Isolation and characterization of full-length cDNA clones coding for cholinesterase from fetal human tissues

(pseudocholinesterase/acyethylcholinesterase/Xenopus oocyte bioassay/complete amino acid sequence/sequence conservation)

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ABSTRACT To study the primary structure and regulation of human cholinesterases, oligodeoxynucleotide probes were prepared according to a consensus peptide sequence present in the active site of both human serum pseudocholinesterase (BtChoEase; EC 3.1.1.8) and Torpedo electric organ "true" acetylcholinesterase (AcChoEase; EC 3.1.1.7). Using these probes, we isolated several cDNA clones from Agt10 libraries of fetal brain and liver origins. These include 2.4-kilobase cDNA clones that code for a polypeptide containing a putative signal peptide and the N-terminal, active-site, and C-terminal peptides of human BtChoEase, suggesting that they code either for BtChoEase itself or for a very similar but distinct fetal form of cholinesterase. In RNA blots of poly(A) RNAA from the cholinesterase-producing fetal brain and liver, these cDNAs hybridized with a single 2.5-kilobase band. Blot hybridization to human genomic DNA revealed these fetal BtChoEase cDNA clones hybridize with DNA fragments of the total length of 17.5 kilobases, and signal intensities indicated that these sequences are not present in many copies. Both the cDNA-encoded protein and its nucleotide sequence display striking homology to parallel sequences published for Torpedo AcChoEase. These findings demonstrate extensive homologies between the fetal BtChoEase encoded by these clones and other cholinesterases of various forms and species.

Two types of cholinesterases are capable of rapidly hydrolyzing the neurotransmitter acetylcholine in humans. These are the "true" acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7) and the "pseudo-" or butyrylcholinesterase (BtChoEase; acetylcholine hydrolyase, EC 3.1.1.8), distinguished by their substrate specificity and sensitivity to selective inhibitors (1). Both enzymes are rare ubiquitous proteins that exist in parallel arrays of multiple molecular forms with similar kinetic properties. The molecular forms differ in the number of catalytic subunits (2), in their level of hydrophobicity (3) and mode of glycosylation (4), and in their cellular and subcellular localization (1). In the human brain (5), AcChoEase is the major species, accompanied by minor BtChoEase activities. In the blood, amphaphatic AcChoEase dimers are bound to erythrocyte membranes by non-amino acid components (6), whereas soluble BtChoEase tetramers, presumed to be produced in the liver, are present in the serum (7).

Polyclonal and monoclonal antibodies raised against purified human erythrocyte AcChoEase were shown to cross-react with AcChoEase from other tissues and species (8-10) but not with human BtChoEase (10), whereas antibodies against whole human serum were found to precipitate active BtChoEase (11). In addition, peptide sequencing revealed considerable homology between the active site peptides of human serum BtChoEase (7) and the "true" AcChoEase from the electric organ of Torpedo californica (12). Altogether, these observations suggest that various cholinesterases share common domains but may also contain regions specific to particular species and forms. Oocyte microinjection experiments indicate that the polymorphism of human cholinesterases extends to the level of mRNA (13), and individuals with genetically inherited BtChoEase deficiencies display normal AcChoEase activities (14), suggesting that in humans distinct DNA sequences are involved in the synthesis of AcChoEase and BtChoEase. These could code for different AcChoEase and BtChoEase polypeptides or for other proteins, required for various post-translational processing events of a single type of cholinesterase precursor. To directly approach this issue by revealing the primary sequence of human cholinesterases and the regulation of their expression, we searched for cholinesterase cDNA clones by using oligodeoxynucleotide probes prepared according to a consensus peptide sequence present in the active site of both human BtChoEase (7) and Torpedo AcChoEase (12).

METHODS

Poly(A)* RNA was extracted from fetal human brain and liver (18 weeks gestation) and was tested for the presence of cholinesterase mRNA by oocyte microinjection followed by cholinesterase bioassay (13). cDNA libraries (constructed by A. Ullrich of Genentech) were prepared from these RNA preparations and were inserted into the EcoRI site of Agt10, using a polylinker containing restriction sites for EcoRI, Xho I, Sal I, and Sst I. The fetal brain Agt10 library (1.6 × 109 plaque-forming units) was plated out, and nitrocellulose filter copies were prepared and screened with two overlapping oligodeoxynucleotide probes (15). Each probe was designed to complement the predicted mRNA sequence as follows. Probe PROSYN, d[3'-AAACCCCT(AGC)AGCGNC]C, in which N equals A, C, G, or T and only one or the other of the two triplets in parentheses is present, a 17-mer with a 256-fold degeneracy that represents the consensus peptide sequence, Phe-Gly-Glu-Ser-Ala-Gly, present in human serum BtChoEase (7) and in "true" AcChoEase from Torpedo electric organ (12). Probe OPSYNO, d[3'-AAACCCCT(AGC)AGCGCCGC]G, a 29-mer with a 36-fold degeneracy that codes for the peptide Phe-Gly-Glu-Ser-Ala-Gly-Ala-Ser-Val found in human serum BtChoEase (7) and that differs from the parallel peptide of Torpedo AcChoEase by one amino acid (no. 7 in this peptide, Gly in Torpedo) (12). The limitation of codon degeneracy in probe OPSYNO was made.

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Abbreviations: BtChoEase, butyrylcholinesterase; AcChoEase, acetylcholinesterase; BW284C51, 1,5-bis-[4-allyldimethylaminomethyl]-pentan-3-one dibromide; iso-OMPA, tetraisopropyl pyrophosphoramide.

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possible by insertion of deoxyinosine in positions where codon ambiguity permits all four nucleotides (16). Both probes were manually synthesized, using phosphoramidite chemistry (17). Oligodeoxynucleotides were 5'-end-labeled with \([\gamma-^{32}\text{P}]]\text{ATP (5000 Ci/mmol, Amersham; 1 Ci = 37 GBq)}\) and polynucleotide kinase (New England Nuclear). Hybridization was as described (15), followed in the third screen by 3 M tetramethylammonium chloride washes at 53°C (18). Under these conditions, only a single clone in the fetal brain library, designated FBChE12, gave a significant hybridization signal with both probes. The FBChE12 DNA was purified and \(^{32}\text{P}\)-labeled by nick-translation and employed as a probe to screen the fetal brain and liver libraries. Hybridization and washing were performed as previously described (15). Rescreening the fetal brain library resulted in the finding of three more positive inserts, none of which was longer than FBChE12. Out of 1.4 \times 10^6 fetal liver phages, 23 gave positive hybridization signals with \(^{32}\text{P}\)-labeled FBChE12. Of these, 4 phages contained 2.4-kilobase (kb) inserts with identical restriction maps. These hybridized with a C-terminal probe, \([3'-CT^4(AGN;TCX)ACXANCCCGA]\), a 17-mer with a 96-fold degeneracy that codes for the peptide Glu-Ser-Cys-Val-Gly-Leu found in the C terminus of human serum BtChoEase (7). One of these cDNA clones, designated FL39, was used for further characterization and DNA sequencing (Fig. 1).

All experiments were carried out in accordance with the National Institutes of Health Guidelines for recombinant DNA work and with the approval of The Weizmann Institutional Review Board for Human Experimentation.

RESULTS

The initial screening procedure described in Methods resulted in the isolation of a single fetal brain cDNA clone 765 nucleotides in length, designated FBChE12. The nucleotide sequence of FBChE12 that is complementary to probes OPSYN and OPSYNO corresponded exactly to the peptide sequence used to design these oligodeoxynucleotide probes (Fig. 2, amino acid residues encoded by nucleotides 742–759 and 742–771, respectively). FBChE12 was then used as a probe to screen the fetal brain and liver cDNA libraries. Four clones 2.4 kb in length were isolated from the fetal liver library and one of these, designated FL39, was further characterized in comparison with FBChE12. Both clones contained an identical sequence of 693 nucleotides, with the 5' end of the FL39 insert starting at nucleotide 73 of FBChE12 (Fig. 2), suggesting that both cDNAs were derived from similar mRNA transcripts. When the amino acids predicted from the FBChE12 and the FL39 sequence are aligned with the available peptide sequences published for human BtChoEase, the entire coding region for the mature enzyme is defined, starting at residue 1 (nucleotide 160), which corresponds to the N-terminal peptide, and ending at residue 574 (nucleotide 1881), which is the last amino acid residue in the C-terminal tryptic peptide of BtChoEase as determined from amino acid sequencing (7). This sequence also includes the active site tryptic peptide of human BtChoEase, which contains a serine residue that can be labeled by diisopropyl fluorophosphate (7) (Fig. 2, circled). The N-terminal peptide inferred from the FBChE12 and the FL39 sequences is similar to the BtChoEase peptide, except that in our sequence residue 12 is Lys, as in the N-terminal peptide of Torpedo AcChoEase (12), whereas the corresponding residue of human BtChoEase found by amino acid sequencing is Gly (7). The general amino acid composition of the protein encoded by FL39 was very similar to that reported for human BtChoEase (7), with several exceptions, for example, BtChoEase has 7 more proline residues and 6 fewer tryptophan residues; in contrast, the amino acid composition of the FL39-encoded protein clearly differed from the parallel composition derived for erythrocyte AcChoEase (25). In addition, the N terminus of the cholinesterase encoded by FBChE12 and FL39 differs from the peptide reported for erythrocyte AcChoEase (26). All together, this strongly suggests that both FBChE12 and FL39 code for BtChoEase.

The region upstream of the BtChoEase N-terminal residue (nucleotides 88–147) in FBChE12 codes for 20 amino acids characteristic of leader peptides of membrane-associated and exported protein precursors (27). The hydrophobic sequence in this region is rich in large nonpolar amino acids. It is preceded by the tripeptide His-Ser-Lys and terminates with Lys-Ser-His, both composed of polar amino acids. Further

![Fig. 1. Sequencing strategy for fetal BtChoEase cDNA clones FBChE12 and FL39 from human brain and liver. The entire cDNA inserts of FBChE12 and FL39 and their restriction endonuclease EcoRI fragments were isolated and subcloned in the sequencing vectors M13mp10 and M13mp11 (19). Sequential series of overlapping clones were produced by generating deletions of various sizes from both ends of the inserts through the 3'-to-5' exonuclease activity of T4 DNA polymerase (20). DNA sequencing of the resulting M13-FBChE12 and M13-FL39 recombinants was done by the dideoxynucleotide procedure (21), using the universal 17-mer primer (Amersham, no. 4511, indicated by a filled circle at the beginning of an arrow), unique 17-mer primers chemically synthesized from a confirmed cDNA sequence (indicated by arrows beginning with an empty circle), or a universal 17-mer primer synthesized for use in sequencing the deletion-derived clones (20) (indicated by arrows beginning with a vertical line). Confirmed sequences were obtained from both strands of the cDNA as indicated by arrow length and direction. Sequence data were managed by using computer programs (22). Restriction sites for several nucleases were located by computer analysis of the sequence data and confirmed experimentally. The 5' end of the FL39 insert was localized at nucleotide 73 of FBChE12, which was 765 nucleotides long (ends of both inserts are marked by arrows). Sp, signal peptide; Nt, N terminus; Ct, C terminus.]
Fig. 2. Primary structure of the fetal human BtChoEase encoded by FBChE12 and FL39. The 2.4-kb composite nucleotide sequence of clones FBChE12 and FL39 was translated into its encoded amino acid sequence. Nucleotides are numbered in the 5'-to-3' direction, and the predicted amino acids are shown below the corresponding nucleotide sequence. Underlining indicates three amino acid sequences that were found to match peptides present in human serum BtChoEase as shown by peptide sequencing (7). These are the N-terminal peptide (nucleotides 160–225), the active-site peptide (nucleotides 730–765, with a full circle indicating the active-site Ser, residue 198), and the C-terminal peptide (nucleotides 1864–1881). The amino acid sequence of the active-site peptide served as a basis for designing the oligodeoxynucleotide probes with which these cDNA clones were selected (see Methods). Also underlined are a putative ribosome binding site (nucleotides 30–36) and signal peptide (nucleotides 88–147), with three polar amino acid residues at both ends. Seven potential sites for N-linked glycosylation (starting at nucleotides 208, 475, 880, 925, 1180, 1600, and 1615), predicted by the sequence Asn-Xaa-Thr/Ser, in which Xaa represents any amino acid except proline (23), are doubly underlined. His-77 and Asp-129, which are the best candidates to be involved in the active site by comparison with other serine esterases (24), are also circled. Cys residues are encoded in hexagons. The FL39 sequence also includes a long 3' untranslated region, ending with a polyadenylation site and a poly(A) tail.

upstream, the cDNA sequence consists of a fully open reading frame without stop codons, and it includes a putative ribosome binding site (Fig. 2).

The coding region in the DNA (Fig. 3A) and inferred amino acid sequence (Fig. 3B) of the FL39 clone were compared to the parallel sequences recently published for a cDNA clone coding for AcChoEase from Torpedo electric organ (28). This analysis revealed a 53% identity between the corresponding parts of the Torpedo and the human clones, strongly suggesting that they have a common ancestral origin. A higher
level of conservation was found at the amino acid level (Fig. 3B) than at the nucleotide level (Fig. 3A).

The cDNA inserts were 32P-labeled and hybridized with human RNA and DNA. In low-stringency RNA blots loaded with 10 μg of poly(A)+ RNA per lane, 32P-labeled FBChE12 interacted with a single 2.5-kb band of RNA, of a similar size as the FL39 clone. This mRNA was present in fetal brain and liver, but not in the cholinesterase-deficient human epidermoid carcinoma (HEp), which does not express any type of cholinesterase activity (ref. 13, Fig. 4A).

The levels of the mRNAs coding for particular types of cholinesterase in fetal brain and liver were analyzed in parallel by mRNA microinjection into Xenopus oocytes, where AcChoEase mRNA and BtChoEase mRNA are translated to yield their catalytically active enzyme products (13). Considerable production of iso-OMPA-insensitive AcChoEase was observed in oocytes injected with either fetal brain or liver RNA but not with HEp RNA. In contrast, only liver mRNA was capable of producing significant levels of BW284C51-insensitive BtChoEase (Fig. 4A). Thus, the pattern revealed in the RNA blot hybridization (Fig. 4B) is compatible with the levels of both species of cholinesterase mRNA together, but not with BtChoEase mRNA alone.

A DNA blot hybridized with [32P]FBChE12 is presented in Fig. 5. The 32P-labeled cholinesterase cDNA insert hybridized with two distinct human DNA fragments derived by digestion with EcoRI, 4.7 and 2.5 kb in length. When FL39 was used as a probe, an additional band of ca. 10 kb was revealed (not shown). Taking into account the existence of the unique EcoRI site in the original FBChE12 and FL39 cDNA clones (Fig. 1), this indicates the presence of at least one intervening sequence within the gene. Comparison of the signal intensity observed with 20 μg of genomic DNA to that detected with 1.0 ng of agt10-FBChE12 (Fig. 5) indicates that the DNA sequences hybridizing with FBChE12 and FL39 are not present in the human genome in many copies. Parallel

**Fig. 4. In ovo translation and blot hybridization of cholinesterase mRNA from fetal human tissues.** (A) Translatable cholinesterase mRNAs as measured in microinjected *Xenopus* oocytes. Poly(A)+ RNA was prepared from fetal brain and liver (18 weeks gestation) and from cholinesterase-deficient human epidermoid carcinoma (HEp) grown in nude mice (13) by extraction in guanidine thiocyanate followed by two rounds of oligo(dT)-cellulose chromatography (15). Fifty nanograms of each poly(A)+ RNA was injected into each of 10 oocytes for a 20-hr incubation at 17°C (13), and the resulting cholinesterase activity was determined by measuring the rate of hydrolysis of [3H]acetylcholine (5, 13) in oocyte extracts and incubation medium, in the presence of 0.1 mM tetraisopropyl pyrophosphoramide (iso-OMPA) or 10 μM 1,5-bis(4-allyldimethylammonium)phenyl)pentan-3-one dibromide (BW284C51) so as to selectively block the activities of BtChoEase and of AcChoEase, respectively (13, 14). The background release of [3H]acetate in oocyte-free samples was subtracted. Activities induced in oocytes per μg of injected RNA were calculated in nmoi of acetylcholine degraded per hr, and the activity measured in control sham-injected oocytes is presented in parallel. (B) RNA blot hybridization of cholinesterase mRNAs with 32P-labeled human cholinesterase cDNA. FBChE12 DNA was prepared as described in Methods and was 32P-labeled by nick-translation (30). Ten-microgram samples of poly(A)+ RNA from fetal brain and liver and from HEp were fractionated by agarose gel electrophoresis, blotted onto a nitrocellulose filter, hybridized with the 32P-labeled DNA probe, and washed four times, 20 min each, at 50°C in 0.45 M NaCl/0.045 M sodium citrate/0.1% NaDodSO4 (31). The filter was exposed for 10 days, using an intensifying screen (Cawo). Electrophoretic migration of ribosomal RNA (28S and 18S) is marked. RNA blot hybridization with 32P-labeled FL39 DNA gave similar results (not shown).

**Fig. 5. DNA blot hybridization with 32P-labeled human cholinesterase cDNA.** The indicated amounts of human and mouse genomic DNA and of the agt10 phage DNA carrying the FBChE12 insert (clone 12a) were digested with the enzyme EcoRI (E) with or without either BamHI (B) or Xho I (X), electrophoresed in a neutral 0.8% agarose gel, and blotted onto a nitrocellulose filter (15). Hybridization was performed at 42°C for 48 hr with 3 × 10^6 dpm of 32P-labeled FBChE12 DNA at a specific activity of 2 × 10^6 dpm/μg, in 50% (vol/vol) formamide/10% dextran sulfate/0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5, 750 mM NaCl/75 mM sodium citrate/75 μg of herring sperm DNA per ml, adjusted with HCl to a final pH of 6.5. The blot was washed in 15 mM NaCl/1.5 mM sodium citrate at 30°C (four times, 30 min each), and exposed for 3 days with an intensifying screen.
analysis of mouse DNA revealed a significant but not intense hybridization, suggesting the existence of homologous cholinesterase DNA sequences in other mammalian species.

**DISCUSSION**

The polypeptide inferred by the nucleotide sequences of FBChE12 and FL39 shares amino acid sequences with human BtChoEase (7), which is, indeed, present in the fetal human brain (5) and liver (14). In addition, the FBChE12 and FL39 fragments appear to be derived from a relatively small region in the human genome. Under low-stringency conditions, they hybridized with equal efficiency with mRNA from preparations rich in either fetal AcChoEase mRNA or BtChoEase mRNA. The most straightforward explanation for these findings is that these clones were reverse-transcribed from BtChoEase mRNA.

According to these findings, the cholinesterase(s) encoded by the FBChE12 and the FL39 clones are produced from one or a few genes but not from a multigene family. Differential splicing or alternative transcription of a single gene, or independent expression of a few related genes, can direct the synthesis of several highly homologous cholinesterase mRNAs, differing in limited domains [e.g., N terminus (26)]. This is compatible with the finding of intervening sequences within this human gene.

**Note Added in Proof.** While this manuscript was being processed, we received a manuscript describing the complete amino acid sequence of human serum cholinesterase as determined by peptide sequencing by Lockridge and co-workers (32). The complete identity of the two sequences as derived by DNA and by peptide sequencing proves beyond doubt that the isolated cDNA codes for human BtChoEase, and we thank Dr. Lockridge for providing us with this information at the galley proof stage.

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