Protein CutA Undergoes an Unusual Transfer into the Secretory Pathway and Affects the Folding, Oligomerization, and Secretion of Acetylcholinesterase*

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The mammalian protein CutA was first discovered in a search for the membrane anchor of mammalian brain acetylcholinesterase (AChE). It was co-purified with AChE, but it is distinct from the real transmembrane anchor protein, PRiMA. CutA is a ubiquitous trimeric protein, homologous to the bacterial CutA1 protein that belongs to an operon involved in resistance to divalent ions (“copper tolerance A”). The function of this protein in plants and animals is unknown, and several hypotheses concerning its subcellular localization have been proposed. We analyzed the expression and the subcellular localization of mouse CutA variants, starting at three in-frame ATG codons, in transfected COS cells. We show that CutA produces 20-kDa (H) and 15-kDa (L) components. The H component is transferred into the secretory pathway and secreted, without cleavage of a signal peptide, whereas the L component is mostly cytosolic. We show that expression of the longer CutA variant reduces the level of AChE, that this effect depends on the AChE C-terminal peptides, and probably results from misfolding. Surprisingly, CutA increased the secretion of a mutant possessing a KDEL motif at its C terminus; it also increased the formation of AChE homotetramers. We found no evidence for a direct interaction between CutA and AChE. The longer CutA variant seems to affect the processing and trafficking of secretory proteins, whereas the shorter one may have a distinct function in the cytoplasm.

The major molecular species of AChE in mammalian brain consists of membrane-bound tetramers (1). In these hetero-oligomers, four catalytic subunits, corresponding to the AChE-T variant that possesses a C-terminal t peptide (2), are associated with a hydrophobic 20-kDa protein (3, 4). This protein has now been cloned and named PRiMA (proline-rich membrane anchor) (5). However, before the characterization of PRiMA, another protein was identified independently by different groups as a component of AChE preparations purified from mammalian brain (6, 7). This protein was called CutA because of its homology with a bacterial protein (Cu2+ tolerance A), derived from an operon involved in resistance to copper and other divalent metal ions (8). CutA apparently exists in all organisms (9), and although its function is unknown, its structural homology with the bacterial and plant P-II nitrogen regulatory protein suggested a role in signal transduction (10). In the human brain, it has been proposed to be involved in the proliferation and survival of glial cells (11).

Although CutA was first thought to represent the membrane anchor of AChE, co-expression experiments showed that this was not the case. The presence of both CutA and PRiMA in AChE preparations was established by sequencing tryptic peptides and detection in Western blots by different antibodies; in contrast with PRiMA, CutA was not disulfide-linked to AChE subunits, as shown by electrophoresis in SDS-polyacrylamide gels under nonreducing conditions (7).

Despite the presence of CutA in at least three different preparations of affinity-purified PRiMA-anchored AChE (6, 7), it was not possible to demonstrate a direct association between the two proteins. In addition, there is no obvious correlation between the expression of CutA and that of membrane-bound AChE, because CutA is expressed in brain and also in all other mammalian tissues. However, stable transfection with an antisense construct designed to block expression of CutA was found to suppress the membrane anchoring of AChE in a murine neuroblastoma cell line, N18TG2 (7). Unless some other modification occurred during the derivation of this cell line, this suggested that CutA might play a role in the assembly of AChE-T subunits with PRiMA.

The structure of bacterial and mammalian CutA was analyzed by crystallography and found to contain a trimeric core, in both cases (10, 12, 13). Each subunit of CutA contains two cysteines, within the trimeric region. In the trimer, the cysteines are distant from each other and cannot form intra- or intercatenary disulfide bonds. It was suggested that CutA might play a role in the control of oxidoreduction in the cell (10). However, the subcellular localization of CutA has not been firmly established.
CutA, Acetylcholinesterase, and Secretory Trafficking

Transcripts encoding CutA present alternative 5' regions; the splicing pattern is more complex in the human than in the mouse, but in both cases one variant contains three in-frame ATG codons, and reports differ on the identity of the translation initiation site. A CutA protein starting at the first methionine might contain a secretion signal peptide, but the prediction is ambiguous. Alternatively, a recent immunofluorescence study concluded that CutA is imported into mitochondria (9).

We therefore studied the subcellular localization of CutA and the effect of its expression on the synthesis and fate of AChE. We present evidence that CutA is partly cytosolic and partly secreted, some of it retaining its N-terminal peptide. In addition we show that co-expression with CutA influences the trafficking of AChE in the secretory pathway and the assembly of AChE oligomers.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs and Site-directed Mutagenesis**—All constructs were expressed in the pEF-Bos vector (14, 15). Mutagenesis was performed according to the method of Kunkel et al. (16). Rat AChE* subunits were tagged with an HA epitope (YPYDVPDYA), inserted before the stop codon at the C terminus of the t peptide of rat AChE*.

**Transfection and Culture of COS Cells and Treatment with Metabolic Inhibitors**—COS cells were transfected by the DEAE-dextran method, as described previously (17), using up to 2 μg of pEF-BOS vector per 60-mm dish. After transfection, COS cells were incubated for 3–4 days at 37 °C in a medium containing 10% serum (Inotek, Dottikon, Switzerland), which had been pretreated with 10−6 M soman to inactivate serum cholinesterases. In some experiments, transfected COS cells were treated with an inhibitor of secretion, brefeldin A (30 ng/ml), or with inhibitors of proteasomal degradation MG132 (10 μμM) and clasto-β-lactacystin (50 μM). They were incubated for 8 h with brefeldin A or overnight with the proteasome inhibitors before collection of the culture medium and cells. To study the degradation of cellular proteins, synthesis was blocked with 0.4 mg/ml cycloheximide.

**Extracts from Transfected COS Cells**—Three days after transfection, the cells were collected and homogenized in the extraction buffer (1% Triton X-100; 50 mM Tris-HCl, pH 7.5; 10 mM MgCl2, containing either 1% Brij-97 or 0.2% Triton X-100), and centrifuged in a Beckman SW41 rotor at 36,000 rpm, for 17 h 30 min at 6 °C. Fractions of ~100 μl were collected and assayed for AChE, β-galactosidase, and alkaline phosphatase, as internal sedimentation markers, layered onto 5–20% sucrose gradients (50 mM Tris-HCl, pH 7.5; 20 mM MgCl2, containing either 1% Brij-97 or 0.2% Triton X-100), and centrifuged and at 36,000 rpm, for 17 h 30 min at 6 °C. Fractions of ~100 μl were collected and assayed for AChE, β-galactosidase, and alkaline phosphatase activities, as described previously (19).

**Western Blots**—For analysis of proteins by Western blotting, samples were submitted to electrophoresis in 10 or 7.5% polyacrylamide gels under reducing conditions. We used colored proteins as mass standards (PAGE regular prestained protein ladder SM0671 from Fermentas and HiMark prestained high molecular weight protein standards LC5699 from Invitrogen). After electrophoresis, proteins from the gel were electroblotted with the Bio-Rad mini-Protean II transblot system onto polyvinylidene difluoride membranes (Roche Applied Science) for 1 h 30 min. After transfer, the membrane was saturated with 5% milk powder in a buffer containing Tween 20 (20 mM Tris-HCl; 137 mM NaCl; 0.1% Tween 20; pH 7.6) for 2 h. The membrane was then incubated overnight at 16 °C with appropriate antibodies. The rabbit polyclonal anti-HA antibody and the anti-FLAG M2 and anti-TOM-22 mouse monoclonal antibodies were from Sigma. The mouse monoclonal anti-BiP antibody was from BD Biosciences. These antibodies were used at 1:1,000 dilution. The secondary peroxidase-conjugated anti-rabbit and anti-mouse antibodies, from Jackson ImmunoResearch, were used at 1:10,000 dilution. The immunocomplexes were visualized using the ECL method (Supersignal West Pico kit, from Pierce). Exposure time was usually 1–5 min.

**Cross-linking of CutA Oligomers**—A 100 mM stock solution of the cross-linking agent disuccinimidyl suberate (DSS) from Pierce was prepared in dimethyl sulfoxide. Transfected COS cells were washed in PBS and then incubated in the culture dish at 20 °C for 2 h with PBS containing 1.5 mM DSS. The cross-linking reaction was quenched by addition of 50 mM Tris-HCl, pH 7.5, for 15 min at 20 °C. The cells were then washed in PBS, scraped from the dish, and extracted with the pellet containing nuclei, cell debris, and unbroken cells was discarded. Following a subsequent 6,000 × g centrifugation, the supernatant contained the microsomal fraction, and the pellet containing the mitochondria was resuspended in a “mitochondrial purification buffer” and centrifuged at 20,000 × g for 15 min at 4 °C. The mitochondrial fraction was then pelleted three times in the “storage buffer” and finally resuspended in the extraction buffer, as above, for Western blot analyses.

**Determination of AChE Activity**—AChE activity was determined by the colorimetric method of Ellman et al. (18), using acetylthiocholine as substrate. Enzyme samples (usually 10 μl) were added to 0.2 ml of Ellman assay medium, and the reaction was monitored at 414 nm with a Labsystems Multiskan RC automatic plate reader (Helsinki, Finland); the optical density was recorded at 0.5-s intervals over a period of 10 min. Under those conditions, 1 μM corresponds to the hydrolysis of 22.5 pmol of acetylthiocholine.

**Sedimentation Analysis in Sucrose Gradients**—For sedimentation analyses of AChE molecular forms, 100–400 μl samples were mixed with Escherichia coli β-galactosidase and alkaline phosphatase, as internal sedimentation markers, layered onto 5–20% sucrose gradients (50 mM Tris-HCl, pH 7.5; 20 mM MgCl2, containing either 1% Brij-97 or 0.2% Triton X-100), and centrifuged in a Beckman SW41 rotor at 36,000 rpm, for 17 h 30 min at 6 °C. Fractions of ~100 μl were collected and assayed for AChE, β-galactosidase, and alkaline phosphatase activities, as described previously (19).
extracted buffer, as above. The extracts were analyzed by SDS-PAGE and Western blotting.

**Mass Spectrometry Analyses**—Protein bands were excised manually from Coomassie Blue-stained gels, collected, and stored in microtubes containing 1% acetic acid before analysis. After reduction (10 mM dithiothreitol; 100 mM NH₄HCO₃) and protein alkylation (55 mM iodoacetamide; 100 mM NH₄HCO₃), the samples were digested with 9–12 ng/ml trypsin (Roche Applied Science), for 2 h at 37 °C, in 50 mM NH₄HCO₃, 5 mM CaCl₂ (20). Proteolytic peptides were solubilized in 10 μl of 2% aqueous formic acid (v/v), desalted, and concentrated using a ZipTip® C18 microcolumn (Millipore, Molsheim, France).

For MALDI-TOF/TOF analysis, 0.3 μl of each sample was deposited with 0.6 ml of a CHCA matrix solution (acetonitrile/H₂O, 6:4 v/v, 0.1% trifluoroacetic acid v/v). MS analysis was performed with a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applera Applied Biosystems, Framingham, MA) in the positive ion reflectron mode, and MS/MS data were obtained using 2 keV of collision energy. External calibration was achieved with a standard peptide mix (Proteomix peptide mix4, LaserBioLabs, Sophia Antipolis, France). Autolysis products of trypsin were used for internal calibration. The top seven peptides were selected automatically for the MS/MS analysis. GPS Explorer™ software version 2.0 (Applera Applied Biosystems) was used to create and search files. Data were searched against Swissprot from UniProtKB release 9.6 using Mascot 2.2 (Matrixscience). Search parameters were as follows: 2 miscleavages, taxonomy restricted to Mus musculus, accuracy 50 ppm in MS, 0.3 Da in MS/MS, oxidation of methionines, and carbamidomethylation of cysteines. Validation was performed manually using an in-house data base containing the sequences of the specific constructions of interest, and considering N-terminal acetylation as an additional potential modification.

**RESULTS**

**Human and Mouse CutA Variants**—Fig. 1A shows an alignment of the human and mouse genes encoding the CutA isoforms/variants. These isoforms possess a very conserved region involved in the formation of a trimer and differ only by their N-terminal regions, within each species. Human CutA presents three isoforms, one of which has no equivalent in the mouse. The shorter of the other two isoforms, which exist in both species, is contained in the longer one but starts at a downstream methionine encoded by a different exon (Fig. 1B); in the mouse, the corresponding methionines are Met₁ and Met₂₄ (the residues are numbered according to the longer protein) (Fig. 1C). A downstream in-frame ATG codon, encoding Met₄₄, is located in the same exon and might also serve as a translation initiation site (6). It should be noted that in some transcripts, the ATG codons corresponding to these methionines are preceded by upstream ATGs, which are not in-frame with the CutA coding sequence, and are therefore not considered as translation initiation sites (Fig. 1A).

In this study, we focused on the mouse CutA isoforms, and we considered three possible variants, starting at the three in-frame methionines, Met¹, Met¹⁵, and Met⁴⁴, that precede the trimerization domain. To study the production and distribution of CutA, we transfected COS cells with mouse CutA containing FLAG or HA epitopes at the N- and C-terminal extremities (Fig. 1D). As indicated in Fig. 1D, we mutated the three ATG codons in the 5' region of the coding sequence, as well as the two conserved cysteines of CutA, and added an endoplasmic retention motif at the C terminus (KDEL).

**Expression of Endogenous and Transfected CutA in COS Cells and Heavy and Light Components**—Using CutA-specific primers, RT-PCR analysis of mRNA from untransfected COS cells produced an amplimer corresponding to peptide 49–99 from Macaca CutA (data not shown), which is identical to the corresponding fragment of human CutA (Fig. 1, A and C). This indicates that CutA is endogenously expressed in the simian COS cells, in agreement with its ubiquitous tissue distribution (9).

The endogenous Macaca CutA protein could be recognized by an antiserum directed against N-terminal and a C-terminal peptide of mouse CutA, whereas the transfected mouse CutA was labeled with N-terminal or C-terminal epitopes (Fig. 2). SDS-PAGE and Western blots showed a heavy band (H, 20 kDa), which was recognized by antibodies directed against both N- and C-terminal epitopes, and a light band (L, 15 kDa), which was only recognized by antibodies against C-terminal epitopes. The endogenous proteins were not detected in the medium, because of the poor sensitivity of our antibodies, but the epitope-tagged bands were readily observed in the culture medium as well as in cell extracts; the heavy band was predominant in cell extracts and the light band in the medium. The fact that we obtained the same pattern in the cell extracts for the transfected and endogenous proteins shows that the production of both H and L proteins represents a normal processing of CutA and not an aberrant consequence of overexpression.

Both heavy and light bands contain the C terminus of CutA, because they were labeled by antibodies against the anti-C-terminal peptide and against a C-terminal HA epitope (Fig. 2). In contrast, an N-terminal FLAG epitope was found only in the heavy band. Therefore, the heavy band (H) represents the full-length protein, starting at Met¹, whereas the light band (L) does not contain the N-terminal region. The fact that it was not recognized by an antibody directed against the 44–57 peptide indicates that the cleavage probably occurs downstream of the third methionine (Met⁴⁴).

Mass spectrometry showed that the heavy bands from the N- and C-terminal flagged CutA contain the corresponding N-terminal peptides, MDYKDDDDKNWAR and MNWAR, cleaved by trypsin after the first arginine (Arg³). In both cases, the N-terminal methionine was N-acetylated, which may reflect a metabolic stabilization. These peptides were not found in the light bands under identical experimental conditions.

In the culture medium, an N-flagged heavy band could be detected by Western blots. This implies that the longer CutA variant can be secreted without cleavage of an N-terminal signal peptide, as discussed below.

**Effect of Mutations of the Three Potential In-frame Translation Initiation Sites on the Production and Secretion of Long and Short Variants**—The presence of heavy and light components of CutA in the cells and in the medium raised two questions related to the presence of three in-frame ATG codons encoding
A. Alignment of human and mouse CutA genes

B. Schematic organization of transcripts

C. Proteins

D. Mutations in mouse CutA
methionines Met\(^1\), Met\(^{24}\), and Met\(^{44}\) (Fig. 1C). First, could several variants of CutA be produced by alternative usage of these potential translation initiation sites? Second, would any of these possible alternative proteins possess a secretory signal peptide?

The full-length CutA, starting at methionine Met\(^1\), is predicted to possess a secretory signal peptide with a probability of 0.99 by the SignalP 3.0, and the mature protein would start either at Arg\(^{33}\) or Ser\(^{48}\), as indicated in Fig. 1C, but the scores assigned to these potential cleavage sites are much lower than for conventional secreted proteins such as AChE (0.13 versus 0.84). A protein starting at Met\(^{24}\) is also predicted to possess a secretory signal peptide, with a cleavage site before Ser\(^{48}\) (although its score is also low, 0.34) so that the first two possible translation start sites might produce the same final mature protein. In contrast, a protein starting at Met\(^{44}\) is not predicted to possess a secretory signal peptide. The presence of FLAG or HA epitopes, added immediately after Met\(^1\), does not affect the predicted probability of a signal peptide or the cleavage position.

To assess the possible use of the ATG codons for initiation of translation, we mutated them individually and in pairs, in constructs containing a C-terminal HA epitope. The first ATG was mutated to a stop codon, to avoid the possible use of an upstream initiation site, and the downstream ones to serine codons. The resulting mutants were designated according to presence or absence of ATG codons at each position: CutA\(^{(1,24,44)}\) and CutA\(^{(44)}\) produced much higher levels of CutA protein than those containing the first ATG. The mutant containing only the second ATG (CutA\(^{(24,44)}\)) also produced a relatively high level of cellular CutA, which formed a doublet perhaps because of secondary cleavage, but secreted very little CutA. All media were diluted \(\frac{1}{2}\). The fraction of the cellular content released per h was about 1.7% for CutA\(^{(24,44)}\), 0.5% for CutA\(^{(24,44)}\), and less than 0.1% for CutA\(^{(44)}\).

![Figure 1](image1.png)

**Figure 1.** Human and mouse CutA genes and proteins. A, alignment of genomic sequences. The human gene is located on chromosome 17 (B1). The *upper lines* correspond to the human genomic sequence (*capital letters*), and the *lower lines* to the mouse sequence (*lowercase letters*). Vertical bars identify the constitutive exons that are common to human and mouse and are highlighted in yellow; introns are not highlighted. Note that the mouse genome does not seem to possess any region homologous with the first human exon (highlighted in cyan). We have not attempted to align the introns, some of which differ in their length. Potential translation initiation codons (ATG) are highlighted in red; schematic structures of the various transcripts, with the same colors as in A. The nomenclature of the transcripts and proteins (isoforms/variants) corresponds to those of UniProt (human) and Mouse Genome Informatics (mouse). Functional initiation start sites are shown as *full green triangles*, and nonfunctional ones as *open green triangles*; stop codons are indicated as *full red triangles*. C, alignment of the human and mouse proteins. Potential translation initiation sites (ATGs) are shown in green. Note that the first ATG (in the exon shown in blue) of the human transcripts, which produces isoform 1 (starting as MIGS) with transcript A, has no equivalent in the mouse. Although present in all transcripts, it cannot serve as a translation initiation site for CutA in other transcripts, because of frame shifts. The second ATG is used in transcript B, producing isoform 2 (protein starting with MSGG), and the third ATG is used in transcripts C, D, and E, producing the same isoform 3 (protein starting with MPAL). In the same exon, a second ATG is also indicated because it is in-frame and has been proposed to serve as initiation site; the corresponding protein would start with MASG. In the mouse, only two transcripts have been documented by ESTs; they differ by the insertion or not of the exon highlighted in magenta. Transcripts 1, which includes this exon, produces a protein starting as MWNQ (variant 1, homologous to human isoform 2) and containing the following two ATGs, without frameshift. Transcripts 2, without this exon, produces a protein starting with MPAL (variant 2, homologous to human isoform 3). The *doubly underlined* region, strongly conserved between mouse and human, is involved in the trimeric association. The *arrowheads* indicate the most likely cleavage sites for signal peptides of mouse variants 1 and 2, according to SignalP prediction. D, mutations made in mouse mCutA. Point mutations are shown by *capital letters above* the sequence, indicating residues that replaced the original ones. In some mutants, the first ATG was mutated to a stop codon, indicated as X, and the second and third ATGs were mutated to serine codons; the corresponding mutants are colored according to the remaining methionines, e.g. CutA\(^{(1,24,44)}\) for the wild type. Introduced peptide motifs are indicated in brackets: a FLAG epitope (DYKDDDDK) at the N terminus (after Met\(^1\)) or at the C terminus, an HA epitope (YPYDVPDYA), and an endoplasmic reticulum retention motif (KDEL). The peptides identified by mass spectrometry from the heavy (20 kDa) component are *underlined*. Note that our clones contained a mutation (G4A), which did not modify the predicted probability of a signal peptide. Antibodies “anti-N” and “anti-C” were prepared against synthetic N-terminal (44–57: MASGSPPSPQPPAS) and C-terminal (164–177: QVTESV5NSGTALP) peptides, respectively.
CutA, Acetylcholinesterase, and Secretory Trafficking

responding to the other two methionines of CutA (Met149 and Met150) are preceded by out of frame ATGs. We find that the heavy component (20 kDa) was produced only by mutants containing the first ATG (CutA(1,44), CutA(1,24), and CutA(1)), whereas others produced only lighter components.

The mutant possessing only the first ATG (CutA(1)) produced mostly the heavy component, but also some light component, both in the cells and in the medium (Fig. 3). This shows that the L components may result from post-translational proteolytic cleavage of the full-length H protein. The fact that similar L components were obtained with CutA(24) and CutA(44) suggests that they may also be produced by the use of the downstream ATGs. A post-translational cleavage of these shorter proteins might explain why the L components produced by the different CutA mutants appear very similar or identical.

Initiation of the translation at the second or third ATGs appears likely because the level of the L component was markedly higher for the wild type CutA, possessing its three ATGs, than with CutA(1), possessing only the first one. In addition, the CutA(1) and CutA(24) mutants were produced about five times less than the wild type, whereas the cellular level of CutA(44) was about 20-fold higher than that of CutA(1). This may reflect different rates of protein translation, because the third ATG may be a more efficient initiation start site.

Whereas none of the three possible translation initiation codons is included in a classical Kozak consensus environment ((A/G)CCATGG) (21), only the third one is followed by a G. However, mutating nucleotides around the first ATG (TGGATGC to TGCAATGG) only increased the level of cellular CutA(1) about 3-fold (not shown), far from that obtained for CutA(44).

Fig. 3 shows that all mutant CutA proteins were released in the medium as follows: about 1.5% of the cell content per h for CutA and CutA(1), 0.2% for CutA(24), and 0.1% for CutA(44). This is markedly lower than the release of AChE (about 15%/h). The heavy component (H) was found in the medium, for CutA as well as for mutants containing the first ATG start codon (CutA(1), CutA(1,24), and CutA(1,44)), but the light component (L) was predominant. Because the release of the heavy component occurs without cleavage of a signal peptide, it might reflect a leakage from damaged cells.

We therefore examined the effect of brefeldin A, an inhibitor of secretion. After 8 h of incubation with brefeldin A, the cellular level of CutA did not change significantly, but we observed an additional component slightly heavier than the H band and a weak reduction of the secreted protein (Fig. 4). We also studied a CutA mutant to which we added a C-terminal KDEL peptide, downstream of the HA epitope (CutAHA-KDEL). The cellular level of this mutant was lower than that of CutAHA, and the fraction of cellular content released per h was not reduced, perhaps because the KDEL motif did not act as a functional endoplasmic reticulum retention signal, as discussed below in the case of AChE mutants (Fig. 10, A and B). However, both heavy and light bands formed by CutAHA-KDEL appeared as doublets in Western blots (Fig. 4). The heavier components, with an apparent mass about 2 kDa higher than the H and L bands observed for the wild type, probably resulted from a post-translational modification because H corresponds to the full-length protein.

Subcellular Localization of CutA, Specific Release of Cytosolic Proteins by Digitonin, and Purification of a Mitochondrial Fraction—The release of CutA into the medium suggests that it travels through the secretory pathway. However, the N-terminal region of wild type CutA remains partially uncleaved, and it is not unambiguously predicted to function as a secretory signal peptide. Moreover, the CutA(44) mutant, starting at the third methionine, has clearly no secretory signal peptide. In addition, CutA was reported to be localized in mitochondria and not in the secretory organelles (9). It was therefore necessary to establish the subcellular localization of CutA.

COS cells were transfected with CutA together with a secreted protein (AChE) and a cytoplasmic protein (β-galactosidase). They were first treated by digitonin, to permeabilize the plasma and outer mitochondrial membranes, and then by Triton X-100, to release the content of the secretory organelles (Fig. 5, A and B). The selectivity of the two steps was established by the release of β-galactosidase, a cytoplasmic enzyme, by digitonin, and the release of AChE, by Triton X-100. Triton X-100 is a resident endoplasmic reticulum protein, by Triton X-100. The light and heavy chains of CutA were solubilized by digitonin and by Triton X-100, indicating a cytoplasmic and a microsomal localization, respectively.

This was confirmed by CutA mutants containing a single 5’ ATG (CutA(1), CutA(24), and CutA(44)). The heavy chain produced by the CutA mutant possessing only the first ATG (CutA(1)) appeared to reside in the secretory pathway. The light chains produced by CutA mutants starting at the second and third ATGs (CutA(24) and CutA(44)) were mostly released in the first step, indicating a predominantly cytoplasmic localization.

To assess the possible localization of CutA in mitochondria, we prepared mitochondrial, microsomal, and cytosolic fractions from COS cells expressing CutA or its mutants by differential sedimentation (Fig. 5C). To verify the nature of these
fractions, we used the endogenous proteins TOM-22 as a mitochondrial marker, BiP as an ER marker, and β-galactosidase as a cytosolic marker. The distributions of these proteins in each fraction were compared with those of a total extract, obtained by direct solubilization of the cells with 1% Triton X-100. This confirmed that the heavy CutA component is present in the microsomes and that the light component is cytosolic, and we found no enrichment of CutA in the purified mitochondrial fraction. These results clearly indicate that the heavy chain is mostly located in the endoplasmic reticulum and in the secretory pathway, whereas the light chain is mostly cytoplasmic.

**Trimeric Associations between Full-length and Cleaved CutA**—Intact cells expressing CutA or its mutants were treated with a cell-permeable reagent, DSS, and analyzed by SDS-PAGE and Western blotting. In the case of wild type CutAHA, in addition to the light (L) and heavy (H) bands, cross-linking produced three bands, which appeared to represent dimers LL, LH, and HH, as well as heavier bands, probably trimers LLL, LHH, LHH, and HHH (Fig. 6). The CutA(1) and CutA(44) mutants mostly produced HH and LL dimers, as expected.

Thus, the heavy and light CutA components may form mixed trimers, in agreement with the fact that they both possess the trimerization domain. This also shows that they are at least partially contained in the same compartment or that cleavage of H to L occurred during the experiment.

**Effect of Transfected CutA on the Production and Secretion of AChE and Influence of C-terminal Peptides**—We noticed that the cellular and secreted levels of AChE activity decreased markedly when AChE was co-expressed with CutA, in a dose-dependent manner (Fig. 7). This effect was stronger for the AChE T variant, possessing a C-terminal t peptide than for a truncated AChE T mutant, lacking this t peptide. We observed the reduction of AChE level with CutA, with its mutant CutA(11), starting at the first methionine, and with a CutA mutant possessing a C-terminal KDEL retention motif, but much less with mutants CutA(24) and CutA(44) which are mostly cytoplasmic. This suggests that the CutA protein acts in the secretory compartment and affects the biosynthesis, the degradation, and perhaps the oligomerization of AChE T, because these processes depend on the C-terminal t peptide of AChE T.
or when its free cysteine was mutated to a serine, so that the C-terminal tetrapeptide CSDL was replaced by SSDL (mutant “T-S”) (not shown). We also analyzed various mutants in which the aromatic residues of the t peptide, which play a critical role in the oligomerization, secretion, and degradation, were modified (22). The effect was reduced when the segment containing the seven aromatic residues of the t peptide was deleted, when these residues were displaced (scrambled) so that they could not form a cluster in an amphiphilic α-helix, or when they were replaced by leucines (not shown). This confirms that mutations that affect the trafficking and oligomerization of AChE also modify the effect of CutA on the production of AChE.

We showed previously that AChET subunits are partially misfolded, that even correctly folded active subunits are degraded through the ERAD pathway, and that the aromatic residues of the t peptide play an important role in these processes (23). In effect, the inhibition of proteasome activity by MG132 and clasto-β-lactacystin increased the cellular level and the secretion of AChET and of AChEΔ, (truncated mutant lacking the C-terminal t peptide). A fixed amount of vector encoding AChE and its mutants (0.5 μg/60-mm dish) was co-transfected into COS cells with variable amounts of vector encoding CutA or its mutants. AChE activities are expressed as percent of the control. The effects of wild type CutA (■) and CutAΔ1 (♀) were identical, whereas that of CutAΔ44 (○) was much lower, despite the fact that this mutant was expressed at a 20-fold higher level (see Fig. 3).

FIGURE 7. Effect of co-transfection with CutA on the production of AChE. This figure illustrates the effect of CutA and its mutants CutAΔ1 and CutAΔ44 on the production and secretion of AChET (with the t peptide), and AChEΔ (truncated mutant lacking the C-terminal t peptide). A fixed amount of vector encoding AChE and its mutants (0.5 μg/60-mm dish) was co-transfected into COS cells with variable amounts of vector encoding CutA or its mutants. AChE activities are expressed as percent of the control. The effects of wild type CutA (■) and CutAΔ1 (♀) were identical, whereas that of CutAΔ44 (○) was much lower, despite the fact that this mutant was expressed at a 20-fold higher level (see Fig. 3).

or when its free cysteine was mutated to a serine, so that the C-terminal tetrapeptide CSDL was replaced by SSDL (mutant “T-S”) (not shown). We also analyzed various mutants in which the aromatic residues of the t peptide, which play a critical role in the oligomerization, secretion, and degradation, were modified (22). The effect was reduced when the segment containing the seven aromatic residues of the t peptide was deleted, when these residues were displaced (scrambled) so that they could not form a cluster in an amphiphilic α-helix, or when they were replaced by leucines (not shown). This confirms that mutations that affect the trafficking and oligomerization of AChE also modify the effect of CutA on the production of AChE.

We showed previously that AChET subunits are partially misfolded, that even correctly folded active subunits are degraded through the ERAD pathway, and that the aromatic residues of the t peptide play an important role in these processes (23). In effect, the inhibition of proteasome activity by MG132 and clasto-β-lactacystin increased the cellular level and the secretion of AChET and of CutA, showing that are both degraded by ERAD (Fig. 8). However, we observed that AChE was increased by the same factor when expressed alone or with CutA, suggesting that CutA does not increase ERAD (not shown). We further found that when protein synthesis and secretion were blocked, the first order rate of decrease of AChE activity was the same in cells expressing only AChET, or AChET with a dose of CutA, which reduced the cellular activity by about 90% (Fig. 9).

FIGURE 8. Effect of proteasome inhibitors on cellular and secreted CutA and AChE. Cultures of transfected COS cells were treated for 8 h with the proteasome inhibitors MG132 and clasto-lactacystin-β-lactone, before collecting the medium and extracting the cells. A and B, proteasome inhibitors increased the levels of AChET protein (A) and activity (B) in the cells and even more markedly in the medium. AChE activity was expressed as percent of untreated controls. C, proteasome inhibitors strongly increased the levels of CutA protein in the cells and in the medium. In the case of the CutAΔ44 mutant possessing a C-terminal ER retention motif KDEL, which produced doublets for the H and L bands, the increase was particularly marked for the heavier components of the doublet.

FIGURE 9. The first order rate of decrease of AChE activity, in the absence of protein synthesis and of secretion, was not affected by co-expression with CutA. AChET was expressed in COS cells alone (0.5 μg of DNA/60-mm dish) (●), or with CutA (2 μg of DNA/60-mm dish) (□). The cells were treated at time 0 with cycloheximide (CHX, 200 μg/ml) to block protein synthesis and with brefeldin A (BFA, 20 μg/ml) to block secretion. The residual activity, normalized to 100 at time 0, is plotted on a semi-logarithmic scale as a function of time.

Taken together, all these observations strongly suggest that CutA does not exert its effect on the biosynthesis of AChE, because this would not distinguish AChETΔ, AChETΔ, and its mutants, or on its degradation (ERAD), but interferes with folding in a t peptide-dependent manner. This hypothesis is consistent with the fact that rates of recovery of AChE activity by neosynthesis after irreversible inhibition of the cellular enzyme
by the membrane-permeable organophosphate inhibitor soman were proportional to the steady state levels (not shown). The C-terminal tetrapeptide of AChET (CSDL) resembles an ER retention signal and contains a cysteine, which might be involved in the retention of unassociated subunits (24). Because this motif seems to play a role in the effect of CutA, we analyzed AChE mutants containing a classical endoplasmic reticulum retention signal (KDEL), as shown in Fig. 10A. We found that the retention was strongly dependent on the preceding peptidic sequence (Fig. 10B). For example, retention was quite efficient for a mutant in which the t peptide, with its cysteine replaced by a serine, was followed by a FLAG epitope and the KDEL motif (“T-fl-K”) but very weak in the case of the “T-7L-K” mutant, in which the KDEL motif terminates a t peptide in which the seven aromatic residues of the t peptide were replaced by leucines, and the KDEL motif replaced the original CSDL tetrapeptide. The KDEL motif did not cause retention when placed immediately downstream of the catalytic domain (AChE<sub>Δ,K</sub>), probably because of steric hindrance because of the vicinity of a large organized domain. In contrast, the KDEL motif exerted a very efficient retention effect when separated from the catalytic domain by a spacer of 22 residues derived from the H variant (“H-22-K”); this mutant can form dimers but no tetramers.

In the presence of CutA, the secretion of KDEL-containing mutants such as H-22-K and “T-S-fl-K” was increased, whereas the cellular activity was decreased (Fig. 10C). Therefore, CutA facilitates the secretion of retained KDEL-containing AChE mutants. Because T-S-fl-K forms tetramers whereas H-22-K cannot, this effect is not related to oligomerization. The fact that CutA increases the secretion of KDEL-containing proteins confirms that it affects trafficking in the secretory route.

**Effect of CutA on the Level of BiP in COS Cells**—We observed that transfection with AChET increased the level of BiP (data not shown), as expected because a significant fraction of newly synthesized AChET polypeptides are misfolded (2, 22). Their presence is expected to induce an ER stress (“unfolded protein response”) and up-regulate BiP. In contrast, transfection of COS cells with CutA significantly decreased the level of BiP (Fig. 11), suggesting that it participates in the disposal of incorrectly folded proteins in the ER.

**Effect of CutA on the Tetramerization of AChET Subunits**—We examined whether CutA might affect the oligomerization of AChET subunits. When they were expressed alone in COS cells, they produced mostly monomers (G<sub>1</sub>) and dimers (G<sub>2</sub>),...
with a small proportion of tetramers (G₄), representing about 2% of the total cellular activity and 4.5% of the secreted activity. When AChET subunits were co-expressed with CutA (1 μg of DNA/dish), the proportions of tetramers were increased to 4.5 and 26%, respectively (not shown).

The increase in the level of secreted G₄ was particularly marked for mutants that possessed a C-terminal KDEL retention motif, as illustrated in Fig. 12 for T-S-fl-K. This retention signal may facilitate the assembly of AChET subunits into tetramers because of their accumulation in the ER; however, it does not prevent the secretion of tetramers, probably because it is masked in the oligomeric structure, as noted in a previous study (25). Thus, CutA facilitates the oligomerization of AChE subunits containing the t peptide, particularly when they are retained in the ER.

Because CutA has been proposed to participate in the membrane anchoring of AChE by the transmembrane protein PRiMA, we also studied the effect of CutA on the recruitment of AChET tetramers by PRiMA (19, 26) and also by an N-terminal fragment of cholinesterase-associated collagen Q, called Qs (17, 27). The tetramers were mostly membrane-bound when associated with PRiMA and secreted when associated with Qs. We found that co-expression with CutA decreased the total AChE activity, as shown in Fig. 7, but had no detectable effect on the proportions of AChET tetramers formed with either PRiMA or Qs (not shown).

Cysteines of CutA Are Not Involved in Its Effects on AChE Processing—The effects of CutA clearly differ between AChET and T-S, suggesting a possible interaction with the free cysteine of AChET, perhaps the transient formation of mixed disulfide bonds. To explore this possibility, we replaced the two cysteines of CutA by serines. The resulting CutA mutants reduced the activity of copper ions (29). It has also been proposed to act in signal transduction (10, 12).

We were interested in CutA because it had been co-purified with membrane-bound AChE from mammalian brain (6, 7) and appeared somehow involved in the anchoring of this enzyme through its associated transmembrane protein PRiMA (7). It has been proposed to act as a modulator of the redox state (10) and might thus contribute to the formation of disulfide bonds between AChET and PRiMA; in that case, its conserved cysteines would play a key role. However, the subcellular location of CutA had not been characterized, and a recent report suggested that it is located in mitochondria (9). Our first concern was therefore to define in which subcellular compartment CutA actually resides, to understand how it could affect the processing of AChE. This issue was complicated by the fact that CutA presents several isoforms that differ in their N-terminal peptides, some of which might function as a secretory signal peptide. Three isoforms have been described in the human, only two of which also exist in the mouse (Fig. 1). They result from the use of two possible ATG translation initiation codons, so that the longer one includes the shorter one; in addition, a third in-frame ATG might produce a still shorter variant. We focused our study on mouse variants starting at Met¹, Met⁴⁴, and Met⁴⁴.

The Long CutA Variant Enters the Secretory Pathway and Can Be Secreted without Cleavage of a Signal Peptide, Whereas Shorter Variants Are Mostly Cytoplasmic—We found that a transcript encoding the longer wild type variant of mouse CutA produces two proteins of ~20 kDa (H) and 15 kDa (L), which differ by the length of the N-terminal region. The L component is produced by post-translational proteolytic cleavage of the full-length protein. It may also result from initiation of translation at downstream ATGs, combined with proteolytic cleavage, because mutants possessing only the second and/or third ATG produce apparently identical L components. Thus, the CutA(24,44) mutant, which corresponds to the mouse variant 2, produces the L form of CutA.

Although the codon corresponding to Met⁴⁴ is always preceded by another ATG, it may be used as a translation initiation site because it is closer to a Kozak consensus. In this respect, it is interesting to note that translation of human CutA transcripts does not start from the first ATG encountered by ribosomes as they scan the mRNA, which are not in-frame with the coding sequence of the CutA protein (Fig. 1, A and B); for example, the ATG used to initiate translation of transcript A to produce isoforms 1 is also present but not used in the other transcripts that produce isoforms 2 and 3.
By introducing N- and C-terminal epitopes, and by mass spectrometry, we showed that the heavier component corresponds to the full-length protein, whereas the shorter one lacks an N-terminal peptide, either because translation is initiated at Met24 or Met44, because of a post-translational cleavage, or possibly a combination of the two processes. Mutants starting at Met1, Met24, or Met44 were all labeled with a C-terminal epitope and thus contained the same C-terminal peptide.

Using a selective permeabilization of the plasma membrane with digitonin, as well as subcellular fractionation, we showed that the heavy component is located in the secretory pathway, whereas the shorter component is mostly in the cytoplasm. This is consistent with the fact that the full-length protein was secreted, although its rate of secretion was less than 2% of the cellular content released per h, much lower than that of AChET (about 15%).

The transfer of this protein through the secretory pathway was confirmed by an analysis of constructs in which an AChE catalytic domain was fused downstream of the CutA protein; transfected cells expressing CutA-AChE or CutA(1)-AChE fusion proteins produced AChE catalytic activity. This implies that the protein was transferred into the endoplasmic reticulum, where it was correctly folded and acquired intra-molecular disulfide bonds. The processing of this protein in the secretory pathway was confirmed by its N-glycosylation and secretion.

CutA proteins starting at Met1 are predicted to possess potential signal peptides, but this is ambiguous because the cleavage site has a very low score. In fact, the N-terminal peptide of CutA (starting at Met1) remains uncleaved in the cellular and in at least a fraction of the secreted protein; cleavage of an N-terminal fragment occurred as a secondary event, independently of its transfer into the endoplasmic reticulum and secretion, because it progressed with time in the culture medium. Although the prediction of a signal peptide was stronger for proteins starting at Met24, they were much less efficiently secreted than those starting at Met1. These observations indicate that CutA proteins follow an unusual processing in the secretory pathway. This was also shown by the fact that treatment with brefeldin A or addition of a C-terminal KDEL motif did not inhibit secretion of CutA proteins as efficiently as that of AChE. In addition, this partial inhibition did not increase but rather decreased the cellular content, suggesting that intracellular degradation was activated. In this respect, it was interesting to find that, in contrast with wild type CutA which produced two bands in SDS-PAGE, corresponding to heavy and light components, the mutant possessing a C-terminal KDEL motif produced two doublets, each presenting a slightly heavier component (about 2 kDa), which may result from a post-translational modification, perhaps because of a prolonged residence in the secretory pathway.

CutA Affects the Folding, Oligomerization, and Secretion of AChE—We found that CutA, particularly the variants starting at Met1, affects the level of cellular and secreted AChE activity. This effect was much more marked for AChE mutants in which the cysteine or the aromatic residues of the t peptide were modified, or for a truncated AChE mutant, AChE(H9004), which only produces secreted monomers. We have previously shown that such mutations do not affect the synthesis of AChE polypeptides, but do affect their folding and degradation (22). In the case of AChE...
CutA, Acetylcholinesterase, and Secretory Trafficking

mutants possessing a C-terminal KDEL retention motif, CutA reduced the cellular activity but increased the residual secretion, showing that it does not simply reflect a competition for the cellular biosynthetic machinery. We did not find any influence of CutA on the degradation of AChE, either by following the fate of the enzyme after blockade of synthesis by cycloheximide and of secretion by brefeldin A, or by analyzing the increase in the cellular and secreted levels upon inhibition of proteasome activity. It thus seems likely that CutA acts at the level of protein folding, in agreement with the fact that the t peptide affects the proportion of AChE polypeptides that fold properly into a catalytically active conformation (22). This is consistent with the fact that CutA reduces the level of BiP, suggesting that it may assist in the disposal of misfolded proteins.

Co-expression with CutA increased the formation of tetramers, and their secretion, particularly in the case of mutants that lacked the C-terminal cysteine and normally produced a low proportion of tetramers. Therefore, the presence of CutA in the secretory compartment affects the oligomerization of AChET subunits. These effects were much less marked for CutA mutants starting at Met24 or Met44, which are mostly cytoplasmic. It is likely that only the small fraction that somehow enters the secretory pathway may interfere with AChE processing.

Thus, the presence of CutA proteins in the secretory pathway affects the processing, probably the folding, and the oligomerization of AChE. We found no effect on endogenous alkaline phosphatase, suggesting that CutA specifically affects a subset of secreted proteins, including AChE. The cysteines of CutA were not required for these effects. This seems to rule out an influence of CutA on disulfide bond formation, in agreement with the fact that it facilitated the oligomerization of AChET mutants lacking the C-terminal cysteine, such as T-S-fl-K.

Because CutA was discovered in connection with membrane-bound AChET tetramers, which are associated with the transmembrane protein PRiMA, we analyzed the possible effect of CutA on the formation of tetramers induced by PRiMA or by another AChE-associated protein, Qs, which also interacts with the t peptides through a proline-rich motif (5, 19, 26, 30). In co-transfected COS cells, CutA did not appear to affect the recruitment of AChET subunits into tetramers by these proteins. However, this may simply reflect the specific cellular conditions of overexpression and does not exclude a possible role of CutA in the physiological anchoring of AChE in the mammalian brain.

In a recent study, we found that the \(\text{ACHET}_T\)-PRiMA complex may incorporate additional small proteins, because it forms a doublet in SDS-PAGE under nonreducing conditions (26). However, co-expression with HA-labeled CutA showed that this complex is not directly disulfide-linked with CutA. In the data banks, CutA is usually described as an “acetylcholinesterase-associated protein.” This is clearly not the case.

In fact, we were not able to demonstrate any direct interaction between CutA and AChE, by co-immunoprecipitation, even after cross-linking in intact cells. This suggests that the effects described here are probably based on an indirect influence of CutA on the environment of the secretory compartments; thus, the presence of CutA in purified preparations of the AChET-PRiMA complex from mammalian brain may have been coincidental.

CutA May Exert Distinct Roles in the Secretory Pathway and in the Cytoplasm—The hypothesis that the longer CutA variant plays a role in protein folding and trafficking in the secretory pathway is consistent with the observation that it reduces the level of BiP in the cells. It may contribute to the disposal of misfolded proteins. The fact that CutA seems to affect the processing of secreted proteins at several levels suggests that it may not interact directly with these proteins, as a classical chaperone, but rather indirectly through some influence on the environment of the secretory compartments.

Because the short CutA variants remain mostly cytoplasmic, they may play a different role, possibly in the regulation of transcription, as suggested by the structural homology of CutA with the trimeric PII signal transduction factors, which bind nucleotides and control nitrogen metabolism in procaryotes and plants (31). The unusual and diverse processing and localization of CutA variants in mammalian cells therefore raise interesting questions about their possible functions.

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