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Hydrolysis of the IciA protein, an inhibitor of DNA replication initiation, by protease Do in *Escherichia coli*

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The 33 kDa IciA protein, an inhibitor of replication initiation of the *Escherichia coli* chromosome, was found to be specifically cleaved to 27 kDa fragment by protease Do, the *htrA* gene product. The 27 kDa polypeptide could no longer interact with the *oriC* region, and therefore the cleavage-site is likely to reside within the N-terminal DNA-binding domain of the IciA protein. In addition, protease Do was found to localize primarily to the cytoplasm although it also could bind to membranes through an ionic interaction. These results suggest that intracellular breakdown of the IciA protein by protease Do may provide a potential mechanism involving the regulation of initiation of DNA replication in *Escherichia coli*.

IciA protein; DNA replication inhibitor; *oriC* DNA; Protease Do

1. INTRODUCTION

The IciA protein, a dimer of 33 kDa subunits, binds specifically to the *oriC* region that contains three 13-mers in tandem, the opening of which is the main event that must precede the onset of replication of the *E. coli* chromosome [1,2]. This binding blocks the opening and limits action of the key initiator DnaA protein. Therefore, the IciA protein has been suggested to play a critical role in the regulation of initiation of replication in *E. coli* chromosome, the key stage in the cell cycle [1,2]. Because critical, regulatory proteins generally have short half-lives and intracellular breakdown of these proteins is known to play important roles in regulation of metabolic and developmental circuits [3], identification of a protease(s) that may specifically hydrolyze the IciA protein should be of interest in further understanding the regulatory mechanism.

Protease Do is a serine endoprotease with an unusual high molecular mass of about 550 kDa [4]. This enzyme consists of identical 10–12 subunits of 51 kDa, which in part are processed to smaller polypeptides with 46 and 48 kDa sizes [4]. Protease Do has been shown to distinctly hydrolyze the purified Ada protein and therefore suggested to involve in the regulation of the adaptive response to alkylating agents [5,6]. In addition, it has recently been shown that the enzyme is identical to the *htrA* gene product and disruption of the gene results in a decrease in the overall rate of intracellular proteolysis and the loss of the cell's viability at high temperatures [7–9]. Therefore, it has also been suggested that protease Do may play an important role in intracellular protein breakdown and is essential for survival at high temperatures [7–9].

In an attempt to clarify further the physiological functions of protease Do, we tested whether the protease can hydrolyze the purified IciA protein. In the present study, we show that the IciA protein is specifically cleaved into 27 kDa fragment by protease Do but not by other soluble proteases in *E. coli* [10–12], including the ATP-dependent proteases La and Ti [13,14]. We also show that the cleavage-product of the IciA protein can no longer bind to the *oriC* region containing the tandem 13-mer repeats.

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2. MATERIALS AND METHODS

2.1. *Materials*

E. coli strain JC7623, its null mutant for protease Do, and the cell harboring the protease Do gene in a multicopy plasmid [7] were grown to late-log phase in Luria broth. The cells were suspended in 10 mM Tris-HCl (pH 7.8) containing 5 mM $MgCl_2$ and disrupted by French pressing at 14,000 psi. The solutions were then centrifuged at 30,000 × *g* for 1 h, and the resulting supernatants, dialyzed against the same buffer, were referred to as the cell extracts. The IciA protein was purified from the extracts of an *E. coli* strain (MC1061) harboring the *iciA* gene in a multicopy plasmid, as described [15]. Antibodies against the IciA protein and protease Do were prepared by injecting the purified proteins into albino rabbits.

In order to purify the 27 kDa cleavage-product of the IciA protein, 100 μg of the protein was incubated overnight at 37°C with 50 μg of the purified protease Do, 50 mM Tris-HCl (pH 7.8), and 5 mM $MgCl_2$. The mixtures were then loaded on a DEAE-cellulose column

(0.5 × 1 cm) equilibrated with the same buffer. While protease Do was recovered in the unbound fractions, the 27 kDa fragments were obtained by eluting the column with 0.2 M NaCl.

2.2. *Assays*

Proteolysis was assayed by incubating the IciA protein at 30°C or 37°C for 1–4 h with appropriate amounts of proteases Do, 50 mM Tris-HCl (pH 8), and 5 mM $MgCl_2$. After the incubation, the samples were electrophoresed on 12% (w/v) polyacrylamide gels containing sodium dodecyl sulfate (SDS) [16]. The gels were then stained with Coomassie R250 or subjected to immunoblot analysis using anti-IciA antiserum. Gel-shift assay was performed using the ^{32}P-labelled DNA fragments (198 bp), that contain the three 13-mers of the *oriC* region, as described [1,15].

2.3. *Subcellular fractionation*

E. coli strain JC7623 was grown in Luria broth to mid-log phase and the subcellular fractions were prepared as described by Nossal and Heppel [17]. After obtaining the osmotic shock-fluid (periplasmic fraction), the cells were resuspended in 10 mM Tris-HCl (pH 7.8) and 5 mM $MgCl_2$ and disrupted by sonication. After removing the cell debris by centrifugation at 2,000 × *g* for 10 min, the supernatant was again centrifuged at 30,000 × *g* for 30 min. The resulting supernatant was referred to as cytoplasmic fraction. The pellet, which represents membrane fraction, was resuspended in the same buffer.

3. RESULTS AND DISCUSSION

In order to determine whether the IciA protein is sensitive to degradation by any soluble protease(s) in *E. coli*, the cell extracts were prepared and incubated with the purified protein. As shown in Fig. 1, more than 70% of the IciA protein was degraded in 2 h by the extract obtained from wild-type cells (lane b). This degradation reached to near completion when incubated with the extract from the cells overproducing protease Do (lane d). On the other hand, the extract from a null mutant for the protease degraded the IciA protein much less significantly than that from the wild-type cells (lane c). These results clearly suggest that protease Do is mainly responsible for the hydrolysis of the IciA protein.

Noteworth was the finding that the hydrolysis of the IciA protein by the extracts containing protease Do generates a 27 kDa product, that can be detected by Coomassie staining (Fig. 1A) but not by immunostaining with anti-IciA antiserum (Fig. 1B). To determine whether the 27 kDa polypeptide is indeed derived from limited proteolysis of the 33 kDa IciA protein, the purified protease Do was incubated with the protein. Fig. 2 shows that the 33 kDa IciA protein is distinctively cleaved to 27 kDa polypeptide (lane c), suggesting that the antigenic site may reside within the remaining 6 kDa fragment. The smaller fragment, however, could not be detected by either Coomassie staining or immunoblot analysis of the gels containing higher concentrations of polyacrylamide (data not shown). Therefore, it appears that protease Do may degrade further the 6 kDa product into oligopeptides and/or amino acids. On the other hand, little or no cleavage was observed by incubation of the IciA protein with other proteases purified from *E. coli* extracts [10–14], including proteases Re, Mi, Fa, So, La and Ti (data not shown).

The IciA protein has been shown to contain a helix-turn-helix motif near its N-terminus, that may be in-

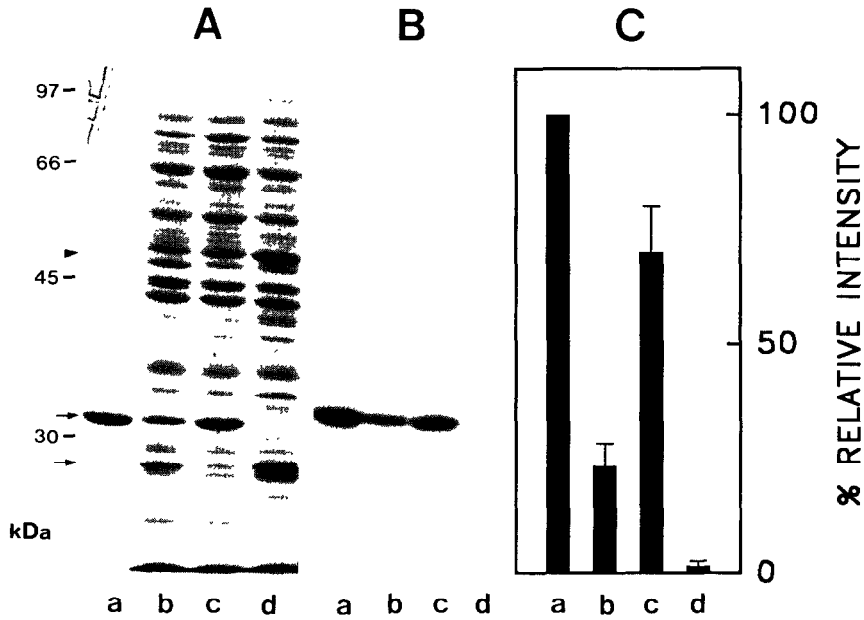

Fig. 1. Hydrolysis of the purified IciA protein by *E. coli* extracts. The purified IciA protein (4 μg each) was incubated alone (lane a) or with 50 μg of the extracts obtained from *E coli* strain JC7623 (b), its null mutant for protease Do (c), and the cell harboring the protease Do gene in a multicopy plasmid (d). After the incubation at 37°C for 2 h, the samples were electrophoresed on 12% slab gels containing SDS. Proteins in the gels were stained with Coomassie R250 (A) or transferred to a nitrocellulose membrane (B). The membrane was incubated with anti-IciA antiserum and then with ^{125}I-labelled protein A and autoradiographed. The bands in the autoradiogram were scanned using a densitometer (C) The triangle indicates protease Do, and the arrows show the IciA protein and its cleavage-product.

volved in specific DNA-binding with the *oriC* region [2]. To examine if the cleavage product of the IciA protein is still capable of binding to the DNA, the IciA protein was incubated with an excess of the protease and then with the ^{32}P-labelled *oriC* fragment and subjected to gel-shift assay. We also tested the DNA-binding of the purified 27 kDa polypeptide (see Fig. 2, lane d). As shown in Fig. 3, neither was able to interact with the DNA (lanes c–e) unlike the IciA protein that had been incubated under the same condition but in the absence of protease Do (lane b). Therefore, the cleavage site of the IciA protein seems to exist within the N-terminal DNA-binding domain, if indeed the helix-turn-helix motif is responsible for the binding to the *oriC* region.

The physiological role of the IciA hydrolysis, however, remains unclear. And yet it is tempting to speculate that the intracellular breakdown of the IciA protein by protease Do may play an important role in the control of the protein availability inside the cells and thereby in the regulation of replication initiation of the *E. coli* chromosome. For participation of protease Do in the possible, regulatory pathway, the enzyme should at least be localized to the cytoplasmic space of *E. coli*. Although protease Do was initially isolated from the soluble extract of the cells [4], it has also been reported that the enzyme (identified as DegP or HtrA) is a periplasmic or membrane protein and contains a putative signal sequence at its N-terminus for processing [8,9,18,19].

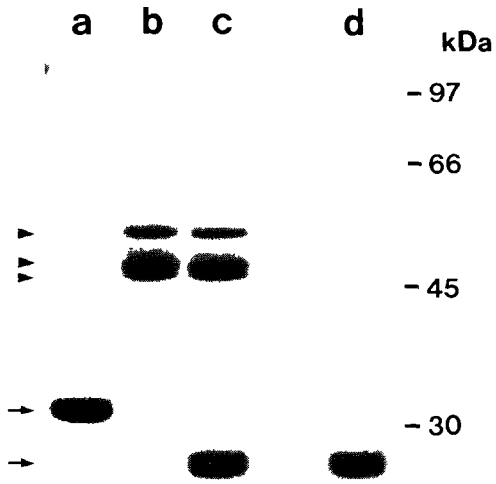

Fig. 2. Hydrolysis of the IciA protein by the purified protease Do. The IciA protein (4 μg each) was incubated at 37°C for 4 h in the absence (lane a) and presence of 2 μg of protease Do (c). The protease alone was also incubated as above (b) After the electrophoresis of the samples, proteins in the gels were stained with Coomassie R250. Lane (d) shows the purified 27 kDa cleavage-product of the IciA protein. The triangles indicate protease Do, and the arrows show the IciA protein and the 27 kDa product

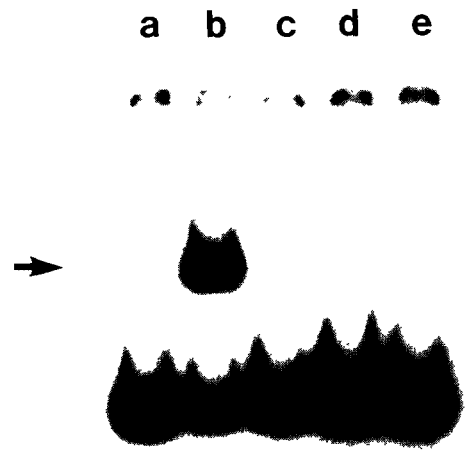

Fig. 3. Specific binding of the IciA protein and its cleavage-products to the DNA fragment containing the three 13-mers of the *oriC* region. The ^{32}P-labelled DNA fragments were incubated alone (lane a), with 5 ng of the IciA protein (b), with 5 ng of IciA previously incubated for 1 h at 37°C with 50 ng of protease Do (c), or with 5 (d) and 20 ng of the purified 27 kDa fragment (e). The incubations were performed at 30°C for 30 min, and the resulting samples were subjected to gel-shift assay. The arrow indicates the shifted DNA band.

To clarify these discrepant results, we performed the subcellular fractionation of protease Do. Fig. 4 shows that the cytosolic and membrane fractions contain more than 95% of the total enzyme (lanes c and d), suggesting that protease Do in the periplasmic fraction (lane b) may be from contamination of the other fractions. In addition, approximately half the amount of protease Do in the membrane fraction could be released into the soluble fraction by a simple washing of the membranes

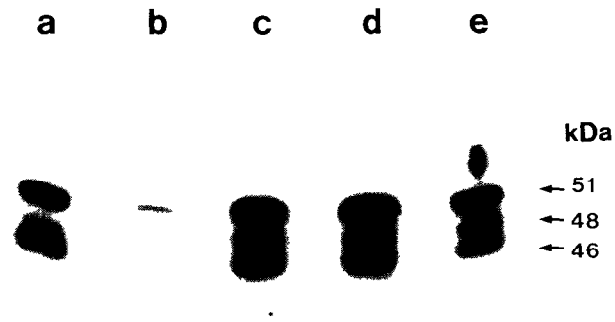

Fig. 4. Subcellular localization of protease Do *E. coli* strain JC7623 was grown to mid-log phase and its subcellular fractionation was performed as described in section 2. The resulting periplasmic (lane b), cytoplasmic (c) and membrane fractions (d) were concentrated to an equal volume (10 ml) by ultrafiltration. Aliquots (50 μl each) of the fractions were then electrophoresed in 10% slab gels in the presence of SDS and subjected to immunoblot analysis using anti-protease Do antiserum. The membrane fraction was washed once with the same volume of 0.2 M KCl, and its supernatant (50 μl) was treated as above (e). Lane (a) shows the purified protease Do.

with 0.2 M KCl (lane e). Therefore, protease Do appears to be capable of binding to membranes through an ionic interaction and hence can exist in both soluble and membrane-bound forms but may not be an integral protein. Of interest is the finding that both the cytosolic and membrane fractions contain the 46 and 48 kDa polypeptides as well as the 51 kDa subunits of protease Do. During purification the protease from the soluble extracts of *E. coli* (i.e., in the absence of membranous fraction), the 51 kDa proteins were found to be gradually degraded into the 46 and 48 kDa polypeptides (data not shown). Moreover, the prolonged storage of the purified enzyme preparation at 4°C also generated the smaller polypeptides from the 51 kDa subunits. Therefore, it appears likely that the 46 and 48 kDa polypeptides are generated by autolysis of the 51 kDa protease Do but not necessarily by processing of its putative N-terminal signal sequence [8,9] for transport or incorporation into membranes. However, mutational analysis is required for clarification of the role of the N-terminal hydrophobic sequence of the IciA protein.

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