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Identification and Characterization of a RecA-like Protein Induced by DNA Damaging Agents in Fluorescent *Pseudomonas* sp.

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A RecA-like protein (RecAps) was identified from fluorescent *Pseudomonas* sp. and the inducible nature of the protein was characterized in detail. It was shown by dose-response and time-course experiments using two DNA damaging agents, nalidixic acid and mitomycin-C, that the cellular level of RecAps protein was increased 3-8 fold compared to that of the control. The most effective doses of nalidixic acid and mitomycin-C for the protein induction were 30 μg/ml and 0.3 μg/ml at the treatment time point of 150 min, respectively. The enhanced level of RecAps protein was gradually decreased to the control level after 10 hr in normal medium. Interestingly, the cellular level of RecAps protein was increased by the same DNA damaging agents even when cell growth was completely inhibited by treatment with 170 μg/ml of chloramphenicol, an inhibitor of protein synthesis, suggesting that new protein synthesis is not required for the induction of RecAps. All these results suggest that a typical SOS repair function driven by RecA-like protein is conserved in *Pseudomonas* sp. cells as in *E. coli*.

Homologous recombination is an important process for DNA repair, generation of genetic diversity, and cell cycle-dependent events in cells (Walker, 1984; Cox and Lehman, 1987). General steps in homologous recombination involve bringing together two homologous DNA molecules, matching for the homology, and then exchanging the DNA strands between them (Sandler et al., 1996). In *Escherichia coli* (*E. coli*), this homologous recombination is centrally performed by *recA* gene product, RecA protein. In *in vitro* system, RecA protein was shown to make ATP-RecA-single-stranded DNA (ssDNA) filaments, form joint molecules between ssDNA and homologous double-stranded DNA, and then promote branch migration accompanied with ATP hydrolysis (Kowalczykowski and Eggleston, 1994; Friedmanohana and Cohen, 1998). RecA protein *in vivo* has been known to play an essential role in genetic recombination, DNA repair and coprotease activity in response to DNA damage thereby resulting in SOS response, prophage induction and LexA cleavage (Cox and Lehman, 1987; Kowalczykowski and Eggleston, 1994). The *recA* gene is highly conserved in bacteria, eukarya (Benson et al., 1994; Bishop, 1994) and even in archaea (Sandler et al., 1996; Rashid et al., 1997). Currently, approximately 65 *recA* genes from different bacteria have been cloned and sequenced (Sandler et al., 1996). Phylogenetic analysis of the bacterial *recA* genes has also been achieved (Lloyd and Sharp, 1993; Karlin et al., 1995).

A number of bacteria have shown to induce physiological responses or new proteins or both in response to UV-light and 4-nitroquinoline-1-oxide which both induce the SOS response in *E. coli* (Lovett et al., 1994; Ewing, 1995). Eukaryotic organisms also seem to show similar responses that are induced by the same agents which operate the SOS system in *E. coli*. DMC1, Rad51, and Rad57 proteins in *Saccharomyces cerevisiae* (*S. cerevisiae*) are like RecA types related to the *E. coli* RecA protein (Angulo et al., 1985, 1989; Bishop et al., 1992; Shinohara et al., 1992; Story et al., 1993; New et al., 1998). The *S. cerevisiae* RecA-like proteins are involved not only in the recombination event but also in the repair of DNA damage (Shinohara et al., 1992; Story et al., 1993). In addition, other RecA-like proteins, Rad51 homologues, have been isolated and characterized from chicken, mouse and human cells (Bezzubova et al., 1993; Yoshimura et al., 1993; Takahashi et al., 1994; Cartwright et al., 1998). These investigations suggest that the structure and/or the function of RecA protein may be conserved throughout prokaryotes and

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eukaryotes as well (Bishop et al., 1992; Ogawa et al., 1993; Story et al., 1993).

Although much is investigated about the role of RecA protein in DNA repair and recombination, little is known about the inducible nature of the protein after treatment with DNA damaging agents in bacteria. Therefore, the present study has been performed to examine the possible existence of SOS response similar to that of *E. coli* in *Pseudomonas* and also to characterize the inducible nature of the RecA-like protein of fluorescent *Pseudomonas* sp. YP cells against DNA damaging agents.

Materials and Methods

Bacterial strains and growth conditions

E. coli strains HH49 (*△lacU169 proA⁺ lon araD139 rpsL supF △(srlR-recA)*306::Tn10 (pMC9)) and AB1157 (F⁻ *thr1 leu6 proA his4 thi1 argE3 lacY galK2 ara14 xyl15 mtl1 tsx33*) were kindly provided by Dr. Jeffrey W. Roberts (Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853, U.S.A.). *Pseudomonas* sp. YP cells were obtained from Dr. Y. Park (Department of Biological Science, Chosun University, Kwangju 501-759, Korea). All culture supplies were purchased from Difco Laboratories. *E. coli* cells were grown in Luria-Bertani (LB) medium (1% Bacto-tryptone, 1% NaCl, 0.5% yeast extract) at 37℃ with vigorous shaking. *Pseudomonas* cells were cultured in King's B medium as described previously (Kho et al., 1995).

Chemical reagents and antibodies

Acrylamide, chloramphenicol, nalidixic acid (NA), and mitomycin-C (MMC) were purchased from Aldrich. Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Sigma. The polyclonal rabbit anti-*E. coli* RecA antibodies were prepared as described previously (Lee et al., 1994). The secondary antibody and nitrocellulose paper used in Western blot analysis were purchased from Hyclone and Millipore, respectively.

Preparation of protein sample used for SDS-polyacrylamide gel electrophoresis

Fifty microliters of Laemmli lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2.0 mM EDTA, 15% sucrose, 10% glycerol, 3% SDS, 0.7 M 2-mercaptoethanol, 0.01% bromophenol blue) were added to 10^8 cells and boiled for 5 min. After centrifugation at 12,000 rpm for 10 min in a micro-centrifuge, the supernatant was collected and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Polyacrylamide gel electrophoresis and Western blot analysis

SDS-PAGE was carried out as described by Laemmli (1970). The proteins fractionated on SDS-PAGE were electro-transferred to a nitrocellulose (NC) filter (Millipore Co., HAHY type, 0.45 μm in pore size) in a transfer buffer (20 mM Tris-base, 150 mM glycine) at 45 V for 2 h as described by Towbin et al. (1979). The NC filter was washed briefly with TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) and then incubated in TBS containing 3% non-fat milk for 2 h with gentle agitation. The *E. coli* RecA antiserum was added at a dilution of 1 : 500 and incubation was continued for another 2 h with agitation. The blots were incubated with a 1 : 2,500 dilution of anti-rabbit IgG coupled to alkaline phosphatase (Hyclone) in TBS containing 3% non-fat milk for 1 h at room temperature. The blots were then rinsed twice as before and stained with AP solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$) containing 165 μg/ml of BCIP and 330 μg/ml of NBT. The band intensities on the blot were measured by a densitometric scan.

Results

Identification of a RecA-like protein (RecAps) from Pseudomonas *sp. cells by Western blot analysis*

When *E. coli* AB1157 (*recA⁺*) cells were treated with 0.3 μg/ml of mitomycin-C for 150 min, a 38 kDa RecA protein was induced by the DNA damaging agent as expected (Fig. 1A). However, as shown in the same figure, *E. coli* HH49 cell (*recA*-deficient mutant) contained no RecA protein. When *Pseudomonas* cells were treated with the same dose of mitomycin-C for 150 min (Fig. 1A), a 42 kDa RecA-like protein was also detected on Western blot probed with polyclonal antibodies against the *E. coli* RecA protein. With preimmune serum, no protein was detected from the cell extract of *Pseudomonas* (data not shown). As shown in Fig. 1B, the RecAps protein was also induced in response to another DNA damaging agent, nalidixic acid. When *E. coli* AB1157 and *Pseudomonas* cells were treated with 30 μg/ml of nalidixic acid for 150 min, the *E. coli* RecA and the RecAps proteins increased up to 9.5- and 6.5-fold, respectively. These results suggest that a RecA-like protein exists in *Pseudomonas* sp. PY cells as in *E. coli* (Cox and Lehman, 1987; Lovett et al., 1994; Kowalczykowski and Eggleston, 1994).

The inducible nature of RecAps protein from Pseudomonas sp. cells by DNA damaging agents

In order to further characterize the inducible nature of *Pseudomonas* RecA-like protein (RecAps) by DNA damaging agents, dose-response and time-course experiments were carried out (Figs. 2 and 3). To analyze the dose-dependent inducibility of the RecAps protein by nalidixic acid and mitomycin-C in *Pseudomonas* cells, cells were grown to an exponential growth phase and treated with 1 to 70 μg/ml of nalidixic acid or 0.01 to

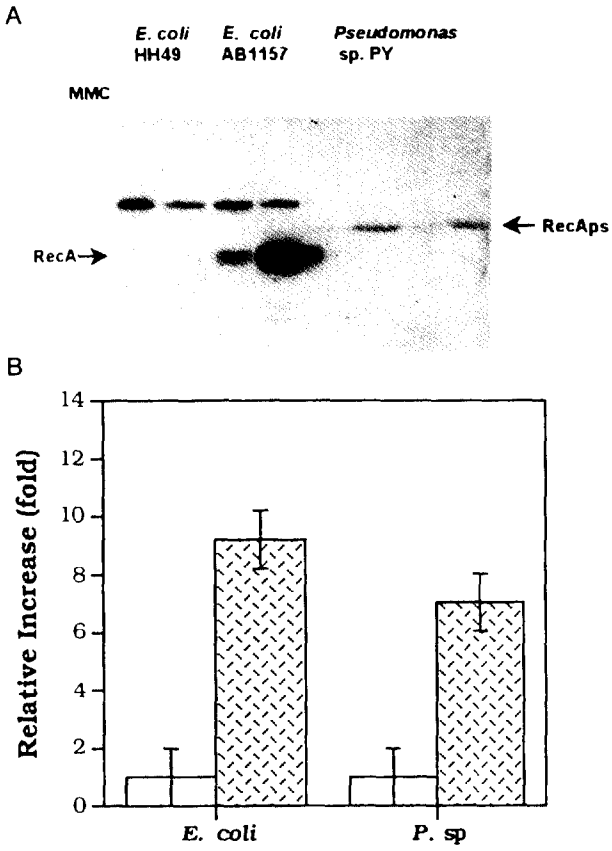

Fig. 1. Detection of a RecA-like protein (RecAps) from *Pseudomonas* sp. (*P.* sp.) cells. The RecAps in *P.* sp. was identified by immunological cross-reactivity between the *P.* sp. and *E. coli* RecA proteins (A) and the induction patters by nalidixic acid of *E. coli* RecA and *Pseudomonas* RecAps proteins was compared (B). In the upper panel, *E. coli* HH49 (*recA*$^-$), *E. coli* AB1157 (*recA*$^+$), and *Pseudomonas* cells were mock-treated (-) or treated (+) with 0.3 μg/ml of mitomycin-C (MMC) for 150 min. In lower panel, *E. coli* and *P.* sp. cells were treated with 30 μg/ml of nalidixic acid (NA) for 150 min. Cellular extracts were prepared and analyzed by Western blotting with antiserum raised against the *E. coli* RecA protein. The relative increases of RecA protein by NA (▨) were expressed as fold increase compared to untreated control value (□) after densitometric scanning. The four bands appeared from *E. coli* cell extracts are non-specific ones recognized by polyclonal anti-*E. coli* RecA antibodies.

0.7 μg/ml of mitomycin-C for 150 min. It was shown by Western blot analysis with *E. coli* anti-RecA antibody that the cellular level of RecAps protein was dose-dependently increased by nalidixic acid (Fig. 2A) and mitomycin-C treatments (Fig. 2B). However, when more than 40 μg/ml of nalidixic acid and 0.4 μg/ml of mitomycin-C were treated for 150 min, the induction of RecAps protein was rather inhibited probably due to the cytotoxicity of these chemicals. From these dose-response experiments, it was decided that the most effective doses of nalidixic acid and mitomycin-C for the RecAps protein induction were 30 μg/ml and 0.3 μg/ml, respectively.

Time-dependent accumulation and decreasing decline patterns of RecAps protein by DNA damaging agents in Pseudomonas sp. cells

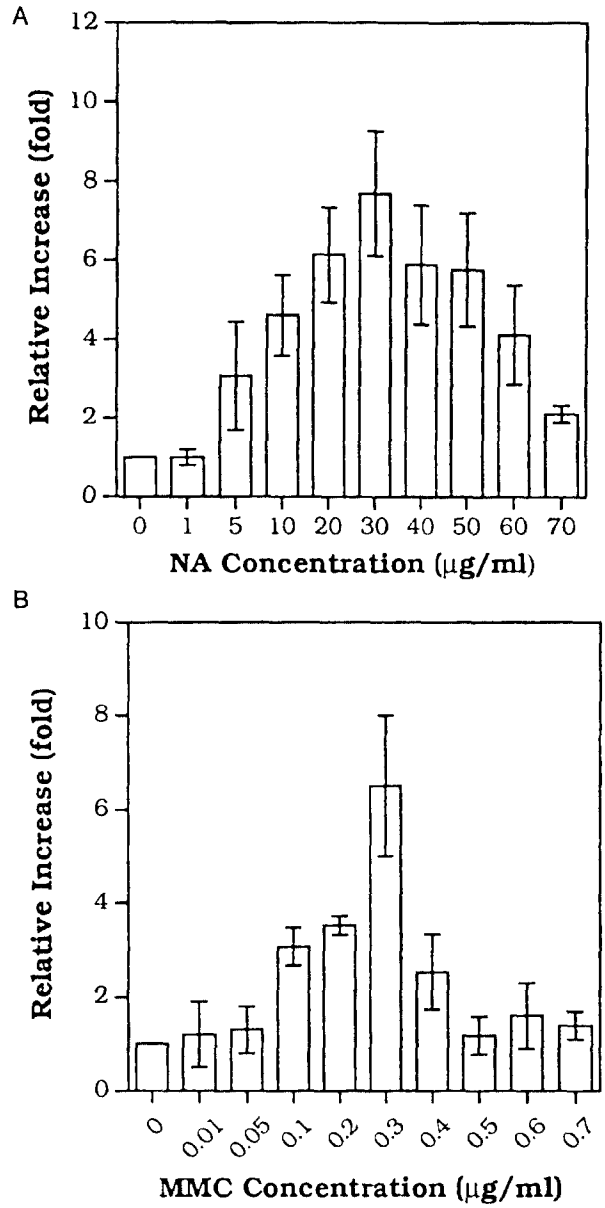

Fig. 2. The inducibility of RecAps protein of *Pseudomonas* sp. by nalidixic acid (NA) and mitomycin-C (MMC). *Pseudomonas* cells grown to an exponential phase were treated with 0 to 70 μg/ml of NA (A) or with 0 to 0.7 μg/ml of MMC (B) for 150 min. Cell lysates were prepared and Western blot analyses were performed with *E. coli* anti-RecA antibody. The relative increases of RecA protein were expressed as fold increase compared to untreated control value after densitometric scanning.

In addition, to examine the time-dependent induction of the RecAps protein against DNA damaging agents, cells were challenged with 30 μg/ml of nalidixic acid or 0.3 μg/ml of mitomycin-C for various periods and Western blot analysis was performed (Fig. 3). The *Pseudomonas* RecA-like protein was increased to maximum when cells were treated for 150 min with 30 μg/ml of nalidixic acid or 0.3 μg/ml of mitomycin-C (Fig. 3A). To examine the persistance of the induced RecAps protein, cells which were treated with 30 μg/ml

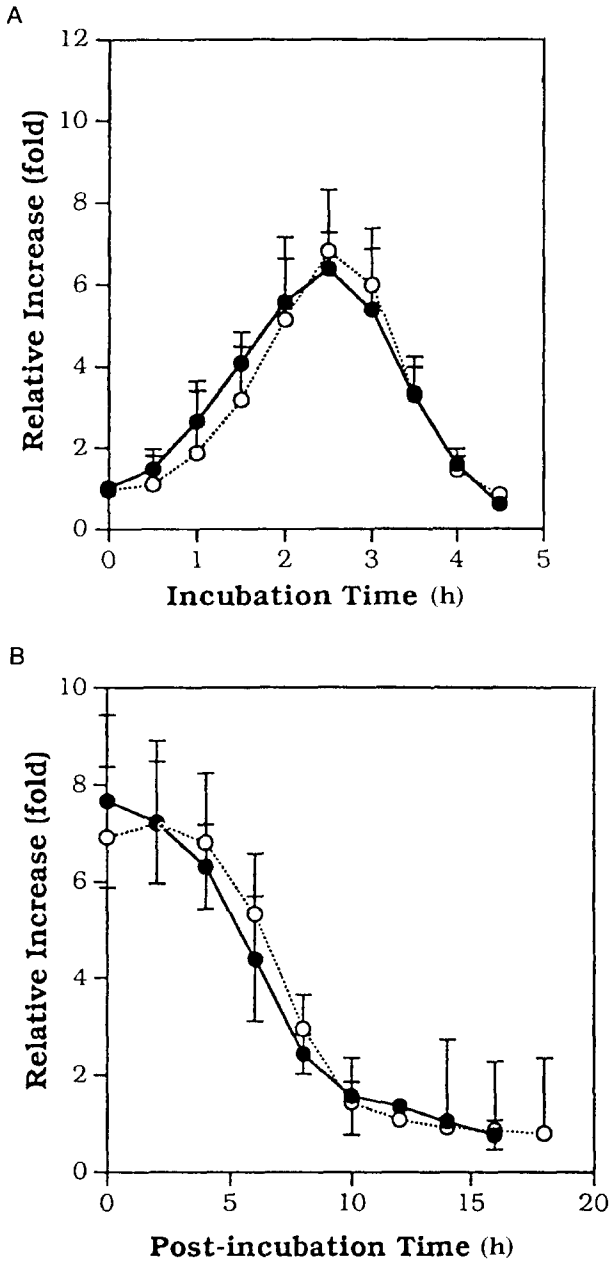

Fig. 3. Time-dependent accumulation and decline of the RecAps protein by nalidixic acid (NA) and mitomycin-C (MMC) in *Pseudomonas* sp. cells. In time-course experiments (A), *Pseudomonas* cells were grown to an exponential phase and treated with 30 μg/ml of NA (○) or 0.3 μg/ml of MMC (●) for the indicated times. In the post-incubation experiments (B), cells were treated with 30 μg/ml of NA (●) or 0.3 μg/ml of MMC (○) for 150 min. After the treatment, cells were further incubated in normal medium for up to 18 hr. At the indicated time points, cells were removed and Western blot analysis was performed with rabbit anti-*E. coli* RecA antibody.

of nalidixic acid or 0.3 μg/ml of mitomycin-C for 150 min were further incubated in normal medium without the DNA damaging agents (Fig. 3B). After harvesting the cells at the indicated time, Western blot analysis was performed using *E. coli* anti-RecA antibody. The levels of RecAps protein induced by the two DNA

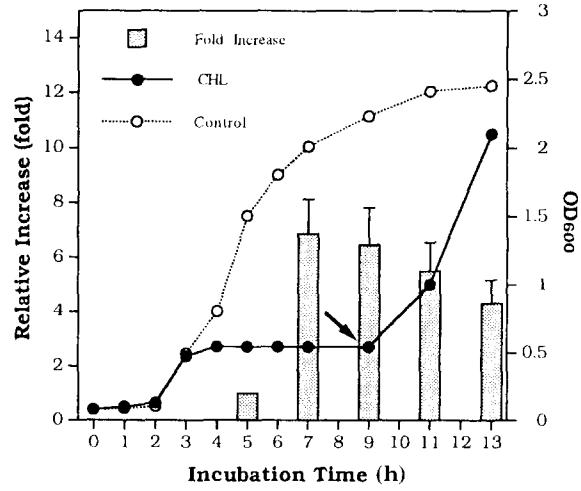

Fig. 4. Effect of chloramphenicol on the induction of RecAps protein of *Pseudomonas* by a DNA damaging agent. Cells were grown to a 0.5 value of optical density at 600 nm and then 170 μg/ml of chloramphenicol (CHL) was added to the culture medium directly. After 2 h (incubation time point of 5 h), 30 μg/ml of nalidixic acid was added for 150 min in the presence of chloramphenicol. Thereafter, cells were collected from the culture every hour and Western blot analysis was carried out. Growth curve of non-treated control cell (○) and cells treated with chloramphenicol (●). Histograms indicate the relative fold increase of RecAps protein of *Pseudomonas*. An arrow indicates time point of the removal of chloramphenicol.

damaging agents returned to a control level at the post-incubation time of 10 h (Fig. 3B).

Effect of chloramphenicol on the induction of RecAps protein by a DNA damaging agent

On the other hand, to determine whether the new protein synthesis is required for the induction of RecAps protein, *Pseudomonas* cells were cultured in a medium containing 170 μg/ml of chloramphenicol to stall cell growth by inhibiting normal protein synthesis. As shown in Fig. 4 (closed circle), cell growth was completely inhibited by the addition of the chloramphenicol. However, when chloramphenicol was removed from the medium, cell growth returned to the normal state. When 30 μg/ml of nalidixic acid was treated for 150 min even in the presence of chloramphenicol, the cellular level of RecAps protein was increased up to approximately 3-fold. This result strongly suggests that the RecAps protein is induced without new protein synthesis when the cell's DNA is damaged as in the case of other proteins related to SOS response (Lovett et al., 1993).

Discussion

The data presented in this study have demonstrated that a SOS-like response triggered by the RecAps protein is also conserved in fluorescent *Pseudomonas* sp. YP cells. The RecAps protein was induced by DNA damaging agents such as nalidixic acid, mitomycin-C (Figs. 1 and 2), and UV-light (data not shown).

The RecAps protein level was increased by DNA damaging agents within 30 min and the RecAps induction lasted for at least 10 h (Fig. 3). In addition, the RecAps protein was still inducible to the DNA damaging agent even when cell growth was suppressed by inhibiting normal protein synthesis (Fig. 3). All these properties are typical in RecA-like proteins controlling SOS response in bacteria (Cox and Lehman, 1987; Ewing, 1995; Rashid et al., 1997) and some eukaryotic cells (Angulo et al., 1985; Shinohara et al., 1992; Lovett et al., 1994).

It has been well established that *E. coli* RecA protein triggers SOS response by cleaving LexA repressor when it was activated by molecular signals generated by DNA damage or by inhibition of DNA replication (Cox and Lehman, 1987; Ennis et al., 1989; Lovett et al., 1994; Kowalczykowski and Eggleston, 1994). When SOS response is initiated by the activated RecA protein in *E. coli*, a number of damage-inducible genes (*din* genes) are expressed to mediate DNA repair and mutagenesis (Walker, 1984). Therefore, it would be interesting to know that the RecAps protein also has the same regulatory function to lead SOS response in *Pseudomonas* cells and the expression of *recA*-like gene encoding RecAps protein is also repressed by LexA-like protein in the normal state as in *E. coli*. If the RecAps protein has a function as a SOS initiator like the *E. coli* RecA protein, it would have coprotease activity to cleave the LexA-like protein.

In order to elucidate the RecAps protein function related to SOS response and homologous recombination the following experiments should be performed: (1) RecAps protein should be purified and characterized in detail in respect to its intrinsic protease activity to LexA protein, RecAps-ssDNA helical filament formation ability, joint-molecule formation activity, and strand exchange activity accompanied with ATP hydrolysis; (2) a *recA*-like gene encoding RecAps protein have to be cloned and analyzed whether the gene has structural similarity to other *recA* genes and also contains a SOS box in its 5'-flanking region on which the LexA protein can be bound (Rashid et al., 1996; Sandler et al., 1996).

In this laboratory, a putative *recA*-like gene encoding RecAps protein has already been cloned from *Pseudomonas* sp. cells. The *recA*-like gene of *Pseudomonas* is now under investigation to determine the expression pattern of the RNA transcript against DNA damaging agents and for the nucleotide sequence of the gene.

Acknowledgements

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