The catalytic subunits of asymmetric and hydrophobic forms of acetylcholinesterase arise from a single gene by alternative mRNA splicing. Each protein is encoded in three exons, with exons 1 and 2 encoding sequence common to both forms and exons 3A and 3H specifying unique carboxyl-terminal domains. We examined the expression of cDNAs for the two forms by transient transfection in COS-1 cells. The catalytic subunit of the asymmetric form expressed by transfected cells exhibits low activity and is retained within the cell. The cDNA encoding hydrophobic acetylcholinesterase directs the synthesis of enzyme with much greater activity, which is expressed on the outer surface of the cell membrane and can be released by phosphatidylinositol-specific phospholipase C. A mutant truncated acetylcholinesterase which lacks either carboxyl-terminal sequence encoded by the alternative exons is secreted into the medium. An exon 1-3H fusion mutant, created by deletion of coding exon 2 from the hydrophobic form cDNA, is glycophspholipid-linked. The 30-amino acid carboxyl-terminal domain specified by exon 3H appears necessary and sufficient to direct glycophspholipid attachment. Thus, homologous expression of wild-type and mutant acetylcholinesterase proteins indicates that the carboxyl-terminal domains specified by alternative coding exons determine the cellular dispositions of acetylcholinesterase.

Acetylcholinesterase (AChE) occurs as multiple molecular structures which differ in their hydrodynamic properties and modes of extracellular disposition (1). The polymorphic species of AChE can be divided into two classes. One class appears as associations of multiple catalytic subunits that are disulfide-linked to one or more structural subunits (2-5). The best characterized heteromeric forms contain 4, 8, or 12 catalytic subunits disulfide-bonded to a collagen-containing filamentous tail in association with the basal lamina. These forms have been termed the asymmetric or "A" forms because of their dimensional asymmetry. A tetramer of catalytic subunits disulfide-bonded to a collagen-containing A form and the glycophspholipid-anchored H form from Torpedo californica are identical through amino acid 535 and diverge thereafter. The unique carboxyl terminus of H-AChE consists of the dipeptide Ala-Cys, to which the glycophspholipid is attached (9). The divergent carboxyl-terminal domain of the asymmetric catalytic subunit extends for 40 amino acids. Analysis of Torpedo mRNA (10, 11) revealed a divergence in AChE nucleotide sequences in the corresponding coding region. Sequencing of genomic clones (11, 12) has identified two exons, 3A and 3H, which are responsible for the carboxyl-terminal divergence between asymmetric and hydrophobic catalytic subunits. Both A-AChE and H-AChE share the sequence encoded by exon 1 (leader peptide and first 480 amino acids) and exon 2 (amino acids 481-535); alternative usage of the third coding exons, 3A and 3H, respectively, gives rise to unique carboxyl-terminal regions. In the case of the glycophspholipid-anchored form, exon 3H encodes the nascent 30-amino acid carboxyl terminus from which a 28-amino acid peptide is cleaved complementary with addition of the glycophspholipid (9-12). Despite the abundance of the native hydrophobic form of acetylcholinesterase in electric organ, isolation of its cDNA has proved difficult. The screening of a T. californica electric organ library which resulted in eight full-length A form cDNAs yielded nearly 100 candidate clones, and none were cDNAs specifying H-AChE (13). Sikorav et al. (10) also found sequences encoding the catalytic subunit of the asymmetric species to predominate, but were able to find a fragment of the cDNA encoding the hydrophobic form in Torpedo marmorata. The low abundance (~10% of AChE mRNA in electric organ) and the existence of extended tandem repeat sequences in the 3'-noncoding region (12) may account for this result. We circumvented the lack of full-length sequence encoding H-AChE by constructing a cDNA from existing genomic and cDNA clones.

To examine the biosynthesis, secretion, and properties of AChE, we have expressed by transfection A and H form cDNAs and nito directed mutants in COS-1 cells. The expression of these sequences provides final confirmation that alternative mRNA processing is responsible for structural divergence in the acetylcholinesterases. The cellular dispositions of the mutant enzymes also reveal some of the requirements for membrane association and secretion of AChE.

**MATERIALS AND METHODS**

**Nucleic Acid Sequences**—Unless otherwise indicated, manipulation of nucleic acid sequences were performed using commercially
obtained reagents, employing standard techniques (14, 15) or methods specified in manufacturers' protocols.

The Torpedo cDNA encoding the A form was obtained from a partial EcoRI digest (0.1 unit/μg of DNA, 37 °C, 1 h) of ACh10 clone 14 (cf Refs 11 and 13). The full-length 9.9-kb insert was purified by electrophoresis on a 1% low-melt agarose gel followed by extraction of the excised band.

The H13 clone was previously isolated from a HindIII Torpedo liver genomic library in λZAP by screening with a form cDNA sequence (12). A 3.6-kb PstI-EcoRI fragment of this genomic clone was ligated into vector m13mp19, resulting in subclone 19-3c15. Construction 19-3c15 was obtained from 19-3 by deletion subcloning.

Mutagenesis of 19-3c15 was performed by the method of Kunkel et al. (16) using a primer:template uracil-containing DNA template (~0.1 pmol) was annealed with the mutagenic oligonucleotide at 43 °C for 30 min using a primer/template ratio of 1:1. The extension reaction with T4 polymerase was carried out at 43 °C (17) for 90 min. Deletion mutant 19-3c15/1nt was identified by dideoxynucleotide termination sequencing (17). The Torpedo cDNA encoding the A form was obtained from a HindIII Torpedo liver genomic library in λZAP by screening with a form cDNA sequence (12). A 3.6-kb PstI-EcoRI fragment of this genomic clone was ligated into vector m13mp19, resulting in subclone 19-3c15. Construction 19-3c15 was obtained from 19-3 by deletion subcloning.

The COS-1 cell line (American Type Cell Collection) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a 10% CO2 humidified incubator 24-36 h after transfection. All subsequent experimental procedures were carried out within 6-36 h of incubation as indicated for S-labeled samples.

Construction and Mutagenesis of cDNA Encoding Hydrophobic Acetylcholinesterase—The PstI-Smal fragment of the mutagenized genomic sequence was removed from 19-3c15/1nt and ligated into Bluescript SK+ (Stratagene). The Sty1-BamHI fragment of the insert was band isolated on an NA5 DAE cellulose membrane (Schleicher & Schuell). The 5'-BamHI-Sty1 fragment of the A form cDNA (17) and the two fragments were ligated together into BamHI-digested Bluescript SK+. Successful ligation was determined by restriction analysis of alkaline lysine minipreps of ampicillin-resistant colonies. The full-length construction was excised as a 2.4-kb BamHI fragment and cloned into m13mp18 in antisense orientation with respect to the (+)-strand. The complete construction was confirmed by dideoxy sequencing.

Mutant H form sequences were generated by oligonucleotide-directed mutagenesis in m13mp18. For the substitution mutant, uracil-containing template preparation, annealing by slow cooling from 65 °C, and T4 polymerase extension at 37 °C were carried out according to the Mutagene kit protocol. For deletional mutagenesis, annealing of the mutagenic oligonucleotide was performed at 41 °C. The entire coding sequence of each mutant was confirmed by sequencing.

Transfection of AChE Sequences—A, H, and mutant H form sequences were inserted into the mammalian expression plasmid pBD (18) at BamHI sites flanking the cloning region. Recombinants were screened for proper orientation between the SV40 early promoter and polyadenylation sequence by restriction enzyme analysis. Expression plasmids containing both cDNA and E. coli 16S rRNA genes were transcribed by sequential bandings on cesium chloride/ethidium bromide gradients. The COS-1 cell line (American Type Cell Collection) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a 10% CO2 humidified atmosphere.

In general, cells were plated at a density of 1 × 104 cells/100-mm plate 16-20 h prior to transfection. Plasmid DNA (5 μg) was precipitated in ethanol, resuspended at a concentration of 125 μg/ml in Tris-buffered saline (137 mM NaCl, 5.13 mM KCl, 0.025 mM NaHPO4, 1.35 mM CaCl2, 1.05 mM MgCl2, 24.8 mM Tris (pH 7.5)), and added to 2 volumes of DEAE/dextran solution (10 mg/ml in Tris-buffered saline) premixed to ~50 °C. The transfection mixture, diluted 3-fold in serum-free DMEM, was applied to cells rinsed in serum-free DMEM. After incubation at 37 °C for 4 h, the transfection medium was aspirated and the cells were subjected to a 3-min shock in DMEM containing 10% glycero. Following two rinses in PBS, DMEM/fetal bovine serum (10%) was added and the cells were returned to 37 °C for 30 min. For replicate plateings, cells were seeded at 2.5 × 104 cells/150-mm plate, transfected with 12.5 μg of plasmid DNA in the appropriate volumes, and subcultured 16-24 h later onto smaller dishes.

Except where indicated, cells were transferred to a 28 °C, 10% CO2 humidified incubator 24-36 h after transfection. All subsequent experimental procedures were carried out within 0-36 h of incubation at 28 °C.

Measurement of AChE Activity of Transfected Cells—AChE activity was assayed by the method of Ellman et al. (19) 48-72 h after transfection. Cells transfected on 100-mm plates were incubated at 28 °C for 4 h, and then harvested. Plates were placed on ice and rinsed twice in ice-cold PBS. Cells were scraped from the plates, sediments in an Eppendorf microcentrifuge at 3000 rpm at 4 °C for 1 min, and rinsed twice by resuspending in 200 μl of PBS. Final resuspension was in 200 μl of PBS for measurement of cell-surface activity or in 200 μl of PBS with 1% Triton X-100 for measurement of total cellular activity.

Metabolic Labeling and Immunoprecipitation—Prior to labeling with [35S]methionine (~1000 Ci/mmol; Trasylol-label, ICN), transfected cells were rinsed in PBS and incubated for 1 h in methionine-free DMEM supplemented with 1.5% BSA. For experiments in which cells were chased, cells were harvested in 2 ml of DMEM/BSA (1.5%) and incubated with 10 μCi/radioactive amino acid (35S) for 20 min in 1 ml of DMEM. Samples were immunoprecipitated twice with rabbit polyclonal antibody directed against Torpedo AChE (20) using protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) as the immunoadsorbent. Immunoprecipitates were washed with 10 ml dithiothreitol, electrophoresed in the presence of SDS on 10% polyacrylamide gels, and subjected to fluorography using Enlightning (Du Pont New England Nuclear).

For labeling with [3H]ethanolamine (22.4 Ci/mmol, Amer sham CPG), cells were labeled 15 min with 3 μCi [3H]ethanolam in 3.0 ml of DMEM/fetal bovine serum (10%) per 100-mm plate at 37 °C for 8 h and then transferred to 28 °C. After a total labeling period of 20 h, cells and media were collected and immunoprecipitated as indicated for S-labeled samples.

Treatments with Phenylisothiocyanate-Specific Phospholipase C—Purified PI-specific phospholipase C from Bacillus thuringiensis (21) (300 units/ml) was a gift from Dr. Martin G. Low (Columbia University). Harvested cells, washed as described for AChE activity measurements, were resuspended in PBS, and 50-μl aliquots were incubated with PI-specific phospholipase C at 37 °C. Cells were then sedimented at 14,000 rpm in an Eppendorf microcentrifuge for 5 min, whereupon supernatants were collected, and the cell pellets were resuspended in 50 μl of PBS. For immunofluorescence studies, adherent cells were rinsed twice in PBS and once in DMEM/BSA (1.5%) and incubated in DMEM/BSA (1.5%) with lipase buffer or with PI-specific phospholipase C (5 μl/units) for 90 min at 37 °C.

Immunofluorescence—Treated cells, subcultured onto UV-sterilized poly-D-lysine-coated No. 1 coverslips, were washed briefly and fixed in 4% paraformaldehyde in PBS for 20 min. Cells were then washed twice in PBS/glycerine (0.05 M) followed by a 10-min incubation with 2% normal goat serum (NGS; Organon Teknika-Cappell Corp.) in PBS/glycerine (0.05 M). After a PBS/glycerine (0.05 M) rinse, the cells were incubated for 1 h with a 1:200 dilution of an anti-Torpedo acetylcholinesterase rabbit polyclonal antibody in PBS/glycerine (0.05 M) with 0.5% BSA and then in PBS/glycerine (0.05 M)/NGS (0.5%). The cells were then incubated for 30 min with rhodamine-conjugated goat anti-rabbit IgGl (Organon Teknika-Cappell Corp.) at a 1:200 dilution in PBS/glycerine (0.05 M)/NGS (0.5%) and then subjected to 10-3 min washes in PBS/glycerine (0.05 M)/NGS (0.5%).

Coverslips were mounted onto microscope slides using 90% glycerol in PBS. Specimens were examined on a Zeiss universal microscope equipped with a Nikon UFX camera.

RESULTS

Numbering of sequences is based on the mRNA open reading frame, assigning position 1 to the first nucleotide of the initiation codon and to the first amino acid of the processed protein.

Construction of H-AChE cDNA—The genomic sequence used in the construction of an H-AChE cDNA originated from the 19-kb HindIII clone H13 (Fig. 1). A 3.6-kb m13 deletion subclone of H13, 19-3c15 is defined at its 5'-end by a PstI restriction site, which is coincident with the acceptor site of exon 2. As determined previously by S1 nuclease analysis and sequencing (11, 12), 19-3c15 contains, in addition to the 167-base pair exon 2, exon 3H, specifying the carboxyl terminus of H-AChE, and a 1.5-kb intervening intron.
The constructions for expression in COS cells. All AChE sequences were used in intermediate steps (data not shown) to provide convenient terminal restriction sites for further subcloning. A 3.6-kb PstI-EcoRI restriction fragment of X2001 genomic clone H13 was subjected to deletion subcloning in mp19 and comprises the 2.7-kb insert of 19-3c15 deletion. The 1.6-kb 5'-BamHI-Sty1 restriction fragment of the A form cDNA, isolated from XgtlO clone AChE-14 by partial digestion with EcoRI, was ligated using BamHI-EcoRI adaptors described under "Materials and Methods," created 19-3clUint. The 4~15. Deletional mutagenesis of the intronic sequence of 19-3c15, 2.2-kb A form cDNA, isolated from AChE-14 by partial digestion with EcoRI, was ligated using BamHI-EcoRI adaptors (Pharmacia LKB Biotechnology Inc.) into pCD, resulting in construction SV-A. The 1.6-kb 5'-BamHI-Sty1 restriction fragment of the A form cDNA was joined to the 0.8-kb 3'-Sty1-HamHI fragment derived from 19-3c151nt in a double ligation, giving rise to the 2.4-kb H-AChE cDNA insert of SV-H.

To delete the intron in 19-3c15, site-directed mutagenesis was performed using a 30-mer oligonucleotide (5'-GTTCTCCATCACAAGCTGGTGGGTGGA-3') specific to the presumed splice junction sequence, i.e. complementary to the last 15 bases of exon 2 and the first 15 bases of the exon 3H sequence. Mutagenesis was carried out at 43 °C to favor "clamping" of the 5'-end of the mutagenic 30-mer oligonucleotide to the template, thus looping out the intron during the polymerase extension reaction. One of eight candidates screened by dideoxy sequencing had the desired deletion (19-3c15Aint; Fig. 1). A double ligation centered at the unique Sty1 site in 19-3c15 was performed by a 30-mer oligonucleotide specific to the presumed splice junction sequence, i.e. complementary to the last 15 bases of exon 2 and the first 15 bases of the exon 3H sequence. Mutagenesis was carried out at 43 °C to favor "clamping" of the 5'-end of the mutagenic 30-mer oligonucleotide to the template, thus looping out the intron during the polymerase extension reaction. One of eight candidates screened by dideoxy sequencing had the desired deletion (19-3c15Aint; Fig. 1).

The 5'-portion of the constructed H-AChE cDNA originated from cDNA sequence encoding A-AChE (λACH-E14, Fig. 1). A double ligation centered at the unique Sty1 site in exon 2 joined the 3'-sequence of the mutagenized genomic fragment. The integrity of the resulting H-AChE cDNA sequence was confirmed by dideoxy sequencing. Previous S1 nuclease experiments (12) demonstrated that this construction is also protected in full-length by Torpedo electric organ mRNA.

**Transient Transfection of Torpedo cDNAs**—Transfected COS cells (22) were examined for transient expression of Torpedo AChE cDNAs by immunoprecipitation with polyclonal antibodies directed against Torpedo AChE (20), enzyme assay, and immunofluorescence. In initial experiments during which COS cells were maintained at 37 °C, the A form cDNA was used to test the expression system. Immunoprecipitations of lysates prepared from transfected cells metabolically labeled with [35S]methionine demonstrated the synthesis of Torpedo AChE protein of the expected size (Fig. 2). The identity of the labeled band immunoprecipitated only from cells transfected with cDNA (SV-A) is confirmed in the competition for antibody by native Torpedo AChE, but not by an equivalent amount of BSA. A decrease in apparent molecular weight of the subunit upon treatment with endoglycosidase F indicates that immunoprecipitated protein is glycosylated (data not shown). However, the amount of AChE catalytic activity expressed at 37 °C by cells transfected with the A form cDNA was marginal and found to be only intracellular.

**Expression of Active Enzyme at Reduced Temperature**—Upon transfer to 28 °C for 24 h, AChE activity associated with cells transfected with SV-A is enhanced (Fig. 3). Samples of culture media taken at the end of incubation exhibited no secreted activity. To minimize background activity, culture media were treated with diisopropyl fluorophosphate to inhibit endogenous serum cholinesterases and were diazoyed to remove residual diisopropyl fluorophosphate. In contrast to SV-A transfections, cells transfected with SV-H exhibited considerable acetylcholine hydrolyzing activity upon incubation at 28 °C.

As demonstrated by [35S]labeling (Fig. 3), the initial synthesis of AChE protein at 37 and 28 °C in transfected cells is fairly uniform, although cellular accumulation of the A form at 4 h may be less at 28 °C. At 28 °C, variation in [35S]-labeled protein synthesized from SV-A and SV-H apparently does not account for the >20-fold difference in cell-associated activity (Table I).

The cellular disposition of AChE protein synthesized in COS cells transfected with AChE cDNAs was determined by measuring activity associated with intact and lysed cell samples. Activity expressed from the A form cDNA was only intracellular. Cell-surface activity accounts for ~70% of the

---

**FIG. 1. Constructions used in expression of Torpedo acetylcholinesterase.** Summarized are manipulations which resulted in the constructions for expression in COS cells. All AChE sequences originated from genomic and cDNA clones described elsewhere (11-13). Bluescript (Stratagene) and pGEM (Promega Biotec) plasmids were used in intermediate steps (data not shown) to provide convenient terminal restriction sites for further subcloning. A 3.6-kb PstI-EcoRI restriction fragment of X2001 genomic clone H13 was subjected to deletion subcloning in mp19 and comprises the 2.7-kb insert of 19-3c15 deletion. The 1.6-kb 5'-BamHI-Sty1 restriction fragment of the A form cDNA, isolated from XgtlO clone AChE-14 by partial digestion with EcoRI, was ligated using BamHI-EcoRI adaptors (Pharmacia LKB Biotechnology Inc.) into pCD, resulting in construction SV-A. The 1.6-kb 5'-BamHI-Sty1 restriction fragment of the A form cDNA was joined to the 0.8-kb 3'-Sty1-HamHI fragment derived from 19-3c151nt in a double ligation, giving rise to the 2.4-kb H-AChE cDNA insert of SV-H.

---

**FIG. 2. Immunoprecipitation of AChE protein from [35S]methionine-labeled transfected COS cells.** COS-1 cells were transfected, metabolically labeled with [35S]methionine, and immunoprecipitated. The autoradiogram shows the results of SDS-polyacrylamide gel electrophoresis of reduced lysate samples from cells transfected in the absence of DNA (mock), with vector alone (SV), and with vector with the A-AChE cDNA insert (SV-A). Shown for each are samples of 1/100 volume of the total cell lysate (lanes 1, 4, and 7), lysate immunoprecipitated with normal rabbit serum (NRS; lanes 2, 5, and 8), and lysate precipitated with rabbit polyclonal antibody 80 (lanes 3, 6, 9, 10, and 11). In lanes 10 and 11, immunoprecipitation with antibody 80 was in the presence of 0.5 mM bovine serum albumin or 0.5 mM purified Torpedo A-AChE.
onto six replicate plates (3.2 × 10⁶ cells, two held at 37 °C and two transferred to 28 °C, were labeled 48 h post-transfection, three of the replicate plates from each transfection were transferred to 28 °C. Four hours later, replicate plates of cells, two held at 37 °C and two transferred to 28 °C, were labeled with [³⁵S]methionine for 20 min. Chase in methionine-supplemented medium for 0 or 4 h was followed by harvesting and immunoprecipitation with polyclonal anti-AChE antibody 81. Incubation of one replicate plate at each temperature continued for 24 h, after which the cells were harvested and assayed for total cellular AChE activity. Reduced immunoprecipitates were subjected to electrophoresis and fluorography. The autoradiogram shows a comparison of immunoprecipitated [³⁵S]-labeled AChE from cells transfected with SV-A and later incubated at 28 °C. The numbers at the bottom of each panel indicate acetylcholinesterase activity over endogenous background (absorbance units/minute) in 3.2 × 10⁶ transfected cells measured from the lysate of a replicate plate of cells after 24 h for each condition.

**TABLE I**

<table>
<thead>
<tr>
<th>AChE Activitya</th>
<th>Cell surface</th>
<th>Total cellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV</td>
<td>0.045 ± 0.011</td>
<td>0.055 ± 0.012</td>
</tr>
<tr>
<td>SV-A</td>
<td>0.051 ± 0.015</td>
<td>0.112 ± 0.026</td>
</tr>
<tr>
<td>SV-H</td>
<td>0.953 ± 0.195</td>
<td>1.293 ± 0.304</td>
</tr>
</tbody>
</table>

a Activity is expressed in absorbance units/minute/100-mm plate (10⁶ cells transfected) as the mean ± S.E. of determinations from three or more independent transfections.

**Fig. 3.** Comparison of [³⁵S]methionine-labeled AChE and AChE activities in transfected cells. COS cells (2.5 × 10⁶ cells/150-mm plate) were transfected with SV-A and subcultured 36 h later onto six replicate plates (3.2 × 10⁶ transfected cells/35-mm plate). At 48 h post-transfection, three of the replicate plates from each transfection were transferred to 28 °C. For comparison, the data from cells transfected with SV-H and later incubated at 28 °C are also shown. The numbers at the bottom of each panel indicate acetylcholinesterase activity over endogenous background (absorbance units/minute) in 3.2 × 10⁶ transfected cells measured from the lysate of a replicate plate of cells after 24 h for each condition.

**Fig. 4.** Release of AChE activity from transfected cells by PI-specific phospholipase C. Equal aliquots of cells harvested after transfection with SV-H were incubated at 37 °C intact in PBS with buffer (●) or with 1 unit/ml PI-specific phospholipase C in the same buffer (++) for the time intervals indicated. The relative activity recovered in the supernatants is indicated.

**Fig. 5.** Indirect immunofluorescence analysis of COS cells transfected with SV-H. Transfected COS cells were subcultured onto coverslips and incubated at 28 °C for 24 h. Duplicate coverslips were treated for 90 min at 37 °C with lipase buffer or with 5 units/ml PI-specific phospholipase C. Cells were then fixed intact, treated with rabbit anti-AChE polyclonal antibody 81, and visualized with rhodamine-conjugated goat anti-rabbit IgG antibody. Shown is the surface immunofluorescent labeling of cells transfected with SV vector alone (A) or with SV-H (B-D). Cells in D were incubated with PI-specific phospholipase C prior to fixation (magnification × 197).

**PI-specific Phospholipase C Release of Expressed Hydrophobic Acetylcholinesterase—**As is evident in Fig. 4, ~80% of the surface activity was released into the supernatant of cells treated with PI-specific phospholipase C. The specific action of PI-specific phospholipase C appears to have two phases. A rapid initial release of ~40% of total activity in the first 5 min is followed by a slower accumulation of activity in the supernatant.

**Spontaneous release of activity into the supernatant during incubation of cells in the absence of PI-specific phospholipase C amounted to 10–15% and was not influenced by stabilizing agents such as BSA (data not shown). Endogenous phospholipase or proteases may be acting to release activity. However, since a substantial fraction of the native glycosphospholipid-containing enzyme is readily extracted from electric organ tissue in low ionic strength buffer, some of the hydrophobic enzyme may spontaneously partition into the buffer phase during incubation of the harvested cells. Release may also depend on the lipid composition of the host cell.

To confirm the cell-surface localization and glycosphospholipid linkage of AChE expressed from the H form cDNA, indirect immunofluorescence experiments were performed on transfected COS cells using anti-Torpedo AChE antibodies (Fig. 5). An effort was made to visually screen most of the cells by fluorescence microscopy. None of the cells transfected with SV (vector lacking cDNA insert) showed appreciable fluorescence. The SV-transfected cells incubated in control buffer (Fig. 5A) were indistinguishable from those treated with PI-specific phospholipase C (data not shown). Fluorescent labeling of cells transfected with SV-H is apparent (Fig. 5, B and C). Approximately 5–10% of the transfected cells exhibited significant surface fluorescence. This frequency is consistent with the expected efficiency of transient transfection. The duplicate population of SV-H-transfected cells treated with PI-specific phospholipase C showed a marked decrease in frequency of staining cells. About 1% of the cells (i.e., 10–20% among expressing cells) exhibited discernible surface fluorescence which was not released by the phospholipase. Compared to the fluorescence of cells shown in Fig. 5 (B and C), the residual labeling after phospholipase treatment (Fig. 5D) was more punctate and typically apparent on the margins of cells. This observation suggests that PI-specific
Heterologous Expression of Acetylcholinesterase

**FIG. 6.** Pulse-chase labeling and immunoprecipitation of wild-type and carboxyl-terminal mutant AChE proteins. COS cells were transfected with the indicated vectors and transferred 36 h later to 28 °C. At 48 h post-transfection, [35S]methionine labeling was carried out on replicate plates of cells and chased for intervals of 0, 4, and 24 h. Chase media and cell lysates at each time point were immunoprecipitated with rabbit anti-AChE antibody 80. The autoradiogram shows the results of SDS-polyacrylamide gel electrophoresis of the reduced immunoprecipitates.

**FIG. 7.** [35S]Methionine and [3H]ethanolamine labeling of acetylcholinesterase in transfected cells. Duplicate plates of cells transfected with the vectors indicated were incubated either with [35S]methionine (50 μCi/ml) for 1 h or with [3H]ethanolamine (67 μCi/ml) for 20 h and then processed by immunoprecipitation. Reduced immunoprecipitates were electrophoresed on the same gel and then subjected to fluorography. For 35S, the film was exposed for 24 h; for 3H, exposure was for 4 weeks.

Mutational Analysis of AChE Disposition—The expression of two mutants with gross structural alterations was examined by metabolic labeling with methionine and ethanolamine. A truncated AChE protein which terminates at the exon 2-3H splice junction was created by replacing the Alasn6 codon of the H-AChE cDNA (nucleotides 1669-1671) with a stop codon using the mutagenic oligonucleotide 5'-CTCAACGCCACATGATGTGATGGAGAA-3'. The truncated sequence was expressed from construction SV-HA536* (Table II). A fusion mutant was generated by deleting exon 2 (nucleotides 1502-1669) from the H-AChE cDNA with the oligonucleotide 5'-ACATTCGCAAAGACTGCTTGTGATGGAGAA-3'. The resulting expression vector is designated SV-HAexII (Table II). The fusion at this position creates a threonine at position 479. This position is comparable to the threonine at position 535 in H-AChE.

Mutants SV-HA536* and SV-HAexII were analyzed by pulse-chase labeling in comparison with the wild-type A-AChE and H-AChE sequences. Lysates and media from [35S]methionine-labeled transfected cells immunoprecipitated at three chase intervals are shown in Fig. 6. Initial accumulations of [35S]-labeled protein for the constructions are fairly uniform. By 24 h, [35S]-labeled AChE in cell lysates was diminished for phospholipase C-insensitive H-AChE in transfected cells is localized at discrete sites.

**Table II**

<table>
<thead>
<tr>
<th>Sequences encoded by wild-type and mutant acetylcholinesterase cDNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>The numbers denote amino acid sequence with the amino-terminal amino acid of the mature processed forms assigned as 1. Nucleotide sequence relevant to the mutations is shown, with vertical lines indicating splice junctions in the wild-type coding sequences.</td>
</tr>
</tbody>
</table>

**Phospholipase C-insensitive H-AChE**

**Mutational Analysis of AChE Disposition—**The expression of two mutants with gross structural alterations was examined by metabolic labeling with methionine and ethanolamine. A truncated AChE protein which terminates at the exon 2-3H splice junction was created by replacing the Ala212 codon of the H-AChE cDNA (nucleotides 1669-1671) with a stop codon using the mutagenic oligonucleotide 5'-CTCAACGCCACATGATGTGATGGAGAA-3'. The truncated sequence was expressed from construction SV-HA536* (Table II). A fusion mutant was generated by deleting exon 2 (nucleotides 1502-1669) from the H-AChE cDNA with the oligonucleotide 5'-ACATTCGCAAAGACTGCTTGTGATGGAGAA-3'. The resulting expression vector is designated SV-HAexII (Table II). The fusion at this position creates a threonine at position 479. This position is comparable to the threonine at position 535 in H-AChE.

Mutants SV-HA536* and SV-HAexII were analyzed by pulse-chase labeling in comparison with the wild-type A-AChE and H-AChE sequences. Lysates and media from [35S]methionine-labeled transfected cells immunoprecipitated at three chase intervals are shown in Fig. 6. Initial accumulations of [35S]-labeled protein for the constructions are fairly uniform. By 24 h, [35S]-labeled AChE in cell lysates was diminished for phospholipase C-insensitive H-AChE in transfected cells is localized at discrete sites.

**Mutational Analysis of AChE Disposition—**The expression of two mutants with gross structural alterations was examined by metabolic labeling with methionine and ethanolamine. A truncated AChE protein which terminates at the exon 2-3H splice junction was created by replacing the Ala212 codon of the H-AChE cDNA (nucleotides 1669-1671) with a stop codon using the mutagenic oligonucleotide 5'-CTCAACGCCACATGATGTGATGGAGAA-3'. The truncated sequence was expressed from construction SV-HA536* (Table II). A fusion mutant was generated by deleting exon 2 (nucleotides 1502-1669) from the H-AChE cDNA with the oligonucleotide 5'-ACATTCGCAAAGACTGCTTGTGATGGAGAA-3'. The resulting expression vector is designated SV-HAexII (Table II). The fusion at this position creates a threonine at position 479. This position is comparable to the threonine at position 535 in H-AChE.

Mutants SV-HA536* and SV-HAexII were analyzed by pulse-chase labeling in comparison with the wild-type A-AChE and H-AChE sequences. Lysates and media from [35S]methionine-labeled transfected cells immunoprecipitated at three chase intervals are shown in Fig. 6. Initial accumulations of [35S]-labeled protein for the constructions are fairly uniform. By 24 h, [35S]-labeled AChE in cell lysates was diminished for phospholipase C-insensitive H-AChE in transfected cells is localized at discrete sites.
Expression of Acetylcholinesterase

Collagen-like subunit

Glycophospholipid

FIG. 8. Schematic representation of fates of wild-type and carboxyl-terminal mutant AChE proteins. Depicted are wild-type and mutant AChE proteins specified in the vectors SV-A, SV-H, SV-HA536*, and SV-HαexII which were used in transfection experiments. Amino acid domains as they correspond to AChE coding exons are indicated (cf. Fig. 1). Note that A and H proteins differ only in their most distal carboxyl-terminal regions. The HA536* protein shares the same 535-amino acid sequence but lacks either carboxyl-terminal domain. The HαexII protein has the sequence encoded by exons 1 (residues 1–479) and 3H (residues 536–565) in fusion as a result of the in-frame deletion of exon 2 from its DNA. The results of transfection experiments in COS cells, when taken together, indicate that the A protein that is expressed remains intracellular. As expressed in its native context, however, the AChE subunit assembles into tetramers in association with a collagen-like tail and is secreted. The expressed H enzyme, like its native counterpart, is processed with the attachment of glycophospholipid (denoted by ethanolamine, a polymer of six-membered rings (monosaccharides and inositol) and the diacylglycerol) to a newly defined carboxyl-terminal residue with the cleavage of the hydrophobic terminal peptide and becomes localized on the cell surface. Truncated A536* is secreted to a significant degree, whereas the HAexII fusion protein is processed with glycophospholipid. Thus, the carboxyl-terminal domains encoded by alternative exons each dictate a specific fate other than rapid secretion into the extracellular space.

all three constructions. Only with the mutant SV-HA536* is labeled AChE protein apparent in the medium by 24 h. In a replicate experiment, secretion was evident at both 4 and 24 h (data not shown). However, it is apparent that the appearance of labeled SV-HA536* protein in the medium does not account for all of the loss of its cell-associated immunoprecipitable protein.

Incorporation of [3H]ethanolamine into AChE protein expressed by transfected cells is shown in Fig. 7. Tritium-labeled bands corresponding in migration to β-S-labeled bands are apparent only in the immunoprecipitates of cells transfected with SV-H and SV-HαexII. No immunoprecipitable tritiated AChE protein was apparent in media samples. Samples of [3H]ethanolamine-labeled lysates and media (1/100 of total) were also run without immunoprecipitation. Only single bands of ~65 kDa in all lysates and 45 kDa in all media were detected (data not shown).

Lysate and media samples from cells transfected with SV-HA536* and SV-HαexII did not exhibit AChE activity above background. Although mutant HA536* is secreted into the medium at a significant rate, sufficient protein is retained to suggest that if it were appreciably active, its activity would be detectable in the cell lysate.

DISCUSSION

Expression from cDNAs Encoding Torpedo Acetylcholinesterase—Our initial experiments demonstrated the synthesis of Torpedo AChE protein in COS cells transfected with the asymmetric form cDNA. Virtually no catalytic activity was measurable, despite the fact that a glycosylated protein of the correct size was being produced. The problem remained puzzling until Claudio et al. (23) reported that β-bungarotoxin binding activity of the Torpedo nicotinic receptor, reconstituted by cotransfection of subunits into L cells, could be detected only at reduced temperature. The temperature dependence of function of the expressed Torpedo receptor appears to extrapolate to full binding activity at 15 °C. However, even at 28 °C (the temperature which allowed expression of functional Torpedo receptor channels), activity arising from the A-AChE cDNA transfection was minimal. In contrast, cells transfected with H-AChE cDNA express appreciable cell-surface activity. Torpedo A-AChE and H-AChE are synthesized at comparable rates in COS cells, although the A form sequence may give rise to somewhat less stable protein. Secretion could reasonably account for the paucity of cell-associated activity, but activity and β-S-labeled AChE protein were not detected in media upon expression of the A form at 28 °C.

The proper processing and export of glycophospholipid-linked Torpedo enzyme synthesized by cells transfected with SV-H were demonstrated by several independent means. Most of the AChE activity expressed is on the cell surface, consistent with the immunofluorescence of intact cells. Treatment of the cells with phosphatidylinositol-specific phospholipase C released both enzyme activity and immunoreactivity from intact cells. Finally, AChE synthesized by cells transfected with the H form cDNA is labeled by [3H]ethanolamine. A component of the glycophospholipid anchor, ethanolamine provides a particularly selective label. Biosynthetic incorpo...
r ion of radiolabeled ethanolamine into protein is typically seen only in glycosphospholipid linked molecules (cf. Ref. 24), but recent studies (25) demonstrate that ethanolamine incorporates at glutamate residues in elongation factor 1a.

Carboxyl Terminus of Acetylcholinesterase Determines Its Cellular Disposition—Alternatively spliced messages which differ in their third coding exon give rise to acetylcholinesterases which diverge only in their carboxy-terminal regions. The two enzymes, at least from their native source, are catalytically indistinguishable. This suggests that the divergent cellular localization of A-AChE and H-AChE may be specified by the unique coding exons, 3A and 3H, respectively.

To examine the extent to which the carboxy-terminal sequence encoded by alternative exons contributes to the localization of the AChE forms, two mutant AChE proteins were created. Truncated polypeptide HA536* represents the 535-amino acid sequence which is shared by the two AChE forms. Native A-AChE continues beyond residue 535 for 40 amino acids (19, 26). Precursor H-AChE extends 30 amino acids further (12), whereas the native glycosphospholipid-linked protein terminates after just 2 more residues (9). The site of the nonsense mutation is intentionally coincident with the 3′-splice junction of exon 2, which generates alternative carboxy-terminal coding sequences. Mutant protein HΔexII is the fusion product of AChE coding exons 1 and 3H. The in-frame deletion of exon 2 from H-AChE was created to examine the capacity of exon 3H coding sequence to completely specify glycosphospholipid attachment.

Pulse-chase labeling with [35S]methionine indicates that the mutant AChE proteins differ in their cellular dispositions. The truncated protein HA536* is secreted into the medium, whereas the fusion protein HΔexII, like the A and H wild types, remains cell-associated. Ethanolamine incorporation into HΔexII reveals that it, like wild-type H protein, is glycosphospholipid-linked. It is noteworthy that the mutant AChE proteins are inactive, suggesting that the domains encoded by each coding exon contribute to proper folding and catalytic activity.

As depicted in Fig. 8, several conclusions may be drawn from these results. The carboxy-terminal domains of A-AChE and H-AChE both appear to specify a cell-associated localization. The evidence for this is that a truncated AChE which lacks either carboxy-terminal sequence of the wild-type proteins is secreted into the medium, whereas the wild-type proteins are not. Furthermore, the unique carboxy-terminal domain of H-AChE contains sequence which is necessary and sufficient to specify cleavage and attachment of glycosphospholipid. As has been shown, secretion of the truncated AChE protein demonstrates necessity, whereas glycosphospholipid modification of the AChE fusion protein demonstrates sufficiency. The threonine in the 510-amino acid fusion protein at residue 479 in the same position as Thr55 encoded in exon 2 of H-AChE. The remaining 30 amino acids are encoded by exon 3H. After processing and addition of the glycosphospholipid, only the tripeptide Thr-Ala-Cys is retained. The finding that 3 amino acids plus the processed peptide were sufficient to confer the glycosphospholipid attachment differs somewhat from the conclusions of Caras et al. (27), who proposed that additional sequence in the carboxy terminus of processed decay accelerating factor was necessary for glycosphospholipid attachment. The 30 amino acids encoded by exon 3H could signal the attachment of glycosphospholipid on other cellular proteins. However, this will require confirmation by expression of chimeric fusion proteins.

Why the A form as expressed in COS cells is not secreted at an appreciable rate and is only marginally active is not entirely clear. The A-AChE catalytic subunit normally assembles into tetramers; and for some homopolymeric proteins, oligomerization is necessary for transport out of the endoplasmic reticulum (28). Additionally, the A subunit tetramers become linked to a collagen subunit in the Golgi apparatus (29). Perhaps the carboxy-terminal domain of the A-AChE subunit contains a signal which specifies that it be retained in a subcellular compartment until it is fully assembled. Thus, it may be that catalytic subunits of the A form require specific subunit associations or other processing for activity and secretion, and the COS cell is ill-equipped in this regard.

In situ, it appears that much of the AChE that is synthesized never reaches the cell surface and is inactive. An inactive AChE monomer from Torpedo marmorata electric organ has been described (30). The polypeptide is hydrophilic and soluble in low ionic strength buffer. An inactive monomer of short half-life constitutes 20% of cellular AChE in murine neuronal-like T28 cells (31). Rotundo (32) reports that 70–80% of AChE synthesized in cultured chicken embryo muscle is inactive, retained in the cell, and subject to rapid turnover. Therefore, the synthesis by COS cells of inactive subunits of the Torpedo A form which are retained within the cell may be faithfully reflecting the endogenous expression of AChE as it occurs in some cells.

The mechanisms by which structure and localization of AChE forms are determined are diverse. It is clear, however, that the primary structures of the catalytic subunits play a very significant role. There is precedence for alternative exon usage generating molecules with identical amino-terminal sequence but with different dispositions dictated by distinctive carboxy-terminal domains. Pertinent examples among glycosphospholipid-anchored proteins include neural cell adhesion molecules (33, 34) and murine antigen Qa-2 (35), where alternative mRNA processing also gives rise to integral membrane or soluble proteins.

An intriguing aspect of acetylcholinesterase polymorphism is that alternative coding sequences give rise to catalytically identical enzymes which exhibit unique modes of extracellular attachment. Anchoring of hydrophobic acetylcholinesterase to the cell surface through glycosphospholipid is directed by a carboxyl terminus encoded in a differentially spliced mRNA. Asymmetric acetylcholinesterase, assembled in the Golgi apparatus by disulfide bonding of catalytic and collagen-like subunits, is secreted and tethered extracellularly in the basal lamina. The precise role of the alternative carboxy-terminal sequence of the catalytic subunit in this assembly process remains to be determined.

Acknowledgments—We thank Shelley Camp for nucleic acid sequencing to confirm the integrity of constructions used. We are also grateful to Martin O. Low for providing phosphatidylinositol-specific phospholipase C.

REFERENCES

Heterologous Expression of Acetylcholinesterase


