Human catechol-O-methyltransferase: Cloning and expression of the membrane-associated form

(nucleotide sequence/primary structure/enzymatic activity/inhibition)

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ABSTRACT A cDNA clone for human catechol-O-methyltransferase (hCOMT; S-adenosyl-L-methionine:catechol O-methyltransferase; EC 2.1.1.6) was isolated from a human hepatoma cell line (Hep G2) cDNA library by hybridization screening with a porcine cDNA probe. The cDNA clone was sequenced and found to have an insert of 1226 nucleotides. The deduced primary structure of hCOMT is composed of 271 amino acid residues with a predicted molecular mass of 30 kDa. At its N terminus it has a hydrophobic segment of 21 amino acid residues that may be responsible for insertion of hCOMT into the endoplasmic reticulum membrane. The primary structure of hCOMT exhibits high homology to the porcine partial sequence (93%). The deduced amino acid sequence contains two tryptic peptide sequences (T-22, T-33) found in porcine liver catechol-O-methyltransferase (COMT). The coding region of hCOMT cDNA was placed under the control of the cytomegalovirus promoter to transfet human kidney 293 cells. The endogenous COMT activity, which was ~9.98 units per mg of protein in the untransfected cells, increased to 206 units per mg of protein upon transfection with a plasmid containing the COMT cDNA. The COMT activity of recombinant protein was inhibited competitively (IC50 = 700 nM) by the selective COMT inhibitor Ro 40-7592. An anti-COMT monoclonal antibody recognized, on immunoblots, a major polypeptide with apparent molecular mass of 29 kDa, in reasonable agreement with the predicted molecular mass. The recombinant hCOMT was shown by immunoblot analysis to be mainly associated with the membrane fraction. RNA blot analysis revealed one COMT mRNA transcript of 1.4 kilobases in Hep G2 poly(A)+ RNA.

Catechol-O-methyltransferase (COMT; S-adenosyl-L-methionine; catechol O-methyltransferase; EC 2.1.1.6) is an enzyme that catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to the m-hydroxy group of catecholamines (dopamine, noradrenaline, adrenaline), their metabolites, and l-dopa, thereby inactivating them. The enzyme has a broad substrate specificity accepting as substrate also catechol steroids, α-methylated, and α-metabolites, and dopamine (1). It is widely distributed in various cerebral and extracerebral tissues of all mammalian species (2), including erythrocytes (3). The occurrence of at least two distinct isoforms of COMT has been demonstrated, of which one is soluble (S-COMT) and the other membrane-bound (MB-COMT) and whose relative abundance differs in various tissues and species. The biochemical characteristics of MB-COMT are less extensively investigated. This isoenzyme is localized in the microsomal fraction of different tissues, such as liver (4), brain (5), erythrocytes (6), and appears to be an integral membrane protein structurally distinct from the cytosolic S-COMT (7). Although both forms of COMT catalyze the O-methylation of catecholamines, MB-COMT has a much higher affinity for its substrates (6, 8) and might, therefore, contribute markedly to the metabolism of low concentrations of endogenous catecholamines in central nervous system and peripheral tissues. Studies of COMT activity in erythrocytes suggest that individual variations are genetically determined. Data from segregation analysis provided evidence for the control of COMT activity by a major autosomal locus with two alleles (9). Variations in COMT activity are found in patients with major depression, recurrent and bipolar disorders (10), as well as in children with Down syndrome (11). Differences in the enzymatic activity in human individuals may be involved in the pathogenesis of various psychiatric and neurological diseases (12). To study the molecular genetics of these disorders, it is necessary to isolate the gene or genes coding for cytoplasmic and membrane-bound COMT.

In the present communication, we describe the molecular cloning of a cDNA encoding the human liver COMT. Sequence analysis and expression of the clone revealed that we have isolated the cDNA coding for the membrane-bound enzyme.

METHODS

Construction and Screening of cDNA Libraries. The Agt11 expression library from porcine liver was purchased from Clontech. By immunizing a rabbit with the highly purified soluble form of porcine liver COMT we obtained an antiserum that recognized (in ELISA and in immunoblot) the porcine enzyme and inhibited its activity. Immunoblotting with this antiserum was done according to Young and Davis (13). The Agt11 library from Hep G2 cells was constructed and screened following described procedures (14).

RNA Blot Analysis. Total RNA was extracted from a human hepatoma cell line (Hep G2, ATCC HB 8065) by the guanidinium isothiocyanate/cesium chloride method (15). Poly(A)+ RNA was isolated by oligo(dT)-cellulose chromatography (16).

Poly(A)+ RNA (~5 μg) from Hep G2 cells was denatured with glyoxal, electrophoresed through 1.1% agarose containing 25 mM NaH2PO4 (pH 7.0) (17), transferred to an Amerham Hybond-N filter (RFN 1520N), and hybridized with a 32P-labeled nick-translated cDNA insert of AhCOMT F3.

Construction of Expression Plasmid Carrying Human COMT (hCOMT) cDNA. A 1230-base pair (bp) EcoRI fragment (hCOMT F3) cDNA was subcloned by blunt-ended

Abbreviations: COMT, catechol-O-methyltransferase; hCOMT, human COMT; S-COMT, soluble COMT; MB-COMT, membrane-bound COMT; CMV, cytomegalovirus; mAb, monoclonal antibody.
Two to whom reprint requests should be addressed.
The sequence reported in this paper has been deposited in the GenBank data base (accession no. M59525).
ligation into the BamHI site of expression vector pBC12/CMV (constructed by B. R. Cullen, Duke University Medical Center). The pBC12/CMV vector is composed of a simian virus 40 origin connected to human cytomegalovirus (CMV) enhancer-promoter sequences with a unique BamHI site for cloning. The rat preproinsulin II (r(2)) intron and simian virus 40 polyadenylation signal region were placed downstream to the BamHI site.

Transfection of 293 Cells and Assay of hCOMT. Human embryonic kidney cells (293 cell line, ATCC no. CRL 1573) were maintained in minimal essential medium/8% fetal calf serum. Cells (5 × 10^6) were seeded in 100-mm dishes 24 hr before addition of plasmid DNA (20 μg per dish). Transfection was performed using the modified calcium phosphate precipitation method (18). Forty-eight hours after transfection, cells were collected and homogenized in 10 mM phosphate buffer, pH 7.6/10 mM benzamidine (=10^6 cells per ml). COMT activity was then measured in the homogenate by a radioenzymatic method with 2.5 mM pyrocatechol as substrate and S-adenosyl-L-[3H]-methionine as methyl donor (19). One unit of COMT activity was defined as the quantity of enzyme producing 1 nmol of guaiacol per hr at 37°C. For protein quantification the Pierce assay was used.

Isolation of Microsomes. After homogenization of the transfected 293 cells, a separation of the soluble from the membrane fraction was performed by differential centrifugation. The homogenate was centrifuged at 600 × g for 20 min, and the resulting supernatant was centrifuged at 100,000 × g for 60 min. The pellet containing mitochondria and microsomes was washed twice and finally resuspended in 10 mM phosphate buffer, pH 7.6. The presence of recombinant hCOMT, both in the supernatant and in the pellet, was tested by immunoblotting (20).

Soluble and membrane fractions from Hep G2 were obtained essentially as above.

RESULTS AND DISCUSSION

Protein Sequencing of Porcine Liver COMT. To obtain a partial amino acid sequence of COMT, we purified the soluble protein from porcine liver by using an immunoadfinity chromatography column (unpublished data). The highly purified protein was then subjected to tryptic digestion followed by purification of the tryptic peptides by HPLC. The sequences of two tryptic peptides, T-22: ILQYVLQPAVAGD and T-33: LTLIELPNDAIAIQVVDFA-GLQDWTVVVG, were determined by gas-phase microsequencing (22).

Cloning of Porcine Liver COMT cDNA. A polyclonal antibody directed against highly purified porcine liver S-COMT was used to screen a Agt11 cDNA expression library from porcine liver. In an initial immunoscreening, seven positive clones with inserts ranging between 1100 and 1300 base pairs (bp) were detected. The inserts of five of them crosshybridized to each other. The DNA sequences of the two largest crosshybridizing clones (1 and 7) contained an open reading frame coding for a protein of 186 amino acids with a predicted molecular mass of 20 kDa. The predicted amino acid sequences contained a stretch of 33 amino acids that matches perfectly with the porcine COMT tryptic peptide T-33 obtained by protein sequencing. This result strongly indicated that the isolated cDNA codes for a partial sequence of the porcine COMT.

Isolation and Sequencing of hCOMT cDNA. Using the porcine COMT cDNA as hybridization probe, we isolated its human counterpart by screening a human cDNA library. Because S- and MB-COMT had been demonstrated in the Hep G2 cells, its poly(A)^+ RNA was used to construct a Agt11 cDNA library. An initial screening of 1.2 × 10^6 recombinants at moderate stringency with a probe containing a 370-bp long fragment of the porcine liver cDNA clone (pCOMT F7, Fig. 1a) revealed 10 positive clones with inserts ranging between 1226 and 500 bp. Two largest clones that gave the strongest signal, hCOMT F3 and hCOMT F8, contained 1226-bp and 1150-bp EcoRI inserts respectively. DNA sequence analysis revealed that both clones were identical, except for a larger 5'-untranslated region in AbhCOMT F3.

The nucleotide and deduced amino acid sequences of clone AbhCOMT F3 are shown in Fig. 1b. The nucleotide sequence of clone AbhCOMT F3 (−204 to 1000) contains the largest open reading frame starting at nucleotide residues 1–3 and terminating at nucleotide residues 814–816 with a stop codon (TGA). This 813-bp open reading frame codes for a polypeptide of 271 amino acid residues with a predicted molecular mass of 30 kDa. The first initiation codon (nucleotide position

**Fig. 1.** hCOMT. (a) Restriction map of the cDNA clone hCOMT F3 (bases 1–1226). The thick black bar indicates the hCOMT-coding sequence. The porcine fragment (pCOMT F7, 370 bp), which was used for isolation of the human clone, is indicated below the solid bar. (b) Nucleotide and predicted amino acid sequences of cloned hCOMT. Nucleotides are numbered in the 5' → 3' direction, and numbers are shown at right of the sequence. Nucleotide residue 1 is the adenine of the initiating methionine codon ATG, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The putative mature segment is indicated by the box. The seven cysteines are circled. Tryptic peptides (T-22, T-33) that were isolated and sequenced from porcine liver COMT are overlined; residues differing from those predicted in the human sequence are indicated by a dotted line. * Stop codon.
1-3) is embedded in the sequence TGAAGATG, which does not perfectly match with the consensus sequence CCACCATG frequently found for the eukaryotic translation initiation (23). In the 5' untranslated region there is an in-frame stop codon at the position (-159 to -157). hCOMT F3 contains 204 and 207 nucleotides in the 5' and 3' untranslated regions, respectively. The homologues of the porcine tryptic peptide sequences, T-22 and T-33, were also found in the predicted protein sequence of hCOMT (Fig. 1b).

To confirm the assigned initiator codon, a cRNA was synthesized in vitro by using the pSPT19 vector into which the hCOMT cDNA was subcloned. When added to a rabbit reticulocyte lysate containing [35S]methionine, the cRNA directed the translation of a single labeled polypeptide with an estimated molecular mass of 29 kDa (data not shown), implying that the imposed initiator methionine residue can be recognized by the in vitro translation system.

No potential N-linked glycosylation site (Asn, Xaa, Ser/Thr) (24) was found in the predicted amino acid sequence of hCOMT, which is in good agreement with the observation that when a highly purified porcine liver COMT was incubated with a mixture of endoglycosidases, the molecular size of the COMT (tested by SDS/PAGE) was not reduced (data not shown).

Northern (RNA) blot analysis of poly(A)+ RNA from Hep G2 cell revealed a single band of 1.4 kilobases (kb) when

![Fig. 2. Northern blot hybridization of poly(A)+ RNA from Hep G2 cells. The hybridization probe was the 32P-labeled nick-translated cDNA insert from the AhCOMT F3 clone. Positions of molecular size standards are indicated at left.](image)

either the partial porcine cDNA or AhCOMT F3 cDNA was used as hybridization probe (Fig. 2).

**Hydropathy and Secondary Structure of the Predicted Amino Acid Sequence of hCOMT.** The results of the hydropathy index evaluation of hCOMT protein by the method of Kyte and Doolittle (25) are illustrated in Fig. 3. According to these authors, any protein sequence corresponding to a membrane-spanning domain is characterized by a stretch of at least 18 amino acids exhibiting an average hydropathy index \( >1.6 \). In the hCOMT sequence, the N-terminal region (amino acids 6-26) meets these criteria, indicating that the hCOMT we cloned corresponds to the membrane-associated enzyme. Furthermore, the nature of the N-terminal region was investigated by using the criteria of Von Heijne (26). The amino acids 1-28 of hCOMT contain a short negatively charged N-terminal region (amino acids 1-5), a 21-residue-long highly hydrophobic core (amino acids 6-26), and a positively charged C-terminal region. Such features are not characteristic of known eukaryotic signal peptides (27) but seem rather to belong to a class of N-terminal anchor segments, resulting in an N-terminally anchored protein with most of its mass exposed on the cytosolic face of the endoplasmic reticulum membrane.

**Sequence Comparison.** Comparison of the partial COMT sequence deduced from the porcine cDNA (amino acids 1-186) with the deduced amino acid sequence of hCOMT (amino acids 86-271) (Fig. 4) shows high homology. The amino acid sequences of hCOMT (amino acids 1-186) and porcine (amino acids 86-271) and their translated cDNA sequences are identical at nearly all positions. The regions of identity include the N-terminal region (amino acids 1-5), the most hydrophobic region (amino acids 6-26), and the C-terminal region (amino acids 184-271). The C-terminal region is positively charged and is likely to be membrane-anchored. The sequence identity between the two proteins is high, with 100% identity in the region of identity (amino acids 1-186) and 98.5% identity in the region of identity (amino acids 1-271).

![Fig. 3. Hydrophathy plot of the amino acid sequence of the hCOMT computed according to Kyte and Doolittle (25) at the span setting of 17 amino acids. Solid bar indicates the putative membrane segment. Positive values represent hydrophobic regions, and negative values represent hydrophilic regions.](image)

![Fig. 4. Comparison of the hCOMT primary structure with that of the partial porcine sequence. The predicted amino acids 1-186 of porcine COMT have been aligned with amino acids 86-271 of hCOMT by using the GAP computer program (28). Amino acid sequence obtained from the porcine liver COMT peptide (T-33) is shown over the predicted porcine sequence. Identical residues are boxed. The cysteine residues are circled. A • between residues represents chemically homologous residues.](image)
extent of sequence identity of hCOMT with porcine is 83%, but when the homology is calculated considering conservative amino acid substitutions, the figure is 93%. Nucleotide sequence homology between human and porcine COMT was found to be 83%. Seven cysteines are contained in the human sequence (Fig. 1b), of which at least four are conserved between the human and porcine enzyme (Fig. 4).

Based on substantial evidence obtained by affinity-labeling studies, Borchardt (29) has suggested that two nucleophilic residues, probably sulphhydryl, exist at the active site of COMT and that both are necessary for enzymatic activity.

We attempted to assign the active site in the predicted sequence of COMT. As indicated in Fig. 4, a stretch of 71 amino acids (residues 79–150 of porcine and 164–235 of human sequences, respectively) is identical in the predicted sequences of both species. In particular, the two cysteines, separated by 15 amino acids, in the middle of the conserved stretch might be important for the catalytic activity of COMT. Further studies, such as site-directed mutagenesis, are needed to substantiate this assumption.

**Transient Expression of hCOMT cDNA in 293 Cells.** To confirm that the isolated cDNA indeed encodes hCOMT, the hCOMT F3 cDNA was subcloned into the eukaryotic expression vector pBC12/CMV (Fig. 5a). 293 cells were transfected with the resultant construct, and the COMT activity was measured in the cell homogenate. 293 cells contained very little endogenous enzyme activity, and this baseline level did not change after transfection with a plasmid lacking the hCOMT sequence. In contrast, 293 cells transfected with plasmid pBC12/CMV–hCOMT containing the entire hCOMT sequence showed a 20-fold increase in COMT activity (Fig. 5b). The activity increased linearly as a function of the concentration of the homogenate and of the incubation time (15–90 min).

**Inhibition of hCOMT Activity by Ro 40-7592.** The effect of the two COMT inhibitors, Ro 40-7592 (3,4-dihydroxy-4′-methyl-5-nitrobenzophenone) and tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) on recombinant hCOMT was investigated. Ro 40-7592 is a highly potent, competitive, and selective inhibitor and is presently under clinical trial for the therapy of Parkinson disease. This compound, used in combination with L-dopa and a peripheral decarboxylase inhibitor, is designed to prolong the duration of L-dopa action by preventing its conversion to 3-O-methyl-dopa (21).

The inhibitory effect of Ro 40-7592 on the recombinant COMT activity was analyzed in the homogenate of cells transfected with the pBC12/CMV–hCOMT (Fig. 6). The recombinant enzyme was inhibited in a dose-dependent manner by Ro 40-7592 with an IC₅₀ (~700 nM) found similar to that measured for the native enzyme in various animal tissues (21). With tropolone, a relatively weak inhibitor of COMT, a 50% inhibition of recombinant COMT was observed at a concentration of ~1 mM, again in good agreement with the results obtained with the native enzyme (21).

**Immunoblot Analysis of Expressed hCOMT in 293 Cells.** To characterize the recombinant hCOMT expressed in 293 cells, the homogenates from untransfected cells as well as from cells transfected with plasmid pBC12/CMV–hCOMT were subjected to Western (immunological) blotting using mono-

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**Fig. 5.** Structure of the plasmid carrying cDNA for hCOMT and expression of hCOMT in transfected 293 cells. (a) pBC12/CMV-hCOMT contains the entire coding region of the human COMT (hCOMT F3) ligated into the unique BamHI site of the vector (Klenow filled) as described. (b) Expression of hCOMT in transfected 293 cells. After transfection with the indicated plasmid, a cell homogenate was prepared for the assay of COMT activity. Basal activity was measured in untransfected cells (indicated as none). Results, expressed as COMT activity per protein concentration, are the means ± SEMs of three independent experiments done in duplicate. SV40, simian virus 40.

**Fig. 6.** Inhibition of recombinant hCOMT by the selective inhibitor Ro 40-7592. The homogenates of cells transfected with plasmid pBC12/CMV–hCOMT were tested for COMT activity in the presence of different concentrations of the COMT inhibitor Ro 40-7592. Each value represents the means ± SEMs of four different measurements.
clonal antibody (mAb) Co 60-1B/7. This antibody was raised against S-COMT from porcine liver and was shown to crossreact with human and rat COMT (unpublished data). A predominant immunoreactive band of 29-kDa apparent molecular mass and a weaker band of 25.5 kDa were detected (Fig. 7a) in the homogenates from transfected cells but not in the homogenates from untransfected cells.

To verify that the cDNA clone encodes the membrane-associated form of COMT, the homogenates from 293 cells transfected with plasmid pBC12/CMV-hCOMT were subjected to differential centrifugation. The resulting membrane and cytosolic fractions were tested by immunoblotting with mAb Co 60-1B/7 (Fig. 7b). COMT immunoreactivity was found mostly in the membrane fraction, which indicates that the expressed hCOMT is incorporated mainly into the endoplasmic reticulum of 293 cells. The weaker immunoreactive band of 25.5 kDa was found in the cytosolic fraction. Fig. 7c shows an immunoblot of the cytosolic and membrane fractions from Hep G2 cell for comparison. This cell contains S-COMT and MB-COMT forms of the enzyme, which both react with mAb Co 60-1B/7. The molecular sizes of 29 kDa and 25.5 kDa observed for the recombinant COMT expressed in 293 cell are in good agreement with MB-COMT and S-COMT from Hep G2 cell.

These results suggest that the 25.5-kDa protein may arise from the 29-kDa protein by posttranslational processing. Although a computer search for potential proteolytic cleavage site in the region of interest (amino acids 27–46) predicted cleavage sites for trypsin, chymotrypsin, and proline endopeptidase, we have no clue for the exact nature of the process. For 293 cells we speculate that, because of lack of an efficient processing enzyme, most COMT remains membrane-bound.

CONCLUSION

Our results indicate that the cDNA clone that has been isolated encodes hCOMT. This conclusion is based on the following lines of evidence: (i) the predicted amino acid sequence of the human clone shows a high homology to porcine COMT (93%). (ii) Two tryptic peptide sequences (22 and 33 amino acids) of the purified porcine liver COMT are found in the predicted amino acid sequence of hCOMT. (iii) The expression of the cDNA in transfected cells produced a protein that possesses the full biological activity of COMT in the enzymatic assay and is recognized by anti-COMT mAb in the immunoblot. Moreover, immunoblot analysis showed that the recombinant hCOMT was actually associated with the membrane fraction.

The successful cloning of hCOMT will enable us to produce the enzyme in quantities needed for further structural and functional analysis. Finally, we hope that the cDNA clone will provide valuable tools for the investigation not only of the cellular expression of COMT in various regions of the brain but also of the genetic linkage of COMT alleles with certain diseases of the central nervous system.

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