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 (Cu\(^{2+}\) 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, one cysteine and one cysteine, respectively, with one of the cysteines being part of a disulfide bond (10). In the human brain, it has been proposed 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.
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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% Nusserum (Inotech, 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 μg/ml), or with inhibitors of proteasomal degradation MG132 (10 μM) and clasto-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 2.5 mM benzamidine, a protease inhibitor, at 20 °C for 15 min. The supernatant was collected after centrifugation at 13,000 rpm, for 10 min at 4 °C. The culture media were also collected and centrifuged for analysis of secreted proteins.

Subcellular Fractionations—For selective permeabilization of the plasma membrane, transfected COS cells were first washed in the culture dish with 1 ml of PBS and then treated on ice with 1 ml per 60-mm dish of 150 μg/ml digitonin (in 20 mM HEPES, pH 7, 110 mM K⁺ acetate, 2 mM MgCl₂) for 6 min. The supernatant containing cytosolic proteins was collected, and the cells were washed with 1 ml of PBS. They were then treated with 1 ml of extraction buffer containing 1% Triton X-100.

For preparation of a mitochondrial fraction, we used the Qproteome mitochondria isolation kit from Qiagen, following the manufacturer’s instructions. Briefly, the cells were washed in 0.9% NaCl, pelleted at 1,000 × g, and resuspended in the “disruption buffer” to release cytosolic proteins. A 1,000 × g 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 20-s intervals over a period of 10 min. Under those conditions, 1 μOD 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).

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 μM 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...
downstream in-frame ATG codon, encoding Met44, is located due to the longer protein) (Fig. 1). A downstream 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, Met24, 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 antisera directed against an 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, MDYKDDDDKNNWR and MNWWR, cleaved by trypsin after the first arginine (Arg9). 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 Meteor, Met24, and Met44 (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 Met1, is predicted to possess a secretory signal peptide with a probability of 0.99 by the SignalP 3.0 server, and the mature protein would start at either Arg3 or Ser48, 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 Met24 is also predicted to possess a secretory signal peptide, with a cleavage site before Ser48 (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 Met44 is not predicted to possess a secretory signal peptide. The presence of FLAG or HA epitopes, added immediately after Met1, 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: CutA24,44, CutA1,44, CutA1,24, CutA13, CutA23, CutA43, and CutA44. Note that CutA24,44 corresponds to the splice variant 2 (Fig. 1, B and C). Western blots corresponding to these mutants are shown in Fig. 3. As expected, the CutA1,24-44 mutant, lacking all three ATGs, was not found to produce any CutA-related protein, in agreement with the fact that the codons con-

FIGURE 2. CutA produces heavy and light bands, in cell extracts and in the culture medium. In untransfected COS cells, the anti-C-terminal antibody (anti-C) recognized a heavy band (H) and a light band (L), whereas the anti-N-terminal antibody (anti-N) only recognized the H band. These antibodies were not sufficiently sensitive to detect the CutA proteins in the culture medium. Transfected cells expressing epitope-tagged CutA mutants were grown in 60-mm dishes, incubated with 1 ml of fresh medium for 8 h, and extracted in 1 ml of extraction buffer; we used a 20 times higher volume of medium than of cell extracts for SDS-PAGE for N-FLAG-CutA, and a 4 times higher volume for CutA-HA and CutA-MIGS, both H and L bands were labeled with a C-terminal epitope, FLAG or HA, but only the H band was labeled with an N-terminal FLAG epitope.

FIGURE 1. Human and mouse CutA genes and proteins. A, alignment of genomic sequences. The human gene is located on chromosome 6 and the mouse gene 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 indicate identity. The constitutive exons that are common to human and mouse 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. B, 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. Transcript 1, which includes this exon, produces a protein starting as MNWG (variant 1, homologous to human isoform 2) and containing the following two ATGs, without frameshift. Transcript 2, without this exon, produces a protein starting with MIGS.

Effect of mutations of the three potential ATG translation initiation sites on the production and secretion of light and heavy CutA proteins. The different mutants of CutA carried a C-terminal HA epitope and were labeled with an anti-HA antibody. H indicates the heavy band (about 20 kDa) and L the light band (about 15 kDa). Upper panel, Western blots of cell extracts. Lower panel, Western blots of culture media. The cells were transfected with equal amounts of vectors encoding the various mutants (0.5 μg/60-mm dish), cultured, and extracted in the same way. The samples were diluted as indicated on the figure, and the mutants containing the third, but not the first, ATG (CutA24,44 and CutA44) produced much higher levels of CutA protein than those containing the first ATG. The mutant containing only the second ATG (CutA24,43) 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 1/2. The fraction of the cellular content released per h was about 1.7% for CutA1,24,44, 0.5% for CutA24,43, and less than 0.1% for CutA44.

In untransfected COS cells, the anti-C-terminal antibody (anti-C) recognized a heavy band (H) and a light band (L), whereas the anti-N-terminal antibody (anti-N) only recognized the H band. These antibodies were not sufficiently sensitive to detect the CutA proteins in the culture medium. Transfected cells expressing epitope-tagged CutA mutants were grown in 60-mm dishes, incubated with 1 ml of fresh medium for 8 h, and extracted in 1 ml of extraction buffer; we used a 20 times higher volume of medium than of cell extracts for SDS-PAGE for N-FLAG-CutA, and a 4 times higher volume for CutA-HA and CutA-MIGS, both H and L bands were labeled with a C-terminal epitope, FLAG or HA, but only the H band was labeled with an N-terminal FLAG epitope.

In some mutants, the first ATG was mutated to a stop codon, indicated as a streamlined 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 named according to the remaining methionines, e.g. CutA1,24,44, for the wild type. Introduced peptidic motifs are indicated in brackets: a FLAG epitope (DYKDDDDK) at the N terminus (after Met1) 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 (G44), 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: MASGSPPSPPQPPAS) and C-terminal (164–177: QVTEVSNSGTLAP) peptides, respectively.
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responding to the other two methionines of CutA (Met\(^{149}\) and Met\(^{150}\)) 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 AGCATGG) 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\(_{C}\) (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 (CutA\(_{HA,KDEL}\)). The cellular level of this mutant was lower than that of CutA\(_{HA}\), 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 CutA\(_{HA,KDEL}\) 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.

![FIGURE 4. Effect of brefeldin A and of a C-terminal KDEL retention motif on cellular and secreted CutA proteins. COS cells were transfected with CutA containing a C-terminal HA epitope, and with a mutant containing a KDEL retention motif after the HA epitope (CutA\(_{HA,KDEL}\)). Three days after transfection, the cells were washed, and fresh medium (1 ml) was added, with or without brefeldin A (BFA); it was collected after 8 h, and the cells were extracted in 1 ml of buffer containing Triton X-100. For SDS-PAGE, we used a five times higher volume of medium than of cell extract in the case of CutA\(_{HA}\) (same volumes with and without BFA) and approximately equal volumes in the case of CutA\(_{HA,KDEL}\). Brefeldin A strongly reduced secretion of AChE (not shown) but had little effect on the release of CutA. It did not significantly increase the cellular level but appeared to induce the appearance of a heavier band, slightly above H. In the case of the CutA\(_{HA,KDEL}\) mutant, we observed additional components, about 2 kDa heavier than H and L.

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\(_{T}\) and β-galactosidase, 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 CutA, the trimeric association was seen only when AChE was co-expressed with CutA, in a dose-dependent manner (Fig. 7). This effect was stronger for the AChET variant, possessing a C-terminal t peptide than for a truncated AChET mutant, lacking this t peptide. We observed the reduction of AChE level with CutA, with its mutant CutA(1), 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 AChET, because these processes depend on the C-terminal t peptide of AChET (2).

We found that the effect of CutA was reduced when a FLAG epitope was added at the C terminus of AChET (mutant “T-fl”),
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-lactacystin-β-lactone increased the cellular level and the secretion of AChET, and AChE, (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 CutA(1) were identical, whereas that of CutA(44) 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 CutA(1) and CutA(44) on the production and secretion of AChE T (with the t peptide), and AChE T (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 CutA(1) were identical, whereas that of CutA(44) was much lower, despite the fact that this mutant was expressed at a 20-fold higher level (see Fig. 3).

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 AChE T 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 CutA(1) 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 protease inhibitors and of secretion, was not affected by co-expression with CutA. AChE T 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 AChE T, AChE T, 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 so-
man 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 pep-
tide were replaced by leucines, and the KDEL motif replaced the origi-
nal CSDL tetrapeptide. The KDEL motif did not cause retention when
placed immediately downstream of the catalytic domain (AChE_S-K),
probably because of steric hindrance because of the vicinity of a
large organized domain. In contrast, the KDEL motif exerted a very effi-
cient 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 secre-
tion 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-con-
taining 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 con-
 firms 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 frac-
tion of newly synthesized AChET
polypeptides are misfolded (2, 22).
Their presence is expected to in-
duce an ER stress ("unfolded protein response") and up-regu-
late 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 pro-
teins 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_1) and dimers (G_2),

![FIGURE 10. AChE mutants possessing a C-terminal KDEL motif; effect of CutA on the cellular and secreted
activities. A, C-terminal sequences of mutants with or without a KDEL motif. The last three residues of the
common catalytic domain, encoded by constitutive exons, are shown in boldface type and underlined. Residues
of the t peptide that were mutated or added at the C terminus are underlined. B, effect of a KDEL motif on
ancestors and secreted activities. Mutants are shown in pairs that contain or not the C-terminal cysteine (neces-
sary to form dimers), without a KDEL motif (gray bars) and with a KDEL motif (hatched bars). All activities are
expressed as percent of that produced by the wild type AChET in parallel transfections. C, dose-dependent
effect of co-expression with CutA (I) (four independent experiments) and CutA_S (V) (two independent exper-
iments) on the cellular activity and secretion of mutant H-22-K; secretion increased severalfold although the
acellular activity decreased, as in the case of wild type AChET (not shown). The CutA_44 mutant did not produce
this effect (C).](https://www.jbc.org/content/284/8/5203)

**FIGURE 10.**
with a small proportion of tetramers (G₄), representing about 2% of the total cellular activity and 4.5% of the secreted activity. When AChE₄ 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 AChE₄ 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 AChE₄ 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 AChE₄ 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 AChE₄ and T-S, suggesting a possible interaction with the free cysteine of AChE₄, 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 AChE activity, as shown in Fig. 7, but had no detectable effect on the proportions of AChE₄ tetramers formed with either CutA or Qs (not shown).

Cysteines of CutA Are Not Involved in Its Effects on AChE Processing—The effects of CutA clearly differ between AChE₄ and T-S, suggesting a possible interaction with the free cysteine of AChE₄, 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 AChE activity, as shown in Fig. 7, but had no detectable effect on the proportions of AChE₄ tetramers formed with either CutA or Qs (not shown).

FIGURE 11. Effect of CutA mutants on the level of BiP. BiP was analyzed by Western blots (top panel), in cells expressing various CutA mutants. The levels of BiP were quantified by densitometry of the blots in two parallel series of transfected cells (lower panel). The level of BiP was reproducibly decreased by CutA, CutA(24), and CutAHA-KDEL (CutA(44)), but not by mutants starting with the second or third methionines, or with a mutant in which the three potential initiation ATG codons were mutated (CutA(1)).
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 Met^{24} or Met^{44}, because of a post-translational cleavage, or possibly a combination of the two processes. Mutants starting at Met^{1}, Met^{24}, or Met^{44} 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. Constructs CutA_{(24)}-AChE and CutA_{(44)}-AChE, starting at Met^{24} or Met^{44}, also produced a low level of catalytically active protein, but most of the protein was cytoplasmic, unglycosylated, and inactive. This suggests that a minor fraction was transferred into the secretory pathway, where AChE can fold into its active conformation, and is eventually secreted even though the proteins starting at Met^{44} clearly lack any secretory signal peptide.

CutA proteins starting at Met^{1} 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 Met^{1}) 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 Met^{24}, they were much less efficiently secreted than those starting at Met^{1}. 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 Met^{1}, affects the level of cellular and secreted AChE activity. This effect was much more marked for AChE_{(1)}, possessing a wild type C-terminal t peptide than for mutants in which the cysteine or the aromatic residues of the t peptide were modified, or for a truncated AChE mutant, AChE_{(1)}, 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

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 (AChET)4-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 AChE-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 procaroytes 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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