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The Glu Residue in the Conserved Asn-Glu-Pro Sequence of Endoglycoceramidase Is Essential for Enzymatic Activity

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Endoglycoceramidase (EGCase) is an enzyme capable of cleaving the glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids. We previously cloned the gene encoding EGCase II of *Rhodococcus* sp. M-777 and reported that the deduced amino acid sequence contained the Asn-Glu-Pro (NEP) sequence, conserved as part of the active site of family A cellulases (endo-1,4-β-glucanases) (*J. Biol. Chem.* 272, 19846, 1997). The NEP sequence was also found in the deduced amino acid sequence of the newly cloned EGCase gene of *Rhodococcus* sp. C9. Replacement of the Glu residue in the NEP sequence with Gln or Asp by site-directed mutagenesis caused marked loss of enzymatic activity in both the M-777 and C9 EGCases but did not affect the expression of EGCase protein. This result clearly indicated that the NEP sequence is part of the active site of EGCase, in which the Glu residue plays an important role in the catalytic reaction, possibly in the same manner as in endo-1,4-β-glucanase.

Glycosphingolipids, amphipathic compounds consisting of oligosaccharide and ceramide (Cer) moieties, have been defined as tumor antigens, receptors for microbes and their toxins, and possible modulators of various cellular activities (1). Recently, glycosphingolipids were found to be enriched with cholesterol and GPI-anchor proteins to form microdomains on the plasma membrane of vertebrates (2). Endoglycoceramidase (EGCase: EC 3.2.1.123; also known as Cer glycanase) cleaves the glycosidic linkage between oligosaccharides and Cers of various glycosphingolipids. The enzyme has been reported to be expressed in actinomycetes (3), bacteria (4), leech (5), earthworm (6), clam (7) and rabbit mammary tissue (8). Three isoforms of EGCase (EGCase I, II, and III) differing in molecular weight, pI and substrate specificity, were found in *Rhodococcus* sp. M-777 (9). Recently, the gene encoding EGCase II of *Rhodococcus* sp. M-777 was cloned and sequenced (10). Interestingly, part of the amino acid sequence of EGCase II was homologous to that of the active site region of cellulase (endo-1,4-β-glucanase: EC3.2.1.4), which belongs to family A. In addition, the Asn-Glu-Pro (NEP) sequence, the active site of family A cellulases (11–13), was also conserved in this region of EGCase II (10).

We report here that the NEP sequence is part of the active site of EGCases from *Rhodococcus* sp. M-777 and the newly isolated strain C9, and that the Glu residue in the NEP sequence plays an important role in its enzymatic activity.

MATERIALS AND METHODS

Materials. *Escherichia coli* strain JM109 and expression vector pTV118N were obtained from Takara Shuzo, Co., Japan. TLC plates (Silica Gel 60) were purchased from Merck. Anti-EGCase II antibody was prepared by immunization of a rabbit with purified EGCase II of Rhodococcus sp. M-777, and anti-rabbit IgG-HRP antibody was obtained from Santa Cruz Biotechnology.

Strains. EGCase-producing actinomycetes *Rhodococcus* sp. M-777 was previously isolated from a soil sample (3) and maintained in our laboratory. Strain C9 was newly isolated from a soil sample (K. Sakaguchi, N. Okino, N. Sueyoshi, H. Izu, and M. Ito, unpublished). Strain C9 was assigned to the genus *Rhodococcus* on the results of basis of morphological, biochemical and 16S rDNA analyses.

DNA sequence analysis. Nucleotide sequence analyses were performed using a Big Dye Terminator Ready Reaction Kit (Applied Biosystems) and a DNA sequencer (Applied Biosystems, model 377

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Abbreviations: Cer, ceramide; EGCase, endoglycoceramidase; GM1, Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1,1′Cer; HRP; horseradish peroxidase; LA-PCR, long and accurate polymerase chain reaction; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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FIG. 1. Comparison of amino acid sequences of EGCases and family A cellulases (endo-1,4-β-glucanases). The residues conserved in the active site region are shown in bold type. The Asn-Glu-Pro (NEP) sequence conserved in family A cellulases and EGCases is boxed, and the active centers of cellulases are indicated by an *asterisk* (*). References for sequences used are: Bp, *Bacillus polymyxa* (20); Bs, *Bacillus subtilis* (21); Ra, *Ruminococcus albus* (22); Ct, *Clostridium thermocellum* (23, 24); eg, endo-1,4-β-glucanase.

A). Computer analysis was performed with DNASIS (Hitachi Software Engineering) and GENETYX software (Software Development). The phylogenetic relationship of 16S rDNA was analyzed using Clustal W software (14).

Site-directed mutagenesis. Site-specific mutagenesis was carried out by the oligonucleotide-directed dual amber (15) and LA-PCR technique (16) with a Mutan-Super Express Km kit (Takara Shuzo, Co., Japan) and a 5′-phosphorylated oligonucleotide containing a non-pairing nucleotide at its center as follows: PCD3, 5′-TGATGA-ACGATCCGTGGGGC-3′; PCQ1, 5′-CTGATGAACCAGCCGTGGG-3′; PMD1, 5′-GATGAACGATCCGTTCGGAGG-3′; PMQ1, 5′-CTGATG-AACCAGCCGTTCGG-3′. LA-PCR for site-directed mutagenesis was performed using these primers and expression vectors pTEC6 (for C9) and pTEG3 (for M-777) (10) as the template. Other aspects of the protocol were performed according to the manufacturer's procedures.

Expression of wild-type and mutant EGCases. JM109 cells were infected with wild-type and mutant EGCase genes and grown at 37°C in 100 ml of Luria-Bertani medium containing 100 μg/ml ampicillin until O.D. at 600 nm reached 0.5. Then, isopropylthio-β-D-galactoside was added to a final concentration of 1 mM to induce transcription, and cells were cultured at 37°C for an additional 4 h. Cells were harvested by centrifugation, resuspended in 2.5 ml of lysis buffer (10 mM Tris–HCl, pH 8.0, containing 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride), and sonicated for 4 min. Cell debris was removed by centrifugation (6,000 $\times$ g for 15 min). The obtained supernatants were dialyzed for 12 h against 20 mM sodium acetate buffer, pH 6.0, and then used as crude enzyme preparations.

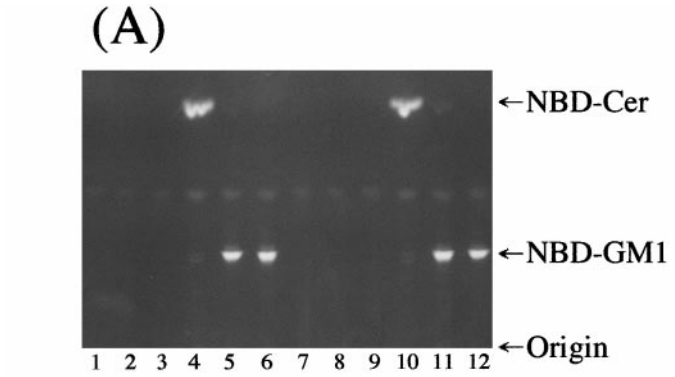
Assays for EGCase activity and protein. The activities of wild-type and mutant EGCases were examined using NBD-GM1 as the substrate as described below. NBD-GM1 was prepared by the method as described in (17). The reaction mixture contained 100 pmol of NBD-GM1 and an appropriate amount of enzyme in 20 μl of 20 mM acetate buffer, pH 5.0, containing 0.2% Triton X-100. Following incubation at 37°C for the times indicated, the reaction was stopped by heating in a boiling water bath for 5 min. The reaction mixture was evaporated with a Speed Vac concentrator (Savant Instruments, Inc.), dissolved in 15 μl of chloroform/methanol (2/1, v/v), and applied to TLC plates which were then developed with chloroform/methanol/0.02% $CaCl_2$ (5/4/1, v/v). The bands of NBD-Cer released by the action of the enzyme and unhydrolyzed NBD-GM1 were quantified with a Shimadzu CS-9300 chromatoscanner (excitation 470 nm, emission 525 nm). The extent of hydrolysis was calculated as follows; hydrolysis (%) = (peak area for NBD-Cer produced) $\times$ 100/(peak area for unhydrolyzed NBD-GM1 + peak area for NBD-Cer produced). Protein content was determined by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard.

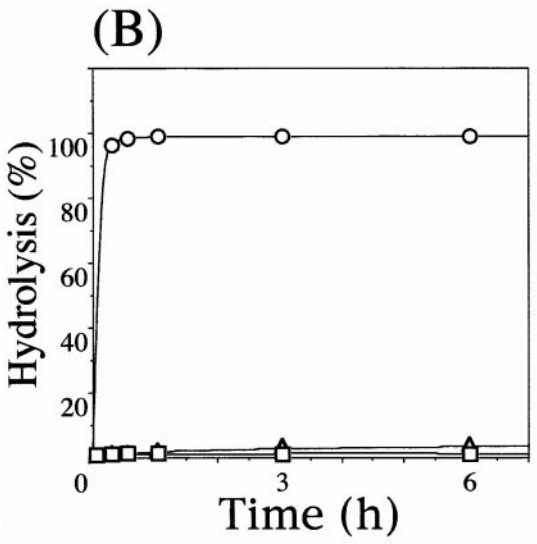
Western blotting. Soluble proteins from transfected *E. coli* JM109 were separated by 10% SDS–PAGE according to the method of Laemmli (18). After electrophoresis, the proteins on the gel were transferred onto a nitrocellulose membrane with a semi-dry blotter. The membrane was then incubated at room temperature with rabbit anti-M-777 EGCase II polyclonal antibody for 60 min. The band corresponding to EGCase was visualized with anti-rabbit IgG-HRP antibody and 4-chloro-1-naphthol as the substrate.

RESULTS AND DISCUSSION

We reported previously that part of amino acid sequence deduced from EGCase II gene of *Rhodococcus* sp. M-777 was homologous to that of the active site region of family A cellulases (endo-1,4-β-glucanases) (10). The Asn-Glu-Pro (NEP) sequence, which is commonly conserved in endo-1,4-β-glucanases, was also found in the M-777 enzyme (10). The Glu residue in the NEP sequence was demonstrated to be the active site of endo-1,4-β-glucanases (11–13). Thus, we examined whether the Glu residue in the NEP sequence of EGCase is crucial for the enzyme activity using M-777 and newly cloned C9 EGCase genes. The C9 EGCase gene was cloned from the genomic library of actinomycetes strain C9, which was assigned to genus *Rhodococcus* but was clearly shown to be different from strain M-777 by 16S rDNA-based phylogenetic analysis. The C9 EGCase gene possessed an open reading frame of 1446 bp encoding 482 amino acids, and showed 78% and 76% homology to M-777 EGCase at the nucleotide and amino acid levels, respectively (K. Sakaguchi, N. Okino, N. Sueyoshi, H. Izu, and M. Ito, unpublished). Interestingly, the NEP sequence was conserved in not only M-777 but also in C9 EGCase (Fig. 1).

Mutations were introduced into the Glu residue in the NEP sequence of both EGCases by site-directed mutagensis. Four mutant EGCase genes were thus constructed and designated as follows: CED (C9 EGCase, Glu224 was changed to Asp), CEQ (C9 EGCase, Glu224Gln), MED (M-777 EGCase II, Glu233Asp) and MEQ (M-777 EGCase II, Glu233Gln). Mutation was confirmed by DNA sequencing using appropriate prim-





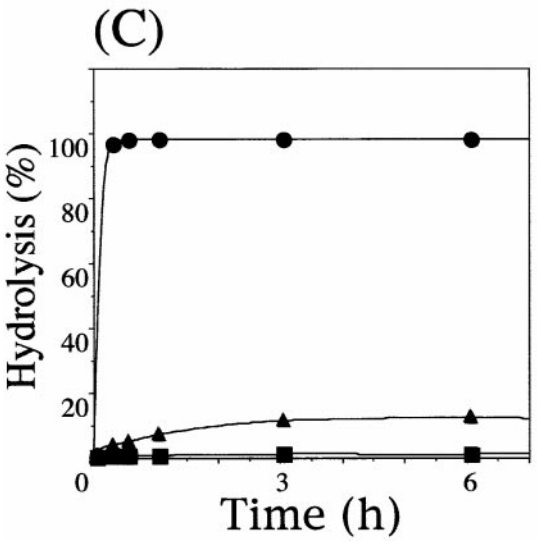


FIG. 2. EGCase activities of wild-type and mutant enzymes. Crude enzymes (70 μg protein) were incubated with 100 pmol of NBD-GM1 in 20 μl of 20 mM sodium acetate buffer, pH 5.0, containing 0.2% Triton X-100 for 16 h (A) or the times indicated (B). The hydrolysis of NBD-GM1 was determined by the method described in Materials and Methods. (A), TLC showing the NBD-Cer released from NBD-GM1 by the action of EGCase. 1, C9 wild-type; 2, CED; 3, CEQ, 4, C9 wild-type + NBD-GM1; 5, CED + NBD-GM1; 6, CEQ + NBD-GM1; 7, M-777 wild-type; 8, MED; 9, MEQ; 10, M-777 wild-type + NBD-GM1; 11, MED + NBD-GM1; 12, MEQ + NBD-GM1. (B), time courses of hydrolysis of NBD-GM1 by C9 wild-type and mutant EGCases. ○, C9 wild-type; △, CED; □, CEQ. (C), the same experiment as described in (B) was performed but M-777 was used instead of C9. ●, M-777 wild-type; ▲, MED; ■, MEQ.

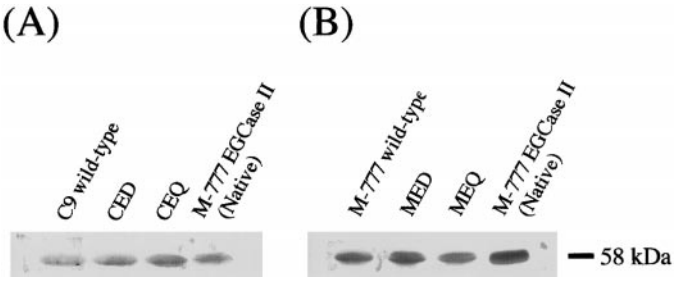


FIG. 3. Expression of EGCase proteins in wild-type and mutant transfectants. Crude enzymes (11 μg of protein) were subjected to 10% SDS–PAGE and transferred onto nitrocellulose membranes, followed by detection with anti-EGCase II polyclonal antibody as described in Materials and Methods. (A), C9 wild-type and mutants; (B), M-777 wild-type and mutants.

ers; Glu–Gln (GAG–CAG), Glu–Asp (GAG–GAT). Wild-type and mutant EGCase genes of M-777 and C9 were constructed in the expression vectors pTEG3 and pTEC6, respectively, which were then used for transformation of *E. coli* JM109 cells. These transfectants were cultured at 37°C for 4 h in a medium containing 1 mM isopropylthio-β-D-galactoside. EGCase activity of cell lysates was assayed using NBD-GM1 as the substrate as described in Materials and Methods. Cell extracts from transfectants containing wild-type EGCase genes hydrolyzed NBD-GM1 to produce NBD-Cer (lanes 4 and 10, Fig. 2A), while those containing mutant genes hardly hydrolyzed the fluorescent substrate even after prolonged incubation (lanes 5, 6, 11, 12, Fig. 2A). The cell extract from mock transfectants containing no EGCase gene did not hydrolyze NBD-GM1 at all (data not shown). Figures 2B and 2C show the time courses for hydrolysis of NBD-GM1 by wild-type and mutant EGCases of C9 and M-777, respectively. It should be noted that EGCase activities of mutants containing CED and MED were slightly higher than those containing CEQ and MEQ (Figs. 2B, 2C).

The levels of expression of EGCases at the protein level were determined by western blotting using anti-EGCase II polyclonal antibody. Recombinant C9 and M-777 EGCases from wild-type and mutant transfectants were stained with the antibody and corresponded to the 58-kDa band of native M-777 EGCase II (Fig. 3). The 58-kDa band was not detected in mock transfectants (data not shown). The level of expression of 58-

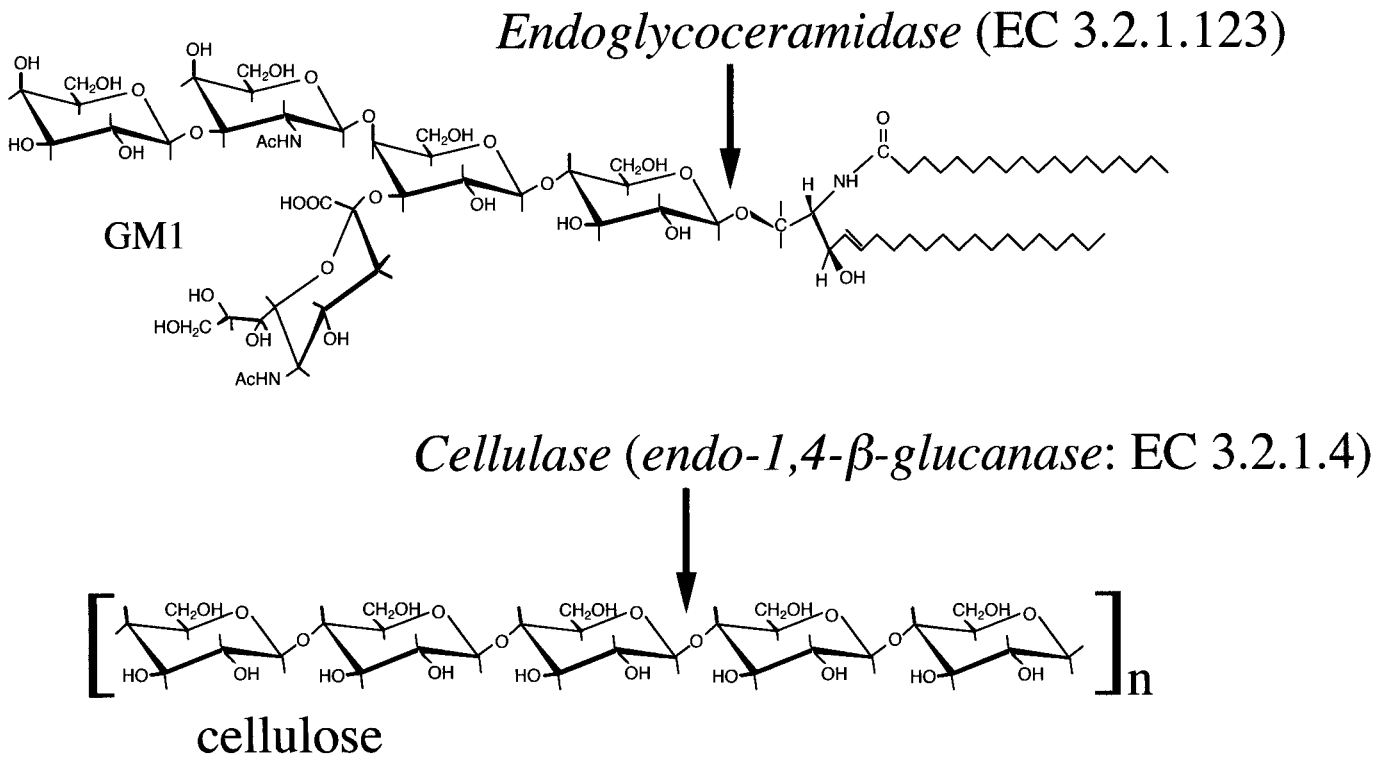


FIG. 4. Points of action of EGCase II and family A cellulase (endo-1,4-β-glucanase).

kDa EGCase protein was almost the same in all transfectants (Fig. 3), although their activities were markedly different (Fig. 2). These results clearly indicated that replacement of the Glu residue in the NEP sequence of EGCase by Gln or Asp strongly reduced the enzyme activity but did not affect expression of the protein.

General acid-base catalysis that involves at least one catalytic carboxylic residue(s) of a Glu and/or Asp was commonly observed in the action of endo-1,4-β-glucanases (19). Furthermore, the Glu residue was shown to be the nucleophile in the active site of endo-1,4-β-glucanases (12). These enzymes hydrolyze the internal β1,4 glucosidic linkage of cellulose, while EGCase II hydrolyzes the internal β1,1′ glucosyl-Cer linkage, but not the β1,1′ galactosyl-Cer linkage, of various glycosphingolipids (Fig. 4). In conclusion, our results strongly suggested that the Glu residue in the NEP sequence is crucial for the hydrolysis reaction of internal β-glucosidic linkage and forms the active site of EGCase, in a manner analogous to that in the family A cellulases. This study also suggested that these enzymes are derived from the same ancestral gene.

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