

Cloning of Antizyme Inhibitor, a Highly Homologous Protein to Ornithine Decarboxylase*

(Received for publication, September 27, 1995, and in revised form, November 15, 1995)

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The degradation of ornithine decarboxylase (ODC) catalyzed by the 26 S proteasome is accelerated by antizyme, an ODC inhibitory protein induced by polyamines. Previously, we have found another possible regulatory protein of ODC degradation, antizyme inhibitor. Antizyme inhibitor binds to the antizyme with a higher affinity than that of ODC, releasing ODC from ODC-antizyme complex. We report here the cDNA sequence of rat heart antizyme inhibitor. The deduced sequence of the protein is highly similar to, but distinct from, sequences of ODCs from various species. Antizyme inhibitor contains amino acid residues required for formation of active sites of ODC, but it completely lacks ODC activity. Antizyme inhibitor has no homology with peptide sequence in the mammalian ODC carboxyl terminus, which is needed for rapid turnover of ODC. It inhibits antizyme-dependent ODC degradation, but, unlike ODC, its degradation is not accelerated by antizyme.

Ornithine decarboxylase (ODC)¹ is a key enzyme in polyamine biosynthesis pathway (1, 2). The turnover of ODC is very rapid and highly regulated (3, 4). The degradation of ODC catalyzed by the 26 S proteasome is accelerated by ODC antizyme (5, 6), an inhibitory protein induced by polyamines (7). Strict regulation of ODC appears to be important for cell growth, because overproduction of ODC is associated with neoplastic transformation (8, 9), whereas overproduction of antizyme inhibits cell growth (10, 11). We previously found in rat liver and heart another possible regulator of ODC degradation, antizyme inhibitor (12). Antizyme inhibitor binds to the antizyme with a higher affinity than that of ODC and releases ODC from the ODC-antizyme complex (12–14). The physiological fluctuation of antizyme inhibitor *in vivo* suggested that it is another regulatory protein that stabilizes ODC by trapping antizyme (14). However, the possibility that antizyme inhibitor is a post-translationally modified product of ODC could not be ruled out. In this report, we describe the cloning and expression

of antizyme inhibitor and show that the sequence of antizyme inhibitor is closely related to, but distinct from, that of ODC.

EXPERIMENTAL PROCEDURES

cDNA Cloning—Oligo(dT)-primed cDNA was synthesized from poly(A)⁺ RNA from the hearts of isoproterenol-treated Wistar rats (10 mg/kg, 2 h) and inserted into λ ZAPII vector (Stratagene) through *Eco*RI adaptors to construct a library. One positive clone was selected from 10⁵ recombinants by screening with a monoclonal antibody to rat heart antizyme inhibitor (14) as a probe. This monoclonal antibody does not react with rat ODC (14). The selected clone, A1, which carried a cDNA insert of about 1.9 kb in length was purified and sequenced. Two more positive clones were selected by plaque hybridization with a probe of the partial length cDNA A1. These clones, A2 and A3, carried cDNA inserts of about 2.2 and 4 kb, respectively, and were sequenced. All the DNA sequences were determined from both strands.

Northern Blot Analysis of Antizyme Inhibitor mRNA—Poly(A)⁺ RNA (5 μ g) from rat tissues was resolved by gel electrophoresis in 1% agarose, 3.6% formaldehyde and blotted to Hybond N⁺ membrane (Amersham). Blots were hybridized with ³²P-labeled antizyme inhibitor cDNA A1 in 1 \times SSC for 16 h at 65 °C, after prehybridization under the same conditions for 1 h. The blots were washed in 2 \times SSPE (1 \times SSPE, 0.18 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA, pH 7.7) and 0.1% SDS at room temperature for 5 min, in 1 \times SSPE and 0.1% SDS at 65 °C for 15 min, and then in 0.1 \times SSPE and 0.1% SDS at 65 °C for 10 min. Under this hybridization condition, antizyme inhibitor cDNA A1 did not hybridize with ODC mRNA, and ODC cDNA did not hybridize with *in vitro* transcribed antizyme inhibitor mRNA. The membrane was exposed to the imaging plate and analyzed by a laser image analyzing system (FUJIX BAS 2000).

Construction of Plasmid-encoding Antizyme Inhibitor-GST Fusion and Preparation of Antizyme Inhibitor Fusion Protein—A1 cDNA lacking the first 14 nucleotides of the coding region was subcloned into the pGEX-4T-3 expression vector (Pharmacia Biotech Inc.). The fusion protein was induced in *Escherichia coli* BL21 with isopropyl-1-thio- β -D-galactopyranoside and purified with glutathione-Sepharose 4B beads (Pharmacia) according to the manufacturer's instructions.

Construction of Plasmid-encoding Antizyme Inhibitor and *In Vitro* Translation—The cDNA fragment contained 10 bp of untranslated 5' sequence, the entire open reading frame, and 3 bp of untranslated 3' sequence was generated by PCR using synthetic primers including *Eco*RI and *Sal*I sites or *Bam*HI site and antizyme inhibitor cDNA A2 as a template, and it ligated into *Eco*RI and *Bam*HI sites of pGEM-3Zf(+) (pGEM-antizyme inhibitor). Transcription/translation of pGEM-antizyme inhibitor was carried out as described previously (10).

Others—ODC activity (14) and ATP- and antizyme- dependent degradation of ODC in an HTC extract (5) were determined as described previously.

RESULTS AND DISCUSSION

A partial length antizyme inhibitor cDNA clone was isolated by screening a rat heart library with a monoclonal anti-antizyme inhibitor antibody (14). Its sequence lacked the 5'-untranslated region and an adjacent portion of the 5'-coding region. Using this cDNA, two full-length cDNA clones, A2 and A3, were isolated. They both contained exactly the same sequence as the partial cDNA, and their sequences were identical except that they had different 5' and 3' end points; 5'UTRs of A2 and A3 were 417 nucleotides and 740 nucleotides, and 3'-untranslated regions of A2 and A3 were 518 nucleotides and more than 1300 nucleotides in length, respectively. These full-length cDNAs contained an open reading frame of 1,344 bp, encoding a polypeptide of 448 amino acids, with a molecular mass of 49,332 Da and pI of 4.49. This molecular mass was similar to that of 51 kDa estimated for purified rat antizyme inhibitor by SDS-PAGE (13). Data bank search showed that the deduced amino acid sequence of antizyme inhibitor had high similarities with ODCs from various species. The amino acid

* This work was supported by grants from the Ministry of Education and Culture of Japan and the Takeda Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: ODC, ornithine decarboxylase; kb, kilobase(s); bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

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Ain 1  MKGFIDNANYSVGLLEDEGTNLGNVIDNYVVE-HTLTGKNAFFVGDLGKIVKKHSQWQNVV
      * * * * *
ODC 1  MGSFTKEE-FDCHILDEGFTAKDILDQKINEVSSDDKDAFYVADLGDVLKKHLRLKAL

60  AQIKPFYVMKCNSTPAVLEILAALGTGACSTKNEMALVQELGVSPENI IYTPCKQASQ
      * * * * *
60  PRVTFYVAVKCNDSRAIVSTLAAIGTGFCASKTEIQLVQLGVPPERLIYANPCQVQSQ
      * * * * *
120  IKYAAKVGVINMTCDNEVELKKIARNHFNKAKVLLHIATEDNIGGEDGNMKGFTLTKNCRH
      * * * * *
120  IKYASNGVQMMTFDSETELMKVARAHKAKLVLRITDDSKAVCRLSVKFGATLKTSRL
      * * * * *
180  LLECAKELDVQIIIGVKFHISSACKYQVYVHALSDARCVFDMAGEFGFTMMMLDIOGGFT
      * * * * *
180  LLERAKELNIDVIGVSPHVGSGCTDPETFVQAVSDARCVFDMGTEVGFMSYLLDIOGGFP
      * * * * *
240  GTE---IQLEEVNHVISPLLDIYFPEGSGIQTISEPGSYVSSAFYLAIVNIIAKKVEND
      * * * * *
240  GSEDTKLKFEITSVINPALDKYFPDSGVRIIAEPGRYVVASAFYLAIVNIIAKKVWKE
      * * * * *
297  KLSSGVEKNGSDEPAFYVYMDGVYGSFASKLSRDINTVPEVHKYKYEDEPLFTSSLWGP
      * * * * *
300  QTGSD-DEDESNEQTLIMYYVNDGVYGSFNCILYDHAHVKALLQKPKPDEKYYSSSIWGP
      * * * * *
357  SDELDQIVESCCLPELSVGDWLIIFDNMGADSLHGSPAFSDTQRPAIYFMMSLSDWYEMQ
      * * * * *
359  TDDGLDRIVERCSLPEMHGDMWLFENMGAYTAAASTFNGFQRPNIYYVMSRSMQWLMK
      * * * * *
417  DAGITSDDAMKNFFAPSCIQLSQEDNFSTEA
      * * * * *
419  QIQSHGFPPEVEEQDVGTLPMSCAQESGMDRHPAACASASINV
      * * * * *

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FIG. 1. **Amino acid sequence of antizyme inhibitor.** A comparison of the amino acid sequence of antizyme inhibitor (Ain) with that of rat ODC. The derived amino acid sequence of the longest open reading frame (nucleotides 418–1761 of antizyme inhibitor cDNA A2) is shown by the one-letter amino acid code. Amino acid identities are indicated by asterisks. Amino acids required for formation of the active site of ODC are boxed in gray. The continuous underline shows the amino acid region of ODC required for antizyme binding. The dotted underlines show the PEST regions of ODC.



FIG. 2. **Expression of antizyme inhibitor mRNA on Northern blots.**

sequence of antizyme inhibitor is compared with that of rat ODC (15) in Fig. 1. The 1–448 amino acids of antizyme inhibitor share 213 identity plus some conservative substitutions with those of rat ODC. Antizyme inhibitor contains all four amino acid residues required for formation of active sites of ODC (16), five of six conserved acidic amino acid residues contributed to active sites of ODC (17) and an amino acid residue necessary for dimer formation (18). It also shows 63% identity with the region of ODC involved in antizyme binding (19). Rat ODC contains two PEST regions (20) in an internal region and near the carboxyl-terminal, respectively. The carboxyl-terminal region of ODC (422–461) including the second PEST region (423–449) has been assigned as an element necessary for the rapid degradation of ODC (6). Antizyme inhibitor lacks this region and does not contain PEST sequences. ODCs of trypanosoma (21), yeast (22), and *Drosophila* (23) also lack this carboxyl-terminal region, but the overall similarity of antizyme inhibitor with these ODCs is lower than that with rat ODC (175, 132, and 140 amino acid identity, respectively). It is interesting to note that rat antizyme inhibitor and rat ODC sequences resemble each other more than yeast ODC resembles the rat ODC sequence.

We previously detected appreciable antizyme inhibitor activity in ODC-induced liver and heart, but only slight activity in kidney (14). Three mRNA species of about 2.2, 2.6, and 3.7 kb were detected in various rat tissues including kidney by Northern blotting (Fig. 2). Antizyme inhibitor cDNA A2 has four possible polyadenylation signals. The two smaller mRNAs may result from alternative utilization of a polyadenylation signal. The size of the largest mRNA is consistent with antizyme

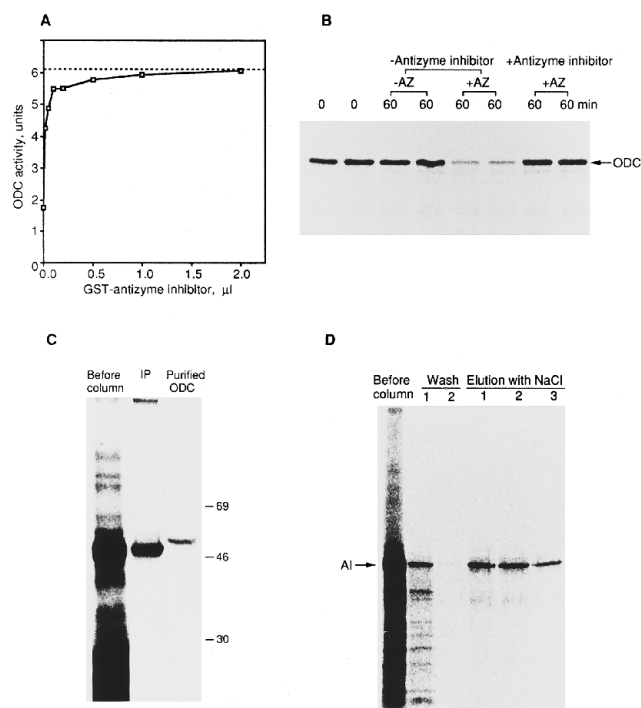


FIG. 3. **Expression of antizyme inhibitor cDNA.** A, reactivation of the ODC-antizyme complex by GST-antizyme inhibitor. ODC (6.10 units) was preincubated with antizyme (4.34 units) at 0 °C for 5 min before addition of antizyme inhibitor. Broken line shows ODC activity before addition of antizyme. B, inhibition of antizyme (AZ)-stimulated ODC degradation in an HTC cell extract by GST-antizyme inhibitor. C, *in vitro* translation of antizyme inhibitor. The products were analyzed by SDS-PAGE before (total) and after immunoprecipitation with a monoclonal anti-antizyme inhibitor antibody (IP). Purified mouse ODC is shown for comparison. D, specific binding of antizyme inhibitor to antizyme. ³⁵S-Labeled antizyme inhibitor synthesized *in vitro* (90 μl) was applied to a column of antizyme-Affi-Gel 10 (0.2 ml). The column was washed with 2 ml (Wash 1) and 1 ml (Wash 2) of 25 mM Tris buffer containing 0.01% Tween 80, and antizyme inhibitor was eluted with 0.3 × 3 ml of the buffer containing 1 M NaCl (Elution with NaCl 1–3). The samples were analyzed by SDS-PAGE.

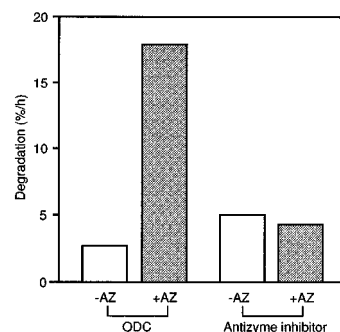


FIG. 4. **Degradation of antizyme inhibitor in an HTC extract.** ³⁵S-Labeled antizyme inhibitor or [³⁵S]ODC was synthesized in a reticulocyte lysate and purified by affinity chromatography with antizyme or anti-ODC antibody as a ligand. The affinity-purified ³⁵S-labeled antizyme inhibitor or [³⁵S]ODC was incubated with an HTC cell extract or phosphate-buffered saline supplemented with an ATP regenerating system, with or without antizyme (AZ). Degradation was monitored as release of trichloroacetic acid-soluble radioactive material. Antizyme inhibitor was degraded slightly even in the absence of the HTC cell extract. This degradation was subtracted from the degradation in the presence of HTC extract. ODC was stable in the absence of the cell extract. Note that degradation of antizyme inhibitor in the cell extract was not accelerated by antizyme.

inhibitor cDNA A3. The biological significance of heterogeneity of antizyme inhibitor mRNA is presently unclear. Treatment with isoproterenol increased the amounts of all three mRNA

species in rat heart, suggesting both transcriptional and translational regulation of expression of antizyme inhibitor.

To verify the authenticity of the cloned antizyme inhibitor cDNA, we expressed it as a glutathione *S*-transferase-antizyme inhibitor fusion protein (GST-antizyme inhibitor). The molecular mass of the product was consistent with the predicted values of 75 and 49 kDa before and after proteolysis with thrombin, respectively. The product expressed inhibited the ODC-inhibiting activity of antizyme; namely, it released active ODC from the inactive ODC-antizyme complex (Fig. 3A). ODC has been shown to be degraded rapidly in a hepatoma tissue culture (HTC) cell extract or a reticulocyte lysate in the presence of ATP and antizyme. The product expressed also inhibited antizyme-dependent ODC degradation in the HTC cell extract (Fig. 3B) as purified antizyme inhibitor from rat heart did (24).

The antizyme inhibitor cDNA was also expressed in an *in vitro* transcription/translation system. The product showed a relative molecular mass of about 50 kDa on SDS-PAGE and was immunoprecipitated with anti-antizyme inhibitor monoclonal antibody (Fig. 3C). Furthermore, the protein was purified by affinity chromatography with antizyme as a ligand (Fig. 3D), and the purified protein inhibited the ODC-inhibiting activity of antizyme (data not shown). These results indicated that this cloned cDNA encodes a functional antizyme inhibitor.

ODC is a homodimer with two active sites formed at the interface between the subunits, each active site being composed of amino acid residues from both subunits. Since the subunit exchanges very rapidly and associates at random, an addition of excess inactive mutant to wild ODC decreases ODC activity by formation of heterodimer between them (16, 25). Like ODC, antizyme inhibitor is a homodimer (12, 13). Antizyme inhibitor-GST fusion and the antizyme inhibitor expressed in an *in vitro* transcription/translation system as described above completely lacks ODC activity, and the fusion has no significant ODC-inhibitory activity (data not shown), suggesting that no appreciable amount of heterodimer is formed between antizyme inhibitor subunit and the ODC subunit.

We examined the stability of antizyme inhibitor *in vitro*. Purified antizyme inhibitor was degraded in the cell extract at a comparable rate to ODC without added antizyme, but, unlike ODC, its degradation was not stimulated by addition of antizyme (Fig. 4). Antizyme binding has been suggested to cause a conformational change in the ODC molecule, resulting in ex-

posure of the degradation signal at the carboxyl-terminal region to the protease (6). The different responses of degradations of ODC and antizyme inhibitor to antizyme binding suggest that the degradation signal of unknown location in antizyme inhibitor molecule may be exposed with or without antizyme.

Recently, antizyme was also found to repress polyamine transport activity (26, 27). Thus, antizyme plays dual roles in preventing excess accumulation of cytotoxic polyamines. The cloning of antizyme inhibitor cDNA makes it possible to examine whether antizyme inhibitor also regulates the function of antizyme in polyamine transport.

Acknowledgment—We thank Drs. T. Hayashi, M. Nishiyama, and K. Tanaka for useful discussions.

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