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Two Distinct Proteins Are Associated with Tetrameric Acetylcholinesterase on the Cell Surface*

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In mammalian brain, acetylcholinesterase (AChE) exists mostly as a tetramer of 70-kDa catalytic subunits that are linked through disulfide bonds to a hydrophobic subunit P of approximately 20 kDa. To characterize P, we reduced the disulfide bonds in purified bovine brain AChE and sequenced tryptic fragments from bands in the 20-kDa region. We obtained sequences belonging to at least two distinct proteins: the P protein and another protein that was not disulfide-linked to catalytic subunits. Both proteins were recognized in Western blots by antisera raised against specific peptides. We cloned cDNA encoding the second protein in a cDNA library from bovine substantia nigra and obtained rat and human homologs. We call this protein mCutA because of its homology to a bacterial protein (CutA). We could not demonstrate a direct interaction between mCutA and AChE *in vitro* in transfected cells. However, in a mouse neuroblastoma cell line that produced membrane-bound AChE as an amphiphilic tetramer, the expression of mCutA antisense mRNA eliminated cell surface AChE and decreased the level of amphiphilic tetramer in cell extracts. mCutA therefore appears necessary for the localization of AChE at the cell surface; it may be part of a multicomponent complex that anchors AChE in membranes, together with the hydrophobic P protein.

Mammalian acetylcholinesterase (AChE)[1] possesses alternative C-terminal R, H, or T peptide sequences that define its post-translational maturation, quaternary associations, and anchoring: $AChE_R$ may generate soluble monomers, $AChE_H$ generates glycolipid-anchored homodimers, and $AChE_T$ generates an array of homo- and hetero-oligomers, including collagen-tailed molecules (1).

In mammalian brain, tetramers of $AChE_T$ subunits are anchored in the membrane through a hydrophobic protein of 20 kDa (2, 3), which has been named subunit P (4). The proportion of membrane-bound tetramers increases steadily during development and becomes predominant in the adult (5). These amphiphilic tetramers (G_4^a) form clusters on the surface of cultured neurons (6) and represent the physiologically active AChE species (7–9). The heteromeric structure of these molecules formally resembles that of collagen-tailed forms; in both cases, the catalytic subunits are linked in pairs by disulfide bonds through the cysteine residue located at position −4 from the C terminus of the T peptide; two subunits are linked directly to each other, and the other two are attached to a structural subunit (10, 11). In the membrane-bound tetramers from mammalian brain, two subunits are linked to subunit P. Thus, electrophoresis of the denatured protein without reduction separates light dimers, with two catalytic AChE subunits only, and heavy dimers, which contain two catalytic subunits linked to subunit P. In collagen-tailed AChE this arrangement is reproduced with the PRAD, the proline-rich attachment domain of the ColQ subunit (12, 13), replacing subunit P. The PRAD not only is linked through disulfides to two catalytic subunits but also interacts noncovalently with each of the four T-peptide sequences in a tetrameric cluster (14). When $AChE_T$ subunits are expressed alone in transfected COS cells or *Xenopus* oocytes, no AChE appears on the cell surface; AChE surface expression does occur when $AChE_T$ subunits are cotransfected with a chimeric membrane-bound PRAD (14, 15). In mice in which the exon encoding the PRAD of ColQ is deleted by homologous recombination, extracts from brain and muscle contain a G_4^a AChE form, suggesting that a gene different from ColQ can be involved in anchoring tetrameric AChE at the cell surface (16).

In order to understand the biosynthetic regulation of physi-

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[1] The abbreviations used are: AChE, acetylcholinesterase; $AChE_R$, $AChE_H$, and $AChE_T$, AChE subunits of type R, H, and T, respectively; EST, expressed sequence tag; mCutA, mammalian copper tolerance analog; GST, glutathione *S*-transferase; GFP, green fluorescent protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HPLC, high pressure liquid chromatography; TID, trifluoromethyliodophenyldiazirine.

ologically active AChE in brain and muscle, it is necessary to analyze its membrane anchoring. We report here the purification and cloning of a 20-kDa protein that was present in several AChE preparations from bovine brain. This protein is homologous to the *Escherichia coli* CutA protein (copper tolerance A) (17) and has already been cloned from various sources. We discuss the structure and expression of the mammalian gene, mCutA, and we compare its homology with proteins from other organisms, including bacteria. We also identify subunit P as a distinct protein by immunoblotting with a specific anti-peptide antibody. Finally, we show that expression of an mCutA antisense mRNA in neuroblastoma cells eliminated AChE accumulation on the cell surface and reduced the level of G_4^a AChE.

MATERIALS AND METHODS

Purification of AChE from Bovine Brain—AChE was solubilized from fresh or frozen bovine caudate nuclei with 1% Triton X-100 and purified by affinity chromatography with an acridinium resin (2). The high purity of the preparations was indicated by the observation that all bands detectable by SDS-polyacrylamide gel electrophoresis and silver staining prior to disulfide reduction contained AChE catalytic subunits.

Isolation of 20-kDa Proteins and Determination of Peptide Sequences—Two approaches were taken to obtain peptide sequences from subunit P. In the first approach, purified AChE was reductively radiomethylated with sodium cyanoborohydride and 10 mM ^{14}C-labeled formalin (Amersham Pharmacia Biotech) by the procedure of Haas and Rosenberry (18) and dialyzed extensively. This procedure converts primary amine groups in lysine and N-terminal residues to radiolabeled dimethyl amino groups. ^{14}C-Methylated bovine brain AChE was reduced by boiling for 5 min in sample buffer containing 40 mM dithiothreitol and 4% SDS and alkylated with iodoacetamide. Preparative electrophoresis was performed in a 6.5-cm cylindrical 10% Tricine gel (approximately 20 ml) with a 1.5-cm stacking gel (19), contained in a model 491 preparative cell, graciously lent by Dr. S. Dyviniak (Bio-Rad). The upper tank buffer was 0.1 M Tris-HCl, 0.1 M Tricine, 0.1% SDS, pH 8.3 (not adjusted). The lower tank buffer was 0.2 M Tris-HCl, pH 8.9, and the elution buffer was 20 mM Tris-HCl, 0.2% SDS, pH 8.9. Electrophoresis was at 20-mA constant current (approximately 50 V) for the first hour and thereafter at 40-mA constant current. Proteins electrophoresing off the lower end of the gel were eluted with elution buffer at the rate of 0.08 ml/min and collected in fractions of 17 drops. Fluorographic analysis of samples of seven pools of fractions after SDS-polyacrylamide gel electrophoresis on slab gels (19) revealed a single diffuse radiolabeled band in each pool with progressively increasing sizes from 3 to 22 kDa, perhaps reflecting varying extents of proteolysis of a 20-kDa anchor component. The pool that contained the highest amount of radioactivity, corresponding to a band of about 16 kDa, was taken for further analysis. The samples were concentrated 5-fold, dialyzed against 10 mM sodium phosphate, 0.01% SDS (pH 7), and denaturated with 30% acetonitrile, which was decreased to 5% acetonitrile by evaporation (20) before digestion with 2% (w/w) trypsin (sequencing grade; Roche Molecular Biochemicals) overnight at 37 °C in 0.1 M *N*-tris(hydroxymethyl)-2-aminoethanesulfonate (pH 8.0), 1.5 mM calcium acetate. Digests were fractionated by HPLC (Beckman Gold, in a Vydac C18 column) with a gradient from 5 to 70% acetonitrile (containing 0.05% trifluoroacetic acid) and water (containing 0.06% trifluoroacetic acid).

A second approach was taken in another series of experiments. Purified bovine brain AChE was selectively reduced with 1 mM dithiothreitol in the absence of denaturants and alkylated with [^{14}C]iodoacetamide. This procedure has been shown to reduce and alkylate only sulfhydryl groups involved in intersubunit disulfide bonding in brain AChE (10). Labeled samples were subjected to electrophoresis in Tris-Tricine gels. Electroblotted bands in the 20-kDa region were sequenced directly, or tryptic peptides were isolated by HPLC and sequenced (10). A similar procedure had been previously used to obtain sequence from the collagen-like subunits associated with *Torpedo* AChE (21).

The sequences of isolated peptide fractions were determined with by gas phase sequencing (Applied Biosystems model 477A). Radiomethylated lysine residues were identified by scintillation counting of aliquots from effluent fractions.

cDNA Libraries and cDNA Cloning—We used cDNA libraries of bovine substantia nigra (gift of Dr. M. Bronstein, NIMH, Bethesda, MD) and reinnervated rat soleus muscle (13). The bovine library was made in pCD3 plasmids, and the rat soleus muscle cDNA library was made in pCDM8 plasmids. Both libraries were grown in *E. coli* in approximately 80 pools of 8000 clones each. Plasmidic DNA prepared from each pool was digested by *Bam*HI (pCD3) or *Xho*I (pCDM8), loaded on an agarose gel, and transferred to a nylon membrane for Southern blotting. The membranes were hybridized in tetramethylammonium chloride (22) with degenerate oligonucleotides deduced from peptide sequences (Table I). Hybridization was performed at 37 °C, and washing was as follows: twice at 4 °C for 30 min in 5× SSC, twice in 3 M tetramethylammonium chloride at 20 °C, and twice at 51 °C for 20 min. in 3 M tetramethylammonium chloride. One positive clone was isolated by serial dilution of the positive pools. Screening of the membranes with a probe derived from this clone yielded several other positive signals, from which the longest were isolated. The cDNA corresponding to the longest insert is described. The BLAST program (23) was used for screening the NCBI and Sanger *Caenorhabditis elegans* data bases (available on the World Wide Web).

DNA Constructs and Expression—For *in vitro* expression experiments, DNA fragments containing the rat and bovine coding sequences were cut between *Xba*I and *Hin*dIII restriction sites and introduced in the pcDNA3 vector (Invitrogen). The fusion with glutathione *S*-transferase (GST) was made using the *Bam*HI site in pGEX-2TK (Amersham Pharmacia Biotech). The FLAG peptidic epitope (DYKDDDDK) was introduced at the C terminus of the rat protein by polymerase chain reaction. GST fusion proteins were expressed in *E. coli* and purified on a glutathione resin (Amersham Pharmacia Biotech) according the manufacturer's protocol.

A 0.785-kilobase pair insert of the complete coding sequence of the rat mCutA was introduced in antisense orientation in the expression plasmid pTracer SV40 (catalogue number V871–20; Invitrogen). This vector controls the production of a fusion protein GFP-zeocin to select the transfected cells.

Preparation of Antibodies—Human erythrocyte AChE that had been purified by affinity chromatography (24) was used to prepare a rabbit antiserum, with the assistance of Dr. Linda Younkin (Mayo Clinic Jacksonville). Since erythrocyte AChE corresponds to $AChE_H$ and contains no separate membrane attachment subunits, this antiserum interacts only with catalytic subunits. It also cross-reacts with AChE from other mammalian species, as shown by immunofluorescence studies of AChE in mouse neuromuscular junctions (25). A rabbit antiserum denoted C17V against a synthetic peptide corresponding to an internal sequence deduced from the cloned mCutA cDNA with an added N-terminal cysteine (CTSIYEWKGKIEEDSEV) was prepared by coupling the peptide to carrier protein (bovine serum albumin) and injecting into rabbits (Biocytex, Marseille, France). Antibodies against a second peptide GVDANSAVEYPMT, denoted G13T, were purified from rabbit antiserum by adsorption on the immobilized peptide and elution at pH 2.8.

Gel Electrophoresis and Western Blots—AChE samples in gel loading buffer (6.25% glycerol, 4% SDS, 30 mM Tris-HCl, 250 mM NaCl, 0.01% bromphenol blue, pH 6.8; with or without 40 mM dithiothreitol as indicated) were incubated at 50 °C for 5 min. Samples were then subjected to electrophoresis in precast Tris-HCl criterion 4–15% linear acrylamide gradient gels (Bio-Rad) with 192 mM glycine, 25 mM Tris, and 0.1% SDS (pH 8.5) as the electrophoresis buffer. Gel electroblotting onto an Immobilon-P membrane (Millipore Corp., Bedford, MA) was conducted in a Bio-Rad Mini Trans-Blot apparatus. After transfer, blots were blocked by soaking 1–3 days in 50 mM Tris-H_3PO_4, 150 mM NaCl (pH 7.5), and 10% newborn calf serum and incubated for 2 h with primary antibodies as described. Blots were then washed for 30 min in blocking buffer containing 0.01% Tween 20, incubated with anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Life Technologies, Inc.) (diluted 1:20,000), and washed further for 3–6 days. The blots were then developed for enhanced chemiluminescence with SuperSignal West Dura (Pierce).

Cell Cultures—COS cells were transfected by the DEAE-dextran method, as described previously (26). The cells were maintained at 37 °C and extracted 2–4 days after transfection.

The murine neuroblastoma cell line, N18TG2 (kindly provided by M. Thomasi and M. Israël), was maintained in a water-saturated atmosphere containing 5% CO_2, and cells were mechanically dislodged for passages. The culture medium (Dulbecco's modified Eagle's medium; Life Technologies) was supplemented with 10% decomplemented fetal bovine serum, 2 mM glutamine, and 1 mM sodium pyruvate. Prior to experiments, cells were grown for 5 days in tissue culture flasks (75 cm^2) or 35-mm Petri dishes without changing the culture medium, which was supplemented with 2% Me_2SO to induce differentiation.

Stable Transfection of Rat mCutA Antisense cDNA—N18TG2 cultures were transfected with either 1 μg of mCutA antisense vector or 1 μg of pTracer-SV40 without the insert as a control, using 3 μl of

TABLE I
Tryptic peptide sequences from purified brain AChE that correspond to mCutA

Radiomethylated AChE was reduced and run on preparative SDS-polyacrylamide gels, and tryptic peptides from fractions corresponding to about 16 kDa were isolated and sequenced as outlined under "Materials and Methods."

FVT*X*PNEKVAKEIAR
AVVEKR
LVPQI
LVPALTDFV
SVHPYEVAEVIALPVEQGNSPYL
QVTESVPDS

Fugene6 transfection reagent (Roche) (27). Stably transfected clones were selected with 300 μg/ml zeocin for ~20 days and visually checked for the expression of the fusion protein GFP-zeocin.

Cell Extracts—The surface of the cell monolayer was washed once with Dulbecco's phosphate-buffered saline (PBS), and cells were then mechanically dislodged in PBS and pelleted. The pellet was homogenized by repeated pipetting and 30 s in a glass-Teflon Potter-Elvehjem homogenizer in extraction solution (25 mM Tris-HCl, pH 7, 10 mM $MgCl_2$, 2 mM benzamidine, 1% Brij 96, 0.8 M NaCl, 20 μg/ml pepstatin, 40 μg/ml leupeptin). The extracts were clarified by a short centrifugation facilitate the determination of AChE activity (28).

Sedimentation Analysis—Sedimentation analysis of AChE forms was performed in 5–20% (w/v) sucrose gradients containing 0.8 M NaCl, 50 mM Tris-HCl, pH 7, 10 mM $MgCl_2$, and 1% Brij 96 that were centrifuged at 40,000 rpm in an SW41 rotor (Beckman Instruments, Fullerton, CA) at 7 °C for 16 h. AChE activity was assayed by the colorimetric method of Ellman *et al.* (28) and normalized to the total extracted protein determined by a BCA assay (Pierce). Sedimentation coefficients were deduced by a linear relationship from the positions of internal marker proteins alkaline phosphatase (6.1 S) and β-galactosidase (16 S) from *E. coli*.

Immunocytochemistry—Microscope slides were coated with poly-L-ornithine (10 μg/ml) in PBS for 48 h at 37 °C. Cells were attached to slides, and the slides were first washed once in PBS and then preincubated in protein block serum-free ready-to-use solution (DAKO) for 15 min at room temperature. Samples were incubated with A63 primary antibody (rabbit polyclonal anti-rat AChE (29)) diluted 1:300 or with the M2 anti-FLAG monoclonal antibody at 7 ng/ml (Eastman Kodak Co.) in Dulbecco's Tris-buffered saline containing 0.1% bovine serum albumin (TBS-BSA) for 1 h at room temperature. The cells were washed and then incubated with the secondary antibody (rhodamine-conjugated anti-rabbit or fluorescein anti-mouse IgG 1:250 in TBS-BSA) for 45 min at room temperature in the dark. After washing, samples were mounted in a glycine-containing mounting solution (Fluoprep, Biomérieux). No staining was observed in controls where the primary antibody was omitted.

RESULTS

Determination of Peptide Sequences of Small Proteins, Co-purified with G_4^a AChE from Bovine Brain—Initial sequence information on 20-kDa proteins that are candidates for subunit P was obtained after selective reduction of AChE purified from bovine caudate nucleus and electrophoresis on Tricine-SDS-polyacrylamide gels. Large quantities of up to 400 μg of protein per lane were necessary to obtain sequences from electroblotted bands in the 20-kDa region. We thus obtained an N-terminal sequence EPQKS*X*SKVTDS and a tryptic peptide sequence GVDANSAVEYPMT. In order to isolate larger amounts of the 20-kDa peptides, we ran radiomethylated and reduced brain AChE on preparative SDS-polyacrylamide gels and collected individual fractions. Several tryptic peptides were isolated and sequenced from a labeled peak fraction corresponding to about 16 kDa, as shown in Table I.

Cloning of Bovine and Rat cDNAs Encoding mCutA—We screened cDNA libraries from bovine substantia nigra with degenerate oligonucleotides deduced from the peptide sequences in Table I. A first screening of the library with oligonucleotides allowed us to identify a partial cDNA clone, which was then used as a probe for a further screening of the bovine library and of a rat soleus library. We thus obtained complete bovine and rat cDNAs. The deduced protein, mCutA, corresponded to 19 kDa and included several sequenced peptides (*underlined* in Fig. 1), indicating that we cloned a 20-kDa protein that was present in our purified AChE preparations. It is noteworthy that the same cDNA was cloned by similar procedures from human brain AChE purified by a different affinity chromatography protocol (30).

Partial cDNA clones (EST) corresponding to mCutA have also been found in a number of mammalian tissues (brain, placenta, and several other organs). Fig. 1 shows the alignment of bovine and rat peptide sequences deduced from cDNAs. The human mCutA gene, also called *CUTA*, is localized in a BAC (AL050332) on chromosome 6 (6p21.3), as reported (30). We analyzed the structure of the human gene by mapping ESTs to the cosmid. We found alternative splicing, generating at least four different transcripts that may be translated into three putative proteins (Fig. 1).

The mCutA cDNA that was found in rat, bovine, and human encodes a hydrophobic N-terminal sequence that is predicted to represent either a cleavable secretion signal, a mitochondrial import signal, or a transmembrane anchor of type II (with a cytoplasmic N terminus). The bovine and rat proteins are very similar, except that in addition to two conserved cysteines, the rat protein contains two additional cysteines, located in the N-terminal hydrophobic sequence. The presence of cysteines in the mature protein might allow the formation of disulfide bonds with $AChE_T$, but the cloned protein contains no peptidic motif resembling the binding domain of the collagen tail, the proline-rich attachment domain, in which two adjacent cysteines are followed by a series of prolines (12, 14).

Western Blot Analysis of AChE-associated Proteins—The mCutA sequence includes the six peptides shown in Table I but not the two peptides EPQKS*X*SKVTDS and GVDANSAVEYPMT from the initial electroblots. Screening of cDNA libraries, rapid amplification of cDNA ends, and reverse transcriptase-polymerase chain reaction analyses did not reveal alternative transcripts, originating from the mCutA gene, that could encode these peptides. In addition, a cosmid containing the human mCutA gene did not contain corresponding sequences. These data suggest that at least two distinct proteins were present in highly purified AChE preparations. In order to understand their relationship with the enzyme, we generated two antisera, one (C17V) to an mCutA peptide and the other (G13T) to the peptide GVDANSAVEYPMT that presumably is in the second protein. With these antibodies, we analyzed purified AChE preparations by Western blots.

Analysis of nonreduced and reduced brain AChE samples on SDS-polyacrylamide gels revealed a variety of components. In a nonreduced sample (Fig. 2*A*, *lane 1*), antibodies against AChE catalytic subunits labeled light monomers (60 kDa), a small amount of heavy monomers (80 kDa), light and heavy dimers (140 and 160 kDa), and higher oligomers, as described previously (2). Disulfide reduction decreased the intensity of most heavy bands and slightly retarded the migration of light monomers (Fig. 2*A*, *lane 2*), in agreement with the presence of three intrasubunit disulfide bonds (1). These patterns correspond to those obtained by silver staining bands of purified brain AChE on SDS-polyacrylamide gels (2).

Fig. 2*B* (*lane 1*) shows that, in the absence of reduction, the polyclonal antibodies G13T, directed against the peptide GVDANSAVEYPMT, mostly labeled heavy dimers and other heavy bands to a lesser degree. This pattern is similar to that previously reported with the monoclonal antibody 132-6, prepared against heat-denatured human brain AChE, which recognizes subunit P when it is disulfide-linked to catalytic sub-

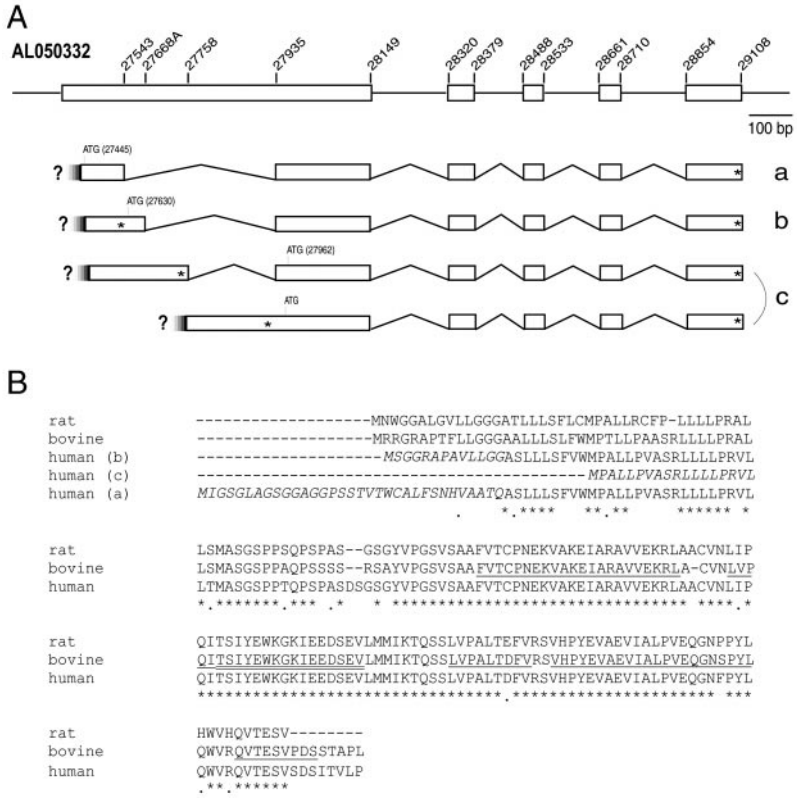

FIG. 1. **A Genomic map of human DNA (AL050332) in the region encoding CutA.** Four alternative splice variants with corresponding exons were found several times in the human EST data base. The limits of the splice sites are noted on the map. The first ATG of the long open reading frame, preceded by a stop codon in frame, is noted. The position of the transcription initiation site is not known, and the *shaded boxes* correspond to the longest ESTs found. The four alternative splice variants may produce three proteins denoted *a*, *b*, and *c*. *B*, amino acid sequences deduced from cDNA clones of mCutAs. The peptide sequences obtained after tryptic cleavage of the labeled 20-kDa protein are *underlined*; each lysine residue indicated in the *singly underlined* sequenced peptides was confirmed to be radiomethylated. The peptide used for antibody production is *doubly underlined*. The alignment includes the three alternative N-terminal sequences deduced from the corresponding annotated ESTs: *a* (DS52710), *b* (AW163654), and *c* (CAB63779).

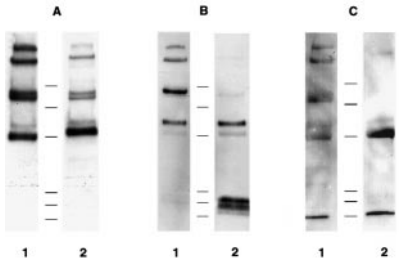

FIG. 2. **Western immunoblots of purified AChE from bovine brain.** *Lanes 1*, nonreduced; *lanes 2*, reduced with dithiothreitol. Samples containing 0.6 μg (*lanes A*), 1.8 μg (*lanes B*) or 16 μg (*lanes C*) of AChE were subjected to gel electrophoresis in 0.1% SDS, and electroblots were incubated with the following antibodies: erythrocyte AChE antiserum (diluted 1:3000) (*lanes A*); G13T antibodies (diluted 1:400) (*lanes B*); C17V antiserum (diluted 1:400) (*lanes C*). Following exposure to horseradish peroxidase-labeled secondary antibodies, bands were developed by enhanced chemiluminescence. The gel migrations of molecular weight standards (Life Technologies BenchMark Prestained Protein Ladder) are indicated by *lines* and correspond to apparent masses of 185.4, 118.9, 62.1, 22.4, 15.4, and 10.1 kDa in descending order.

units (31). In a reduced sample (Fig. 2*B*, *lane 2*), G13T recognized a doublet or a triplet of about 15–17 kDa. The banding patterns in *lanes 1* and *2* of Fig. 2*B* are identical to those in autoradiographs obtained after labeling bovine brain AChE with the hydrophobic photoactivated reagent ^{125}I-labeled TID (2, 3, 32). Since this reagent essentially labels only subunit P (2, 3, 32), these patterns provide strong evidence that G13T recognizes subunit P.

Fig. 2*C* shows that the C17V antiserum, directed against mCutA, recognized a low molecular mass band of around 10 kDa, which was observed in nonreduced as well as in reduced samples and therefore was not disulfide-linked to AChE. Because of the high sensitivity of the enhanced chemiluminescence detection system and the fact that it was not affinity-purified, this antiserum also showed nonspecific adsorption to the heavily loaded AChE bands and to the molecular weight standards (not shown). Nevertheless, the observation of the 10-kDa bands is very significant because they are revealed only by the C17V antiserum.

These experiments confirm that mCutA and subunit P are distinct proteins. In order to understand the relationship between the two proteins and AChE, we performed various biochemical analyses.

The C17V Antiserum Does Not Recognize mCutA in Native G_4^a AChE—We employed sedimentation in sucrose gradients and electrophoresis under nondenaturing conditions, in the presence of various detergents, to examine a possible interaction between C17V antibodies and brain AChE. With both purified enzyme preparations and freshly solubilized extracts, we failed to obtain a shift in the AChE sedimentation coefficient or electrophoretic mobility that would provide direct evidence for the presence of a complex containing G_4^a AChE and mCutA. This lack of antibody interaction may simply be due to the fact that C17V antibodies do not recognize or gain access to the peptide epitope in the native AChE structure.

The mCutA Protein Does Not Interact with AChE in Vitro—In order to test whether mCutA might interact with AChE, we incubated recombinant mCutA-GST fusion protein produced in *E. coli* with a detergent extract from *Xenopus* oocytes expressing $AChE_T$ (13) and with purified bovine brain AChE. No significant AChE activity was retained on microtiter plates coated with a monoclonal antibody against GST or on a GST-binding affinity resin (Amersham Pharmacia Biotech).

Expression of mCutA Protein in COS Cells—We co-expressed the rat mCutA with $AChE_T$ in transiently transfected COS cells. In order to visualize the recombinant protein, we added a FLAG epitope at its C terminus. The C terminus should be maintained in the mature protein, because a C-terminal peptide was recovered in purified AChE (Fig. 1 and Table I). We observed an intense staining in permeabilized cells. However, the FLAG epitope of the mCutA protein was not exposed on the outer surface in nonpermeabilized cells (data not shown), sug-

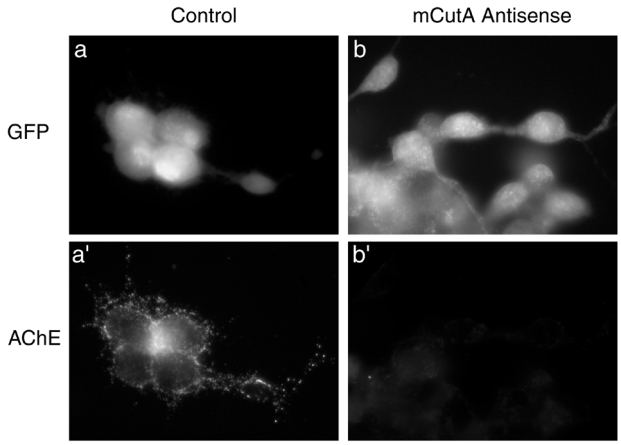

FIG. 3. **Cell surface AChE in neuroblastoma cell line N18TG2.** Cells were transfected with the pTracer vector with or without an mCutA antisense insert. This vector encodes a GFP-zeocin fusion protein, allowing detection of transformed cells by their green fluorescence (*a* and *b*). Cell surface AChE was visualized with A63 antibody on nonpermeabilized cells transfected with the control vector (*a′*). On nonpermeabilized cells transfected with the mCutA antisense vector, almost all A63 antibody labeling disappeared (*b′*).

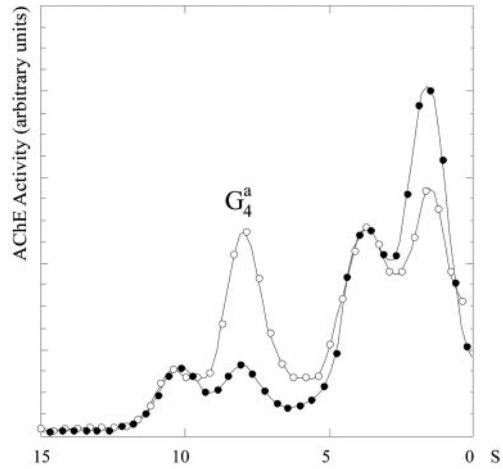

FIG. 4. **Sedimentation analysis of AChE molecular forms in transfected neuroblastoma cells.** Cells transfected with control vector (*open symbols*) or vector with the mCutA antisense insert (*closed symbols*) were extracted in detergent-containing buffer and loaded on 5–20% sucrose gradients containing 1% Brij 96. In these gradients, AChE amphiphilic tetramers (G_4^a) interact with detergent micelles and migrate at 8.5 S, distinct from nonamphiphilic tetramers (G_4^{na}) that do not interact with detergent and migrate at 10.5 S. We observe a specific decrease of the G_4^a form in the cells expressing antisense mCutA, whereas the level of the G_4^{na} form does not change.

```
rat mCutA          --------------MNWGGALGVLLGGGATLLLSFLCMPALLRCFP-LLL
bovine mCutA       --------------MRRGRAPTFLLGGGAALLLSLFWMPTLLPAASRLLL
E. coli_CutA       ----------------------------------------------------
A. thalianaCutA    MASSLTTRLSAVIGSSRRSFPIVGAPCVLSTLSISSLSSSSPFKSGCAQSF

rat mCutA          LPRALLSMASGSPPSQPSPASGSGYVPGSVSAAFVTCPNEKVAKEIARAV
bovine mCutA       LPRALLSMASGSPPAQPSSSSRSAYVPGSVSAAFVTCPNEKVAKEIARAV
E. coli_CutA       -------------------MLDEKSSNTASVVVLCTAPDEATAQDLAAKV
A. thalianaCutA    SVVPLLRSKFSSKAFSSSIRMEESSKTVPSIVVYVTVPNREAGKKLANSI
C. elegans         ----------------------------MVVAYVTAPSKEVAMTVARTT
                                                 * *    . .*  .

rat mCutA          VEKRLAACVNLIPQITSIYEWKGKIEEDSEVLMMIKTQSSLVPALTEFVR
bovine mCutA       VEKRLA-CVNLVPQITSIYEWKGKIEEDSEVLMMIKTQSSLVPALTDFVR
E. coli_CutA       LAEKLAACATLIPGATSLYYWEGKLEQEYEVQMILKTTVSHQQALLECLK
A. thalianaCutA    VQEKLAACVNIVPGIESVYEWEGKVQSDSEELLIIKTRQSLLEPLTEHVN
C. elegans         VTEALAACANVIPEVTSVYKWQGKIEEDQEHVVILKTVESKVEELSQRVR
                   .  .** * ...*   *.* * **.. . *  ...**  *    * . .

rat mCutA          SVHPYEVAEVIALPVEQGNPPYLHWVHQVTESV--------
bovine mCutA       SVHPYEVAEVIALPVEQGNSPYLQWVRQVTESVPDSSTAPL
E. coli_CutA       SHHPYQTPELLVLPVTHGDTDYLSWLNASLR----------
A. thalianaCutA    ANHPYDVPEVIALPITGGSDKYLEWLKNSTRN---------
C. elegans         SLHPAETPCFFTLAIDKITPDFGGWIVDSTNSSTAKH
                   . **    ... *..        *.
```

FIG. 5. **Alignment of rat, bovine, and human mCutA with other CutA-like proteins.** Amino acid sequences from rat and bovine mCutA proteins were aligned with *E. coli* CutA, *A. thaliana* and *C. elegans* CutA isologs, using the ClustalW program (36). *Asterisks* indicate residues that are conserved in all sequences, and *dots* indicate residues that belong to the same class (hydrophobic, charged) in all species.

gesting that mCutA is not a type II transmembrane protein. When co-expressed with $AChE_T$, mCutA did not modify either the cellular distribution of the enzyme or its molecular forms, as determined by sedimentation analysis. These experiments therefore failed to reveal any interaction between the two proteins when co-expressed in COS cells.

Expression of Antisense mCutA mRNA Eliminates AChE from the Cell Surface—To examine whether mCutA is involved in the anchoring of AChE at the cell surface, we used the N18TG2 neuroblastoma cell line that is known to produce AChE (33). We selected cell lines transfected either with an mCutA antisense-containing vector or with the empty vector as a control. This vector contains a GFP-zeocin fusion protein transcription unit to aid in selection of the transfected cells that are labeled with GFP. In the control cells, AChE forms clusters at the cell surface (Fig. 3*a′*). In contrast, this was not observed in cells transfected with the mCutA antisense probe (Fig. 3*b′*). In parallel, the quantity of G_4^a AChE decreased in extracts of cells transfected with the antisense probe (Fig. 4), whereas the total AChE activity per mg of extracted protein was unchanged.

Homology of mCutA with Other Proteins—A search of data banks revealed that numerous sequences homologous to mCutA have been obtained from a large variety of sources. Homologous sequences exist in widely different organisms, such as *C. elegans*, plants (*Arabidopsis thaliana*), and several bacteria, including *E. coli*. In general, these sequences were obtained by systematic analysis of transcripts, and no function is known for the corresponding proteins. In *E. coli*, however, the homologous protein is encoded by *CutA1*, one of the three genes of the *CutA* locus, which is involved in tolerance to Cu^{2+} and other heavy metal ions ("Cu tolerance") (17). In Fig. 5, the bovine and rat mCutA proteins are aligned with *E. coli CutA1* and *CutA* isologs from *A. thaliana* (U78721) and *C. elegans* (from cosmid F35G12).

DISCUSSION

An excellent candidate for a distinct membrane-anchoring subunit in brain AChE was first identified after photolabeling the purified enzyme with ^{125}I-labeled TID. This reagent selectively labeled a 20-kDa protein that was disulfide-linked to catalytic subunits and released by disulfide reduction (2, 3, 10, 32); it was designated subunit P (4). Boschetti and Brodbeck (32) reported that the released TID-labeled subunit P corresponded to a doublet of 20-kDa bands that differed only in their extent of glycosylation. Sequencing of these bands revealed the same N-terminal sequence, EPQKSCSKYTD. To analyze further the structure of subunit P, we characterized AChE-associated proteins by peptide sequencing in highly purified preparations. We reduced the AChE and focused on proteins of about 20 kDa, from which we obtained eight different peptide sequences. An N-terminal sequence EPQKS*X*SKVTDS was very similar to that reported previously (32). Our Western blotting in Fig. 2*B* with antiserum G17T directed against another peptide sequence, GVDANSAVEYPMT, showed that this peptide probably belongs to subunit P, because it is disulfide-linked with AChE subunits, especially in a heavy dimer, and forms a doublet of around 20 kDa after reduction. These observations suggest that a single subunit P protein contains both the peptide sequences EPQKSCSK(Y/V)TD and GVDANSAVEYPMT. We have recently obtained a cDNA clone that contains these sequences in three distinct peptide segments,

indicating that the clone probably corresponds to subunit P.[2]

Our data clearly show that, in addition to subunit P, brain AChE preparations contain another protein that includes the six peptides in Table I. This protein was named mCutA after its homology with the *E. coli* CutA gene, which is involved in tolerance to copper and other heavy metals. The human protein was called CUTA (accession number AF106943), and it was proposed to be involved in survival and/or proliferation of glial cells in the central nervous system (34). We cloned this cDNA from bovine substantia nigra and from rat soleus muscle. The bovine and rat cDNAs are very similar, and the encoded proteins share 84% identity (92% if the hydrophobic N-terminal domain is excluded). The expression of mCutA in mammalian tissues appears ubiquitous, as indicated by Northern blots (not shown) and by numerous ESTs of various origins. Moreover, homologs of this protein exist in all eukaryotes, and their sequence is very well conserved, except for the N-terminal hydrophobic domain (55% between plants and mammals). The bacterial protein CutA is devoid of this hydrophobic domain; CutA is cytoplasmic in *E. coli*. The *C. elegans* gene also seems to lack this domain. In the human gene, this region is encoded by alternative exons, producing at least three different N-terminal sequences, as shown in Fig. 1. We have focused our studies on the variant that was cloned in rats, bovines, and humans.

The mCutA protein does not represent an abundant protein in total brain tissue, according to Western blots (data not shown). Therefore, its detection in several purified AChE preparations, obtained independently in several laboratories, seems unlikely to result from coincidental copurification. It is very improbable that this protein interacts with the three distinct affinity ligands employed in these AChE purifications in a way similar to AChE.

We tried to clarify the relationship between AChE and the two proteins, subunit P and mCutA, in various ways. We were not able to demonstrate the existence of a complex of AChE tetramers with mCutA, possibly because the polyclonal mCutA antiserum C17V does not react with the protein in its native conformation. Analysis of denatured AChE by Western blots showed that, unlike subunit P, mCutA is not disulfide-linked to the catalytic subunit. However, genetic perturbation, by expression of the antisense mRNA, showed that mCutA is involved in the accumulation of AChE at the surface of the neuroblastoma cells. This suggests that mCutA participates in a multisubunit complex that requires the presence of other components, notably the subunit P hydrophobic anchor.

It has been proposed that the organization of AChE and associated proteins is similar in the collagen-tailed forms of the enzyme and in the hydrophobic-tailed tetramers, particularly because light and heavy dimers are present in both cases and because the same catalytic subunit cysteines are involved in disulfide linkages between the catalytic and structural subunits (2, 10). In fact, the present results suggest that the membrane-bound form of brain AChE may be a multicomponent complex. It will be of great interest to unravel its molecular composition and structure. In particular, the characterization of subunit P and the functional relationship between mCutA and AChE will require further studies. Our results suggest that mCutA is involved in the stabilization of AChE at the cell surface and/or the clustering of AChE molecules at the surface of neurons (6). This would be analogous with the case of gephyrin, a ubiquitous protein involved in molybdenum metabolism, which also ensures the postsynaptic anchoring of glycine receptors in neurons (35).

[2] A. Perrier and E. Krejci, manuscript in preparation.

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Two Distinct Proteins Are Associated with Tetrameric Acetylcholinesterase on the Cell Surface

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