genes are required for mutagenesis induced by radiation and many chemicals. Transcription of the SOS genes including recA and umuD,C is repressed by a repressor, LexA protein, and is derepressed by the proteolytic cleavage of LexA facilitated by RecA protein that had been activated by inducing signals produced in the cell by agents that damage DNA. An activated form of RecA protein, RecA*, seems to have roles in SOS mutagenesis other than its known role as an antirepressor. Derepression of the genes involved in SOS mutagenesis such as recA and umuDC in defective chromosomal lexA(Def) mutants does not increase the ability of the cell to perform mutagenesis. Activation of RecA protein is essential to this ability. RecA* facilitates the proteolytic cleavage of several repressors such as λ, P22, and 434 phage repressors and LexA, and Umud protein contains a sequence homologous to the regions surrounding the cleavage sites of these suppressors; therefore, we examined the possibility that Umud protein is cleaved by RecA*. We found evidence that the intact Umud protein was cleaved after mutagenic treatment and that the cleavage was dependent on RecA*. The results suggested that Umud protein may be proteolytically processed by RecA*, and that processed Umud may be the active form of the protein participating in mutagenesis.

Exposure of the Escherichia coli cell to agents that damage DNA or interfere with DNA replication results in the induction of a diverse set of physiological responses, collectively termed the SOS response, that include an increased capacity to perform mutagenesis (1–3). It has been shown that the products of the umuD, umuC, and recA genes are required for mutagenesis induced by radiation or chemical agents (SOS mutagenesis) (3–7). The expression of the umuDC and recA genes and other SOS genes is controlled by a complex circuit involving the recA and lexA gene products (2, 3). LexA protein binds to the operator regions of the recA gene and the umuDC operon and represses the expression of the genes in the uninduced cell (8, 9). Exposure of the cell to DNA-damaging agents generates some inducing signal(s) that activates RecA protein to a form (RecA*) that promotes proteolytic cleavage of LexA at the bond between Ala-112 and Gly-113 (10). The consequence of the inactivation of LexA by proteolytic cleavage is the increased expression of SOS genes, including umuDC and recA (2, 3).

Several lines of evidence suggest that RecA* plays another role in SOS mutagenesis in addition to mediating the cleavage of LexA repressor, a necessary event for the induced expression of the umuDC and recA genes (11–13). In recA* strains, the presence of a defective chromosomal lexA(Def) allele that allows the constitutive expression of the SOS genes at elevated levels does not result in elevated spontaneous mutability, indicating the derepression of the SOS genes is not sufficient for SOS mutagenesis. However, mutator activity is enhanced when recA441 lexA(Def) cells are grown at 42°C, which promotes the cleavage of LexA without DNA damage (11). Ennis et al. (12) measured the mutagenic capacity of the cells by measuring the mutation frequency of UV-irradiated λ phages. They found that mutagenic activity was high only with conditions of the cell that activate the proteolytic function of RecA: either with UV irradiation of the recA* lexA(Def) cell or with the recA730 lexA(Def) cell. These findings suggest that for SOS mutagenesis, RecA* is needed to perform a function in addition to the inactivation of LexA to derepress the SOS genes. Witkin and Kogoma (13) have shown that SOS mutagenesis does not require inactivation of repressors other than LexA. Thus, there are two possibilities for the function of RecA* in mutagenesis that are compatible: (i) RecA* is needed for the cleavage of some protein that then participates in the actual biochemical process of mutagenesis, and (ii) RecA* itself participates mechanistically in SOS mutagenesis.

In this report, we describe experiments designed to test the first of these possibilities. Since RecA* promotes the proteolytic cleavage of several repressors such as λ, P22, and 434 phage repressors and LexA (2, 3), and since the Umud protein contains a sequence homologous to the regions surrounding the cleavage sites of these repressors (14), we examined the possibility that Umud protein is cleaved by SOS induction in a RecA*-dependent way. Indication of the proteolytic cleavage of Umud protein by our earlier work (7) also prompted us to carry out the experiments.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The E. coli strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

General Methods. Methods for DNA manipulation were as described by Davis et al. (21). Transduction with phage P1 and assay of β-galactosidase activity were done as described by Miller (22). NaDodSO4/PAGE was performed as described by Laemmli (23).

Construction of a lacZ-umuD Fusion Gene. pUR1010, which carries a lacZ-umuD fusion gene, was constructed as follows. The 575-base-pair (bp) Xho II fragment of pSK601 containing most of the umuD coding region and a small part of the umuC region (7, 9) was isolated by electroelution and ligated to the BamHI site of pUR290, a plasmid vector for the construction of lacZ fusion genes (20). Two kinds of plasmids in which the Xho II fragment was inserted in opposite orientations were obtained. One of them (pUR1010) could encode a LacZ-UmuD hybrid protein in which amino

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acid 8 of UmuD protein was fused to the carboxyl terminus of functional β-galactosidase.

**Purification of the LacZ-UmuD Hybrid Protein.** To the culture of strain UT481 carrying pUR1010 grown to an OD_{600} of 0.4 in LB medium (1% tryptophol/0.5% yeast extract/0.5% NaCl, pH 7.4), 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to induce the synthesis of the hybrid protein, and the cells were harvested after overnight incubation. The hybrid protein was extracted from the cells and partly purified by affinity chromatography for β-galactosidase as described elsewhere (24). It was further purified by HPLC with an anion-exchange column of Mono Q (Pharmacia). The sample equilibrated with buffer A (40 mM Bistris-HCl, pH 6.5/5 mM MgCl$_2$/10 mM 2-mercaptoethanol; Bistris is [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Sigma)) was put on the column and eluted with a linear 0 to 1 M NaCl gradient in buffer B (40 mM Bistris-HCl, pH 6.5/5 mM MgCl$_2$/5 mM 2-mercaptoethanol). The peak fractions with β-galactosidase activity were pooled and used for immunization. The purity of the hybrid protein was about 95% as judged by NaDodSO$_4$/PAGE.

**Antiserum Preparation.** Antiserum was raised in rabbits against the purified LacZ-UmuD hybrid protein. The serum was assayed for its reactivity with the hybrid protein by the Ouchterlony immunodiffusion method (25).

**Immunoblotting.** Mitomycin C (1 µg/ml) was added to *E. coli* cells at the exponential stage of growth in LB broth (1% tryptophol/0.5% yeast extract/0.5% NaCl, pH 7.4), and the culture was incubated for 2 hr before being harvested. For UV irradiation, the cells were suspended in M9 buffer (22) and irradiated with UV light (20 J/mm$^2$). The cells were incubated in LB broth for 2 hr before being harvested.

Ampicillin (100 µg/ml) was added to the culture medium for strains carrying plasmid pBR322 or pSK601. NaDodSO$_4$/PAGE of the cell lysates was done in 15% gel. Proteins were transferred electrophoretically to a nitrocellulose sheet as described before (26). The sheet was washed three times with buffer C (500 mM NaCl/20 mM Tris·HCl, pH 7.5) and soaked in 5% skim milk in buffer C for 1 hr. After being rinsed with buffer D (0.5% Tween 20 in buffer C) three times, the sheet was incubated with a 1:100 dilution of the antiserum in buffer D containing 1% skim milk for 1 hr at room temperature. After being washed five times with buffer D, the blot was incubated for 1 hr with a 1:1000 dilution of peroxidase-linked anti-rabbit antibody (Bio-Rad) in buffer D containing 1% milk. The sheet was washed five times with buffer D and twice with buffer C. The protein bands were visualized by incubation of the sheet in a solution containing H$_2$O$_2$ and a chromogenic indicator, 4-chloro-1-naphthol (Bio-Rad), as suggested by the supplier. A portion of the sheet for marker proteins was cut and stained with 0.1% amido black in 45% methanol/10% acetic acid.

**Immunoprecipitation.** Exponentially growing cells in M9 medium supplemented with the required nutrients and with an OD$_{600}$ of 0.2 were labeled with [35]methionine (5 µCi/ml; 1 Ci = 37 GBq) for 1 hr, and nonradioactive methionine (1 mM) and mitomycin C (1 µg/ml) were added to the culture. Portions of the sample were taken at different times after the addition and immediately chilled in an ice bath. Cell lysates were prepared, and the protein(s) that reacts with the antiserum was precipitated as described before (27) except that protein A-Sepharose (Sigma) was used instead of the second antibody and the antigen-antibody complexes adsorbed to the protein A-Sepharose were washed several times with RIPA buffer (10% Triton X-100/15% sodium deoxycholate/0.1% NaDodSO$_4$/0.15 M NaCl/0.05 M Tris·HCl, pH 7.2) and then with Heps buffer (10% glycerol/20 mM Heps, pH 7.5). The labeled proteins were separated by NaDodSO$_4$/PAGE and visualized by autoradiography.

**RESULTS**

**Identification of UmuD Protein.** To determine whether the serum against the LacZ-UmuD hybrid protein reacts with UmuD protein, cell lysates of AB1157 with or without pSK601, which carries the *umuD* gene, were analyzed by immunoblotting (Fig. 1). A weak band corresponding to a 17-kDa protein and a strong band corresponding to a 14-kDa protein were specific for the cells treated with mitomycin C that carried a multicopied *umuD* gene (see also Figs. 2 and 3). We suspected that the 17-kDa protein was the intact UmuD protein and that the 14-kDa protein was the proteolytically processed product, since they were detected only in the SOS-induced cells with multicopy *umuD* plasmid and since the molecular weight of the former agrees with that of the UmuD protein that has been identified (6, 7). The size of UmuD based on the DNA sequence is 15 kDa (9, 14), while

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant marker</th>
<th>Ref. or origin</th>
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<tbody>
<tr>
<td>AB1157</td>
<td>recA*, lexA*</td>
<td>15</td>
</tr>
<tr>
<td>GC853</td>
<td>recA*, lexA*, sulB</td>
<td>16</td>
</tr>
<tr>
<td>GW2730</td>
<td>recA441, lexA71::Tn5, sulA</td>
<td>17</td>
</tr>
<tr>
<td>DM2549</td>
<td>del(recA-srlR306, lexA51, sulA)</td>
<td>D. W. Mount</td>
</tr>
<tr>
<td>HRS200</td>
<td>recA*, lexA71::Tn5, sulB</td>
<td>P1 transduction of lexA71::Tn5 from GW2730 to GC853</td>
</tr>
<tr>
<td>HRS201</td>
<td>del(recA-srlR306, lexA71::Tn5, sulB)</td>
<td>P1 transduction of del(recA-srlR306 from DM2549 to HRS200</td>
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<tr>
<td>DM2570</td>
<td>recA*, lexA51</td>
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</tr>
<tr>
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<td>12</td>
</tr>
<tr>
<td>DE274</td>
<td>recA730, lexA51</td>
<td>12</td>
</tr>
<tr>
<td>DM2573</td>
<td>recA430, lexA51</td>
<td>12</td>
</tr>
<tr>
<td>UT481</td>
<td>F'(proAB, lac*), del(lac-pro), ion</td>
<td>18</td>
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del, Deletion.

**Table 1. List of E. coli strains**

<table>
<thead>
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<th>Table 2. List of plasmids</th>
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<tr>
<td>Plasmid</td>
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<td>pUR290</td>
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<tr>
<td>pSK601</td>
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<td>pUR1010</td>
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Amp', ampicillin resistance; Tet', tetracyclin resistance.
Biochemistry: Shinagawa et al.

Fig. 1. Identification of UmuD protein by use of antisem against the LacZ-UmuD hybrid protein. To a culture of AB1157 cells at the exponential stage, mitomycin C (1 μg/ml) was added, and the cells were harvested after incubation for 2 hr. The cellular proteins were separated by NaDodSO4/PAGE and transferred to a nitrocellulose filter by electroblotting. The filter was treated with the antisem, and the proteins that had reacted immunologically with the serum were visualized with the peroxidase-conjugated second antibody. Lanes: 1, size-marker proteins; 2 and 3, AB1157/pSK601; 4 and 5, AB1157/pBR322; 6 and 7, AB1157. The cells in lanes 2, 4, and 6 were treated with mitomycin C; those in lanes 3, 5, and 7 were not. D and D' indicate UmuD protein and its processed product, respectively.

that based on electrophoretic mobility was about 17 kDa (6, 7). Chromosomally encoded UmuD protein was not detected even in the induced cells, probably because of the limited sensitivity of the detection method used here. Some protein bands that appeared in the background may be due to the antibodies raised against the impurities in the antigen preparation used for the preparation of the serum. Others may be due to nonspecific interactions between the proteins during the immunological processing of the blots, since the intensity of the background bands varied considerably for each blot.

RecA*-Dependent Cleavage of UmuD Protein. To examine the genetic conditions for the processing of the 17-kDa UmuD protein, pSK601 was introduced into various strains with mutations related to the SOS system. Neither the 17-kDa nor the 14-kDa proteins were detected in the recA and lexA(Ind-) [Ind-= DM844(7)] strains (data not shown) with or without mitomycin C treatment. The results were consistent with earlier reports that the umuD,C genes are repressed by LexA protein and that LexA protein, but not LexA(Ind-) protein, is inactivated by RecA* (2, 3, 6–8).

In the lexA(Def) strain, only the 17-kDa protein was observed before mitomycin C treatment (Fig. 2, lane 4), and the proteins of both sizes were detected after mitomycin C treatment (Fig. 2, lane 5). This result suggests that the intact UmuD protein was converted to the 14-kDa protein by proteolytic cleavage of the intact protein by the action of RecA*. It was consistent with this hypothesis that the 14-kDa protein was not detected in a lexA(Def) recA strain even after mitomycin C treatment (Fig. 2, lanes 6 and 7).

The above results suggest the possibility that UmuD protein was induced and processed in the cells that had been treated with mutagenic agents. Therefore, we examined whether UV irradiation, which is a potent mutagen and an activator of RecA in vivo, also induces the same phenomena. UmuD protein was induced and processed in the UV-irradiated cells in a RecA*-dependent way as in cells treated with mitomycin C (data not shown).

To further test the hypothesis, pSK601 was introduced into various recA strains with unique properties, and the processing of UmuD in these strains was studied. The recA730 mutant expresses the SOS system constitutively, includ-


Fig. 2. Genetic conditions for the cleavage of the UmuD protein after treatment with mitomycin C. The UmuD protein and its processed product were detected immunologically as in the legend of Fig. 1. Lanes: 1, size-marker proteins; 2 and 3, GC583 (wild type); 4 and 5, HRS200 (lexA::Tn5); 6 and 7, HRS201 [lexA::Tn5, del-recA]). The cells in lanes 3, 5, and 7 were treated with mitomycin C; those in lanes 2, 4, and 6 were not. All of the strains used here carried pSK601 (umud+).

Fig. 3. Influence of recA mutations upon the cleavage of UmuD protein. UmuD protein and its processed product were detected immunologically as in Fig. 1. Lanes: 1, size-marker proteins; 2 and 3, DM2571 (del-recA::lexA51); 4 and 5, DMA2570 (lexA51); 6 and 7, DE274 (lexA51, recA730); 8 and 9, DMA2573 (recA430, lexA51). The cells in lanes 3, 5, 7, and 9 were treated with mitomycin C; those in lanes 2, 4, 6, and 8 were not. All of the strains used here carried pSK601 (umud+).
**DISCUSSION**

Our results here showed that the synthesis and the subsequent proteolytic processing of the UmuD protein are induced in wild-type cells treated with mutagens such as mitomycin C or UV light. The synthetic step could be separated from the processing step in the recA<sup>+</sup> lexA(Def) strain. In such a strain, because of the absence of the functional repressor for the umu operon, the unprocessed UmuD protein was constitutively synthesized at a high level in the absence of inducing treatments. The processing required both the function of the recA gene and treatment that damages the DNA, since little processed product was detected in the recA<sup>+</sup> lexA(Def) cells before the treatment or in the recA lexA(Def) cells either before or after treatment. Processing was observed in the untreated cells of the recA730 lexA(Def) strain, in which the product of the recA730 gene is constitutively in the RecA* state. Thus, the processing was only (and always) observed under the conditions that activate the RecA protein as RecA*. Since the cleavage of the LexA repressor and the repressors of several temperate phages is promoted by RecA* (2, 3), results suggest that cleavage of UmuD protein was also promoted by RecA*.

The difference in size between the UmuD protein and the processed product was about 3 kDa, and UmuD protein contains a Cys-Gly sequence at positions 24 and 25, respectively, in the region that is substantially homologous to the regions of the LexA repressor and the repressors of phages λ, 434, and P22 that include the Ala-Gly bond cleavable by RecA* (9, 14). The bond between Cys-110 and Gly-111 is the cleavage site of φ80 repressor by RecA* (Y. Eguchi, T. Ogawa, and H. Ogawa, personal communication). Therefore, the intact 17-kDa UmuD protein might also be cleaved at this position by RecA* to give the 14-kDa processed product.

The results of the earlier reports (11–13) and of our experiments here show correlation among the activation of RecA protein, proteolytic processing of UmuD protein, and SOS mutagenesis activity of the cell. This correlation was observed in all cases examined. Therefore, the evidence available now is consistent with the hypotheses that the processing of UmuD protein is promoted by RecA* and that the processed protein is the active form involved in SOS mutagenesis.

Burckhardt et al. (29) purified UmuD protein and showed that the cleavage of the protein in vitro was mediated by RecA protein and that self-cleavage of UmuD at alkaline pH was similar to the cleavage of C1 repressor of λ phage and LexA repressor (30–32). The combined results of their work and the present work strongly support the suggestion that UmuD is cleaved in vivo by RecA* itself. Nohmi et al. (33) constructed a mutant umuD gene that coded for the putative cleaved product of UmuD, which is lacking the 24 amino-terminal residues, and demonstrated that the gene product was active for UV mutagenesis. This result suggests that the cleaved product is the active form for mutagenesis and eliminates the possibilities that the cleavage process per se is required for mutagenesis and the cleavage is a way to inactivate UmuD. They also constructed several umuD mutants that coded for UmuD with altered amino acids in the putative cleavage site. All of these mutants showed deficiency in UV mutagenesis, consistent with the idea that the bond between Cys-24 and Gly-25 is the cleavage site of UmuD and that cleavage is required for the activation of UmuD. However, it remains to be seen whether those mutant UmuD proteins are more resistant than the wild-type protein to cleavage by RecA*.

The recA430 mutation is unique in that mutagenesis dependent on the umuDC gene is deficient, but mutagenesis dependent on the mucAB function is proficient (34). Therefore, it is likely that the mutant RecA protein is not deficient in any direct, mechanistic role that may be required for UV mutagenesis. Since UmuD protein was not cleavable in the recA430 lexA(Def) strain (Fig. 3), the defect in SOS mutagenesis may be due to the defect in the cleaving of UmuD protein. The proficiency of MucA,B proteins in recA430 strains could indicate either that MucA is active without processing or that RecA430 can promote processing of MucA but not of UmuD).

The expression of umuD seems to be regulated at two steps, one the transcription step and the other the functional activation step. Dual control may be the means to suppress efficiently the unwanted mutagenic activity of the cell in the absence of DNA damage.

A complex formed by UmuD and UmuC proteins may interact with DNA lesions, which may interfere with DNA replication, and the processing of the UmuD protein by
RecA* may be required for the error-prone replication to proceed past the lesions. The initial complex containing the nascent UmuD protein may be harmful to the cell, since overproduced UmuD,C proteins inhibit DNA replication (17). This model may be essentially an extension of the one proposed by Blanco et al. (34), and it is consistent with the one proposed by Bridge and Woodgate (35).

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