Identification of a Penicillin-Sensitive Carboxypeptidase in the Cellular Slime Mold *Dictyostelium discoideum*

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Penicillin binding proteins (PBPs) are penicillin-sensitive DD-peptidases catalyzing the terminal stages of bacterial cell wall assembly. We identified a *Dictyostelium discoideum* gene that encodes a protein of 522 amino acids showing similarity to *Escherichia coli* PBP4. The *D. discoideum* protein conserves three consensus sequences (SXXK, SXN and KTG) that are responsible for the catalytic activities of PBPs. The gene product prepared in the cell-free translation system showed carboxypeptidase activity but the activity was not detected in the presence of penicillin G. These results demonstrate that the *D. discoideum* gene encodes a eukaryotic form of penicillin-sensitive carboxypeptidase.

Key words carboxypeptidase; *Dictyostelium discoideum*; penicillin binding protein

Penicillin binding proteins (PBPs) play a regulatory role in the terminal stages of bacterial cell wall assembly. Since PBPs are believed to be bacterial specific enzymes, the beta-lactam agents which inhibit the function of PBPs have been used as excellent antibiotics that specifically target bacteria. Twelve PBPs of *Escherichia coli* (PBPs 1a,^{1,2} 1b,^{1,2} 1c,³ 2,^{1,2} 3,^{1,2} 4,^{1,2} 5,^{1,2} 6,^{1,2} 7,⁴ DacD,⁵ AmpC⁶ and AmpH⁶) have been identified and analyzed. Among them, PBPs 4, 5, 6, and DacD have DD-carboxypeptidase activity.⁷ These enzymes cleave the terminal D-alanine from the peptide side chains of murein components.⁵ The considerable sequence identities and structural similarities among these proteins suggest that they diverged from an ancient DD-carboxypeptidase. We screened the genes coding for the DD-carboxypeptidase homologs in the genomic sequence databases to determine the diversity of the enzymes. In this process, we identified a gene encoding a protein homologous to *E. coli* PBP4 in the cellular slime mold *Dictyostelium discoideum*, a well characterized eukaryotic microorganism.⁸

We describe in this report the sequence of the *D. discoideum* gene and the carboxypeptidase activity of the gene product prepared in a cell-free translation system. The enzymatic activity of the gene product was inhibited by penicillin G, thus, the gene was named *pscA* (encoding a Penicillin-sensitive carboxypeptidase).

MATERIALS AND METHODS

Identification of the Gene The *pscA* gene was identified in Genomic Information for *Dictyostelium discoideum* (<http://www.biology.ucsd.edu/others/dsmith/dictydb.html>). cDNA for the gene was provided from the *Dictyostelium* cDNA project Japan (<http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html>).

In Vitro Translation cDNA for the gene was subcloned on pEU3-NII (Toyobo, Japan). The transcripts prepared from the construct were used as the template for the cell-free protein synthesis system PROTEIOSTM (Toyobo, Japan). Transcription and translation reactions were carried out according to the instruction manuals.

Carboxypeptidase Assay The hydrolysis of substrate by the protein was assayed as described previously.^{9–11} Briefly,

the reaction mixture (800 μ l) containing 50 mM Tris-HCl (pH 7.5), 5 mM ZnSO₄, 0.76 mM *O*-hippuryl-DL-beta-phenyl-lactic acid (Bachem, Switzerland), and 6 μ l of *in vitro* translation product was incubated at 20 °C. The enzymatic activity was detected as a change in absorbance at 254 nm.

RESULTS AND DISCUSSION

The nucleotide sequence of *pscA* is shown with the deduced amino acid sequence (Fig. 1A). Comparison of the nucleotide sequences between the genomic DNA and cDNA showed that the gene was not interrupted by an intron. The gene product consists of 522 amino acids. The N-terminal domain of the protein contains charged amino acids and a hydrophobic sequence assumed to function as a signal peptide. Within the C-terminal domain, there is a hydrophobic sequence that potentially functions as a membrane anchor. The presence of these sequences suggests that the gene product would be a membrane-anchored extracellular protein.

A computer search (BLAST Program) was conducted for proteins with amino acid homology to the predicted amino acid sequence of the *D. discoideum* protein. A close fit to the amino acid sequence of the *E. coli* PBP4 was found. The alignment of the *D. discoideum* protein and the bacterial protein is shown in Fig. 1B. These proteins conserve three sites, SXXK, SXN and KTG, that are responsible for the catalytic activities of the PBPs.^{12,13} These observations suggest that the *D. discoideum* protein may be a penicillin interactive carboxypeptidase.

The gene product was prepared in the *in vitro* translation system and its enzymatic activity was assayed by monitoring the hydrolysis of the substrate (*O*-hippuryl-DL-beta-phenyl-lactic acid). The translation product catalyzed the substrate in the absence of penicillin G but the activity was not detected in the presence of penicillin G (Fig. 2). This result indicates that the *D. discoideum* protein is a penicillin-sensitive carboxypeptidase.

Since *D. discoideum* amoebae do not have a peptidoglycan cell wall, the function of the *pscA* in this organism is not known. To determine the importance of the gene in *D. discoideum*, we established a *pscA* null mutant from the axenic strain Ax2. The procedures for the selection and maintenance

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A

TTTTTTTTTTTTTTTTTTCAATTCTGAATAAAAAA

ATGAAAAATTATAAAATATAACATTATTATTAATTATTTCATATTATTATTAATATTATTAGGTCAAATAAAATTTCTTTAAAGATAGTAGACGGAAGTAACGGTAATCAAGATATT
 1 M...K...N...Y...K...I...I...T...L...L...L...I...I...S I L F N I I R S N K I S L K D S D S G S N G N Q D I 40

CAAATTTTAATAAATGATATTTTAAATAATTGTTTCATCAAGTGAAGTAGTTCATGCTTTGGTACACAATGGGTGTTGTAGCAGATATTATACACCAAGTAATGGAGAATTTACAAAT
 41 Q I L I N D I L N N C S S S E S S S C F G T Q W G V V A D I Y T P S N G E F T N 80

ATTTTCAGTTTAAATGAATTACAAGCATTACACAGCAAGTAATACAAAATTTATTAACAACATAGTATTTTCTATACATTTGGTGAAGATTTCAAAGTTTACACCATTTTTCACA
 81 I F S L N E L Q A F T P A S N T K L F T T I S I F Y T F G E D F K V F T P F F T 120

GATAAACCATTTAATAGTGTAGTGGTGGTAGTAGTAATAGTGAATTAGATTTTCATTTGTGTCAAAGGTATGGGTGATCCATCAATGTCAATTGATAATTTAATTGAAGCAGCAAAATTC
 121 D K P F N S V S G G S S N S E L D F I C V K G M G D P S M S I D N L I E A A K F 160

TTTTCATCAAAATCCAAATGAAAAAGTAAATAAATTTATTTGGATACTAGCTTCTATAAATTTGGTAATGGAGTTGATGGTAATTTCCAAGTCTGGGAATGGGAAGATTTAACT
 161 F S S N P T M K K V N K L L L D T S F Y N I G N G V D G N I P S A W E W E D L T 200

TCAACCTATGGTTCATTTCCAACACCATTAATATCAATGAAAAACAATGGATATTTATATCACACCTTCCAATGTCAATTGGTGGTAAACCAACTGCATCATTTAAATATTCTGGTGAA
 201 S T Y G S I P T P L I I N E N T M D I Y I T P S N V I G G K P T A S F K Y S G E 240

GATAAATATTACCAGTTATAATATTAGCAACACCACCACCACAAGTAATAGTTCAACATCAACATTAACATTTTAAATGTCAAGTCAATCAATTTATATTACAGTAATTTGT
 241 D K Y L P V I I L A T T T T T S N S S T S T L N Y S F K M S S Q S I Y I T G N C 280

GATATTAATGGTGGGAATTCAAATTTACAGTACCAATTTTAGATCCTGAGCAATCTTTTAAACAGTATTTCTCAGCATTGTTGGAAGATGGTGGCGTTGAAATTAGTCAACAGCTATT
 281 D I N G G I Q I I T V P I L D P E Q Y F L T V F S A L L E D G G V E I S Q T A I 320

GGTAGTTGTAATTACACTGGTATGGATTATAAATCATTTGAAGTGATTTCAACCGAATTATCAGAAATGTTAAATTATACATTATTAACATCAAAATTTATATGCTGAACTTTTCTC
 321 G S C N Y T G M D Y K S F E V I S P E L S E M L N Y T L L T S N N L Y A E T F L 360

AGACAAATGGGCACTTTTAATTCAGCAGCTTCTGAATCAACACCAACTTATCAAGCTGGTTAGAAATATATTCAACAAACATTATCAATTTCAACATCATATATATACTCAAGTGGATGGT
 361 R Q M G T F N S A A S E S T P T Y Q A G L E Y I Q Q T L S I P T S L Y T Q V D G 400

TCTGGTTTAAAGTAGAAATAATTTATTACACCAAAATCATTAATAACAGTTATTGAAATGTTTATACAAATGTTGGTGATCCACAACATGACTATATCTCATATTACCCGTTGCATCA
 401 S G L S R N N F I T P K S L I T V I E N V Y T N V G D P Q H D Y I S Y L P V A S 440

TTATCAGGTACCTATCGAAACGTTTCATTAATACACCCGAGTGGTATAGTCCATGCAAAAAGTGGTCAATGACTGGTGTAAATTTCTCTAAGTGGTGTCAATTTACCAATGGTCTT
 441 L S G T L S K R F I N T P A S G I V H A K T G S M T G V N S L T G V I L P N G L 480

TCTGATGATCAACAAATTCATATTTCTTTTCAATAATGCAAAATAGTCTGCTCAAAATCTGATATAATTTGATATAATTTGACCAATTTGTAATTTTATTAATAAATTTATTTTA
 481 S D D Q Q N S I F F S I I A N N S P A Q N T D I I D I I D Q I V I L L T K E I L 520

TCATCTTAAAAAATAAATAAATAAATAAAGATAATTAATTTTGT
 521 ...S...S * 522

B

PscA MNYIITLLIISILFNIIIRSNKISLKDSGSGNGNQDITGLINDILNNCSSSESSCFGTQWVADIYTPSNGEITNIESLAECAATTPASNTKIDTTTISIYTGDFVFTPFET 120
 PBP4 MFFS-----SII-----GLTSCIASQAANN--DEY-ITQLPA--SAILANVQKVGASAPAI--D--HSSQ--AP--PASTCKVITAAAIQIGPDF--FTTTIE 87

PscA DLPFNSVSGSSNSLDFICVKGMGDPNSIDNIEAAKFSNPTKKKN--KELDTSTFNIGNVGDNIPSNEMEDTSTVGSITPLIINNTMDIIPSNVISCKPTSSKMSG 239
 PBP4 TR--GNVENVLKGDI--VARFG--ADPTIKRQDERNMVATL--KKSGLNQDGNMLDTSIA-SH--D--KAE--GTPANDITQC--SAPPAATV--L--RNCE--SVSLYSAPKP--GDAGIR 191

PscA EDKLEPVITATTTTSNSSTSTLNYSK--SSQ--SIITGNODI--GGIQ--ITSPILPEQYFLT--SALLEDSCEISQ--AGSCNYTGMDYKSFVISPESMLNYTLTSSNEYA 356
 PBP4 VASYVPYTNISQVRILPRGSAEAQYCE--DV--PGDLNRITLTGLP--RSEPP--PA--A--Q--GASYAGAT--KDE--KQ--GCTWSGTL--RQT--VNEPGTVVASKQSAPLHMLKIMLKSDNEIA 311

PscA FTTFRQGTFFN--SAASESTPTYQAGLEY--QCTLS--PTSLYTQVDSGLSRNNITTKSLTIV--ENVNTVNGVQPHDYISYLVASLSCLSKR--LITPASGIVHAKTGS--TGVSNTLG 473
 PBP4 FTTFRMIGHARFNVPGTWRAGSDAVRQIERQAG--DIGNTI--ADGSLSRNNITTKSLTIV--ENVNTVNGVQPHDYISYLVASLSCLSKR--LITPASGIVHAKTGS--TGVSNTLG 429

PscA VILPGLSDDQNSIF--SIITANNAPANTDID--DQIVILLTK--ILSS 522
 PBP4 VITTTAS--GQRMFAVQY--SGYAVEPADCRNRR--PLVRFESRIYKD--YQNN 477

Fig. 1. Sequence Analysis of the Gene

(A) Nucleotide sequence of the *D. discoideum* gene with the deduced amino acid sequence. Three sites conserved in bacterial PBPs are indicated by dots. The potential signal sequence and anchor domain are indicated by dashed lines. The potential poly (A) signals are underlined. (B) Comparison of the amino acid sequences of the *D. discoideum* protein and *E. coli* PBP4. Identical amino acid residues are shown on the black background. Chemically similar groups of amino acids considered to be (A, G), (S, T), (N, Q), (D, E), (H, K, R), (F, W, Y) and (F, I, L, M, V) are shown on the gray background. The 3 conserved sites are indicated by dots.

Fig. 2. Carboxypeptidase Activity of the *D. discoideum* Protein

Hydrolysis of substrate by the *in vitro* translated *D. discoideum* protein was assayed in the presence (7.6 mM) or absence of penicillin G. The change in absorbance at 254 nm of the reaction mixture was monitored.

of the null mutant, in which the gene was disrupted by the insertion of the blasticidin S resistance gene cassette, were the same as described in a previous report.¹⁴ Two independent clones grew normally in the liquid medium, demonstrating that *pscA* is not essential for cell growth (our observation). Further analysis is currently in progress.

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