Rescue and Stabilization of Acetylcholinesterase in Skeletal Muscle by N-terminal Peptides Derived from the Noncatalytic Subunits*  

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Background: Most newly synthesized acetylcholinesterase molecules are catalytically inactive and rapidly degraded intracellularly; how this is regulated is not known.  

Results: The enzyme is stabilized by noncatalytic subunit-derived peptides and is rescued from ERAD degradation.  

Conclusion: Binding to noncatalytic subunits stabilizes and prevents degradation of acetylcholinesterase in skeletal muscle.  

Significance: Peptides that stabilize target proteins can potentially be used to increase their expression in vivo.

The vast majority of newly synthesized acetylcholinesterase (AChE) molecules do not assemble into catalytically active oligomeric forms and are rapidly degraded intracellularly by the endoplasmic reticulum-associated protein degradation pathway. We have previously shown that AChE in skeletal muscle is regulated in part post-translationally by the availability of the noncatalytic subunit collagen Q, and others have shown that expression of a 17-amino acid N-terminal proline-rich attachment domain of collagen Q is sufficient to promote AChE tetramerization in cells producing AChE. In this study we show that muscle cells, or cell lines expressing AChE catalytic subunits, incubated with synthetic proline-rich attachment domain peptides containing the endoplasmic reticulum retrieval sequence KDEL take up and retrogradely transport them to the endoplasmic reticulum network where they induce assembly of AChE tetramers. The peptides act to enhance AChE folding thereby rescuing them from reticulum degradation. This enhanced folding efficiency occurs in the presence of inhibitors of protein synthesis and in turn increases total cell-associated AChE activity and active tetramer secretion. Pulse-chase studies of isotopically labeled AChE molecules show that the enzyme is rescued from intracellular degradation. These studies provide a mechanistic explanation for the large scale intracellular degradation of AChE previously observed and indicate that simple peptides alone can increase the production and secretion of this critical synaptic enzyme in muscle tissue.

Acetylcholinesterase (AChE) is responsible for the rapid clearance of acetylcholine released from nerve terminals into the synaptic cleft, limiting the activation time of nicotinic or muscarinic acetylcholine receptors. Skeletal muscles and neurons express the same AChE splicing variant, AChEt, capable of forming disulfide-bonded homodimers with each other or with the noncatalytic collagen tail, or Q (ColQ), or the transmembrane PRiMA subunits (1–4). The ColQ subunit produces the extracellular matrix-associated ColQ-AChE, and the transmembrane PRiMA subunit produces the membrane-bound PRiMA-AChE forms, respectively (5, 6). The C-terminal domain of AChEt binds to the N-terminal region of PRiMA or ColQ containing the 17-amino acid proline-rich attachment domain (PRAD) in a four to one stoichiometry (7). Two cysteine residues at the N terminus of the PRAD bind covalently to one cysteine residue at the C terminus of each of two AChE catalytic subunits to form linked dimers, which in turn associate with disulfide-bonded AChE dimers to form tetramers. Co-expression of the PRAD peptide with AChEt in COS-7 cells is sufficient to induce AChE tetramerization, and even expression of a PRAD peptide lacking the two cysteines induces stable oligomers as well (8–11).

AChE is a multimeric glycoprotein with 3–4 asparagine-linked oligosaccharides per catalytic subunit added co-translationally in the endoplasmic reticulum (ER) (1–4). Our laboratory showed that it was synthesized as an inactive precursor AChE that subsequently assembled into higher order oligomeric forms (12, 13). Only a small subset, about 20%, of these molecules fold correctly to become catalytically active, subsequently transit the Golgi apparatus acquiring endoglycosidase-H resistance, and eventually reaching the plasma membrane where they are either secreted, attached to the extracellular matrix, or retained on the surface plasma mem-

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3 The abbreviations used are: AChE, acetylcholinesterase; AChR, acetylcholine receptor; ColQ, acetylcholinesterase collagen tail subunit; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; PRiMA, proline-rich membrane attachment; PRAD, proline-rich attachment domain; DFP, diisopropylfluorophosphate; QMC, quail muscle culture; hAChE, human AChE; EMEM, Eagle’s minimum essential medium; RFP, red fluorescent protein.
brane. The remaining 80% consisting of inactive enzyme molecules retain their endoglycosidase-H sensitivity as well as a mostly monomeric state, and they were rapidly degraded by the endoplasmic reticulum-associated degradation system (ERAD) (13). The mechanism(s) responsible for activating the enzyme and preventing its degradation are not well understood but appear to involve conformational maturation and stabilization through further assembly or interactions with noncatalytic subunits (14). Based on the results of those studies on the role of ColQ in the assembly of AChE and its ability to increase AChE expression, we proposed that the noncatalytic subunit ColQ rescues AChE from intracellular degradation (14). From the studies by Massoulié and co-workers (8, 9) showing that PRAD peptides expressed in heterologous cells expressing AChE could induce oligomerization, we hypothesized that the PRAD peptides could also induce correct folding of the enzyme to the catalytically active conformation, rescuing the AChE from intracellular degradation and thereby increasing the levels of active enzyme.

In this study, we show that incubation of skeletal muscle cells with peptides based on the amino acid sequence of the noncatalytic ColQ subunit’s PRAD domain with the addition of the KDEL ER retrieval signal sequence is sufficient to increase catalytically active AChE by several hundred percent. When muscle cells or cell lines expressing AChE catalytic subunits are incubated in the presence of the PRAD-KDEL peptides, the peptides are taken up by the cells and retrogradely transported back to the ER where they induce assembly of AChE tetramers. This appears to occur through increased folding efficiency and induced oligomerization of the catalytic subunits, which in turn rescue the enzyme from intracellular degradation. Pulse-chase studies of radiolabeled AChE show that PRAD peptides dramatically increase the half-life of the enzyme indicating that the synthetic PRAD peptides rescue AChE catalytic subunits from intracellular degradation. Together, these studies indicate that binding of the noncatalytic AChE targeting subunits to the enzymatic subunits provides an important post-translational regulatory step for the expression of the catalytically active enzyme and provides a mechanism for regulating levels of synaptic AChE without changes in the translational rate of the protein. Moreover, they suggest a novel means of enhancing expression of AChE in vivo with potential novel therapeutic applications.

**Experimental Procedures**

**Tissue Culture of Embryonic Muscle**—Primary myoblasts were obtained from the pectoral muscles of 10-day-old quail embryos and plated at $5 \times 10^4$ cells/ml, 2 ml/dish, on 35-mm culture dishes in Eagle’s minimum essential medium (EMEM; Gibco) supplemented with 2% chicken embryo extract, 10% horse serum (GemCell), and 0.1% gentamicin (Gibco) (EMEM 210) unless otherwise indicated. The quail muscle cultures (QMCs) were fed with fresh medium on days 3 and 5 after plating and were used when mature and actively contracting, between days 5 and 7 in vitro. Avian muscle cultures were maintained at 39°C in a water-saturated incubator with 5% CO₂. HEK 293 cells expressing human AChE (hAChE) and the COS-7 cells were grown at 37°C and fed Dulbecco’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin.

**Peptide Treatments**—The peptides used in these studies (Table 1) were based on the human ColQ and PRiMA PRAD amino acid sequences (6, 15, 16), which differ from the mouse by only one amino acid (Thr → Met), and synthesized by GenScript (Piscataway, NJ) at 95% purity. QMCs were incubated with 10 μM PRAD peptides unless otherwise indicated in defined EMEM containing 0.5% BSA, 50 μg/ml fibronectin, 20 μg/ml conalbumin, 5 μg/ml porcine insulin, 30 μM selenium, 100 μM putrescine, 20 μM progesterone. The defined serum-free medium was used in some experiments where indicated to avoid exposing the cells to the high levels of serum cholinesterases present in complete medium.

**Cell Transfection**—pTarget containing the full-length human AChE coding sequence (pTarget-hAChEt) (15) was the generous gift of Drs. Andrew Engel and Kinji Ohno, and pcDNA 3.1 encoding red fluorescent protein with the endoplasmic reticulum retrieval signal KDEL (ER-RFP), a generous gift from Erick Snapp (17), was transfected into COS-7 cells using Exgen 500 (Fermentas) according to the manufacturer’s recommended protocol. **Analysis of AChE Oligomeric Forms and Enzyme Activity Assay**—Tissue culture dishes were rinsed three times with 2 ml of Hanks’ balanced salt solution, and the AChE oligomeric forms were extracted using 500 μl/culture dish borate extraction buffer (20 mM sodium borate, pH 9.0, 0.5% Triton X-100, 5 mM EDTA, 1 mM NaCl, 0.5% BSA) and the protease inhibitors leupeptin (1 μg/ml) and pepstatin (2 μg/ml) (Sigma). At least three separate cultures per group were used in each experiment. Triplicate samples from each experimental group were pooled and analyzed by velocity sedimentation using a Beckman SW41Ti rotor at 36,000 rpm for 16 h on 5–20% sucrose gradients in extraction buffer without BSA. The fractions were collected and assayed for AChE activity using Ellman’s assay.

**Analysis of Newly Synthesized AChE, Recovery from DFP**—QMCs were treated with $10^{-4}$ M of the irreversible cholinesterase inhibitor DFP (Sigma) for 10 min to inactivate all cell-associated AChE as described previously (18). After washing away unreacted DFP three times with 2 ml of Hanks’ balanced salt solution, the cells were allowed to synthesize new enzyme in defined medium for 30, 60, or 90 min in the presence or absence of 10 μM PRAD-KDEL and extracted in 500 μl of borate extraction buffer to determine total AChE activity. In one set of experiments, QMCs were allowed to recover from DFP treatment in the presence of 5 mM DTT for 30 min followed by incubation with or without 100 μM puromycin to inhibit protein synthesis and/or 10 μM PRAD-KDEL for an additional hour after which the cells were extracted for analysis of AChE activity. Total protein was assayed using the Bradford colorimetric assay.

**Western Blots**—Primary QMCs grown on 35-mm culture dishes as described above were extracted in RIPA buffer containing protease inhibitor mixture (Complete-Mini, Roche Diagnostics). After centrifugation, samples containing 50 μg of total protein were run on 10% reducing SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose membranes (Whatman) and blocked with 1% powdered skim milk (Carnation) in phosphate-buffered saline for 1 h. The membranes were incubated for several hours with mouse anti-avian AChE mAb IA2 (1.6 μg/ml) followed by goat anti-mouse peroxidase antibody (Jackson
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Imunochemicals) overnight and developed with West Pico chemiluminescent substrate (Pierce).

Fluorescence Microscopy—COS-7 cells growing on laminin (Sigma)-coated glass coverslips and transfected with ER-RFP were incubated with 10 μM biotinylated PRAD-KDEL peptide for the indicated times. Following fixation with 4% paraformaldehyde in PBS, pH 7.0, for 20 min, the cells were permeabilized with 0.1% Triton X-100 for 3 min and blocked for 20 min with 1% bovine serum albumin followed by incubation for 1 h with Alexa Fluor-488 streptavidin (Invitrogen/Molecular Probes) and Hoechst 33342 to stain the nuclei. Images were acquired with identical exposure parameters using a Leica DMI 6000 fluorescence microscope with planapochromate ×63 NA 1.40–0.60 and ×100 NA 1.40–0.70 oil emission objectives and equipped with a Hamamatsu digital camera using SiSlidebook 4.0 software.

Metabolic Labeling and Immunoprecipitation of Radiolabeled AChE—Five-day-old QMCs plated on 60-mm dishes were incubated for 30 min in methionine- and cysteine-free defined MEMEM containing 0.5% BSA, 50 μg/ml fibronectin, 20 μg/ml conalbumin, 5 μg/ml porcine insulin, 30 μM selenium, 100 μM putrescine, 100 μM progesterone, and 0.33 mCi/ml [35S]methionine and [35S]cysteine (EasyTag Express Protein Labeling Mix, PerkinElmer Life Sciences, specific activity 1175 Ci/mmol), with or without 10 μM PRAD-KDEL peptide. After pulse labeling at 39 °C, cultures were rinsed once and incubated for 30, 60, 90, or 120 min in chase medium containing a 100-fold excess unlabeled methionine and cysteine in the presence or absence of 10 μM PRAD-KDEL peptide. The metabolically labeled QMCs were extracted on ice for 20 min with borate extraction buffer, and the lysates were microcentrifuged for 20 min at maximum speed to remove insoluble material. The solubilized AChE was immunoprecipitated using mouse antiaian AChE 1A2 and protein A-Sepharose (Sigma) as described previously (19). After washing, AChE was eluted from the beads by resuspension in 2X reducing SDS sample buffer and heating. The eluted proteins were separated by SDS-PAGE. The gels were fixed for 1 h in 10% acetic acid and 10% methanol, then dried, and exposed to a PhosphorImager screen (Amersham Biosciences). The images were acquired with a Storm 840 PhosphorImager scanner (GE Healthcare), and the data were analyzed using ImageQuant software (Amersham Biosciences).

Three to four cultures were used per time point, and the experiment was repeated twice with identical results.

Results

PRAD-KDEL Peptide Design and Synthesis—Studies from the laboratory of Massoulie and co-workers (8, 9) have shown that the N-terminal regions of the noncatalytic AChE subunits CoQ and PRiMA are sufficient to induce AChE oligomerization producing the tetrameric AChE form. Systematic deletion studies showed that a 17-amino acid core peptide was sufficient to induce AChE oligomerization when expressed together with the catalytic subunit in heterologous cells (9). In this study, we tested several different synthetic peptides based on the human and guinea pig CoQ PRAD (Table 1). KDEL residues were added to enhance retrograde transport of the peptides and retention in the ER analogous to the retrograde transport of some bacterial toxins such as botulinum and cholera toxins (20, 21) and the A chain of the lethal plant toxin ricin (22, 23). In addition, we had a PRAD-KDEL peptide synthesized with two lysine residues added at the N terminus for conjugating fluorophores, and another PRAD-KDEL peptide with an N-terminal biotin moiety for fluorescence localization studies (sequences not shown). We also tested the PRiMA PRAD that showed identical results to the CoQ PRAD (data not shown), but we chose to use the CoQ PRAD because our work focuses on skeletal muscle cells.

PRAD Peptides Are Retrogradely Transported to the ER—Because the globular AChE forms are translated and assembled in the ER and post-translationally processed in the ER and Golgi apparatus (12), synthetic PRAD peptides would have to be retrogradely transported and physically localized within these intracellular compartments to have an effect on AChE synthesis and assembly. To determine the intracellular distribution of the PRAD-KDEL peptides, COS-7 cells were plated at low density on laminin-coated glass coverslips, transfected with ER-RFP for transient expression to visualize the endoplasmic reticulum, and incubated with 10 μM biotinylated PRAD-KDEL peptide. The cells were then fixed, permeabilized, and the biotinylated PRAD-KDEL peptide labeled with streptavidin Alexa Fluor-488. Cultured COS-7 cells fed with biotin-PRAD-KDEL are able to take up the peptides and retrogradely transport them to the ER where they co-localize with ER-RFP (Fig. 1).

Synthetic PRAD-KDEL Peptides Promote Intracellular Oligomerization of Newly Synthesized AChE—Six-day-old QMCs were treated with 10−4 M DFP, a membrane-permeable organo-
phosphate compound to irreversibly inhibit all cell-associated AChE. After washing away unreacted DFP, the cells were incubated with 100 μM synthetic PRAD-KDEL peptide for 18 h. The distribution of newly synthesized catalytically active AChE oligomeric forms was determined by velocity sedimentation of the cell extracts followed by measurement of AChE enzyme activity. The synthetic PRAD-KDEL peptide was taken up by the myotubes' increasing assembly of AChE tetramers (G4). Monomers, dimers, and AChE tetramers are labeled G1, G2, and G4, respectively. The top of the gradient is toward the right.

The source of catalytic subunits used as a precursor for PRAD-KDEL peptide-induced G4 assembly includes the inactive or rapidly turning over AChE molecules fated to be intracellularly degraded unless organized and stabilized into higher oligomeric forms, as originally described by Rotundo (12). More detailed experimental results supporting this interpretation are presented in Figs. 6 and 7 discussed below. When QMCs were treated with DFP and subsequently incubated with synthetic PRAD-KDEL peptides for 3 h, the intracellular steady state levels of the different AChE forms were very similar to those observed at 18 h after DFP recovery (data not shown).

Synthetic PRAD Peptides Promote Newly Synthesized Human AChE Oligomerization—To determine whether AChE tetramer oligomerization and activation by PRAD peptides is cell type- or species-specific, we tested whether the PRAD peptide could increase active AChE tetramers in COS-7 cells transiently expressing hAChE. Transfected COS-7 cells transiently expressing hAChE secrete most of the active enzyme into the culture medium. The cells were DFP-treated and incubated with 100 μM PRAD-KDEL for 6 h after which the AChE oligomeric forms secreted into the medium as determined by velocity sedimentation (Fig. 3). Treatment with the PRAD peptide resulted in a greater than 400% increase in secreted G4 AChE.

PRAD-KDEL-induced Tetramerization of AChE Is Dose-dependent—To determine the optimal effective concentration of PRAD-KDEL peptide on intracellular AChE oligomerization, we treated mature 6-day-old QMCs with DFP to irreversibly inhibit all AChE activity, and we allowed them to recover in completed medium supplemented with 0.1, 1.0, 10, or 100 μM PRAD-KDEL peptide. For each point, the AChE in total cell extracts from three cultures was pooled, and the oligomeric forms were separated by velocity sedimentation. The total enzyme activity was determined for each gradient, and the G4 AChE form was expressed as a percent of total. Thus, the y axis indicates the percent of the total active AChE assembled into tetramers (G4).
Additional Amino Acids Surrounding the Core mPRAD Do Not Affect PRAD-KDEL Peptide-induced AChE Tetramerization—To determine whether additional amino acid sequences surrounding the core mouse PRAD were necessary to induce AChE tetramerization, QMCs were incubated in the presence of 10 μM of each of the different PRAD-KDEL peptides (for complete sequences see Table 1). No significant differences were detected among the different peptides used (Fig. 5 and Table 2), although the shorter peptides tended to produce slightly higher AChE levels. Representative gradients profiles are shown for the control, PRAD-KDEL, and 2–3-4-PRAD-KDEL peptides in Fig. 5.

PRAD-KDEL Peptides Promote Activation of Newly Synthesized AChE without Changes in Total Enzyme Protein Thereby Increasing Total Catalytically Active AChE—So far, our results show that the PRAD peptides increase AChE oligomerization and stabilization, most likely using the pool of newly synthesized inactive enzyme. To test this hypothesis directly, QMCs were DFP-treated and allowed to synthesize new AChE molecules in the presence or absence of the PRAD-KDEL peptide. The presence of the peptide increased the levels of catalytically active newly synthesized AChE in a time-dependent manner (Fig. 6). To determine whether the PRAD-KDEL peptides interacted only with the newly synthesized AChE molecules, or with those already synthesized, QMCs were incubated in defined medium with 1 mM dithiothreitol (DTT) to prevent protein folding and activation after DFP treatment and allowed to synthesize new AChE molecules in the presence or absence of DTT. This procedure developed by Helenius and co-workers (24, 25) prevents AChE folding and disulfide bond formation and thus does not allow AChE to become catalytically active. This was followed by a 60-min chase with or without the PRAD-KDEL peptide in the presence or absence of 10 μg/ml puromycin to inhibit protein synthesis. The PRAD peptide was capable of increasing total AChE activity by 136% even when protein synthesis was inhibited (+Puro+PRAD, Fig. 7, left panel). These results indicate that the peptides can promote activation of inactive AChE molecules because the presence of the peptides does not change the levels of AChE protein (Fig. 7, right panel).

Synthetic PRAD Peptides Decrease the Intracellular Degradation Rate of Newly Synthesized AChE—Previous studies from our laboratory (12, 13) showed that a large fraction of AChE, about 80%, is catalytically inactive and rapidly degraded intracellularly by the ERAD system. We therefore asked whether the PRAD-KDEL peptide could rescue this pool of potentially activatable enzyme. To determine the half-life of AChE plus or minus PRAD peptides, we metabolically ([35S]methionine) labeled newly synthesized proteins on 5-day-old QMCs for 30 min in the presence or absence of 10 μM PRAD-KDEL peptide. The total labeled proteins were then chased in the presence of 100× methionine and cysteine and 10 μM PRAD-KDEL peptide, and the cultures were extracted at 30-min intervals for up to 120 min. Isotopically labeled AChE was extracted, immunoprecipitated from the chased fractions, electrophoretically resolved, and the gel exposed in a PhosphorImager to determine the intensity of the AChE bands. The AChE half-life is ~60 min in the control group (Fig. 8). Nevertheless, in the presence of PRAD-KDEL peptide there is more than 70% labeled

Additional sequences surrounding the core PRAD peptide do not affect oligomerization of AChE. QMC were DFP-treated and incubated overnight with 10 μM of either the core 17-amino acid PRAD-KDEL peptide or one of the longer versions 2–3-4-PRAD-KDEL peptide (Table 1). The AChE forms in total cell extracts were separated by velocity sedimentation, and the enzymatic activity was assayed. There is no additional effect of increasing the length of the peptide over the core sequence; in fact, the core PRAD-KDEL sequence appears to give the highest percent of tetrameric AChE. Monomers, dimers, and AChE tetramers are labeled G1, G2, and G4, respectively, and the asymmetric AChE is labeled A12. The top of the gradient is at the right.

### Table 2: Effect of PRAD-KDEL peptide length on induced AChE activity

| PRAD PEPTIDE     | G4 AChE activity | % control |
|------------------|------------------|-----------|
| UNTREATED CONTROL| 0.4798           | 100%      |
| 1-2-3-4-5 PRAD-KDEL| 2.1384           | 446%      |
| 2-3-4 PRAD-KDEL  | 2.1710           | 452%      |
| 2-3 PRAD-KDEL    | 2.2609           | 471%      |
| 1-2-3 PRAD-KDEL  | 2.3723           | 494%      |
| PRAD-KDEL        | 2.3748           | 495%      |
AChE 60 min after pulsing and does not decrease significantly thereafter. Quantitation of the immunoprecipitated AChE suggests that PRAD-KDEL peptide can rescue newly synthesized AChE from intracellular degradation increasing the half-life of the enzyme most likely by interacting with the rapidly turning pool (12).

Discussion

Previous studies in our laboratory have shown that about 70–80% of the newly synthesized AChE molecules in muscle cells are catalytically inactive and are rapidly degraded by the ERAD with a half-life of about 45–60 min (12, 13). The remaining 20–30% of the enzyme molecules become catalytically active and consist of both stable and unstable forms that transit the secretory pathway to the cell surface where they are inserted into cholinergic synapses such as the neuromuscular junction or nicotinic and muscarinic synapses in the central and peripheral nervous systems. These studies were confirmed by Chatel et al. (26) using monoclonal antibodies that could distinguish between catalytically active and inactive AChE, and they showed that in chicken brain at least 30% of the AChE was catalytically inactive, an ER resident, and not trafficked in the Golgi apparatus (27). The mechanisms responsible for the activation and stabilization of AChE subunits into catalytically active oligomers are being elucidated. For example, co-assembly of the noncatalytic ColQ and PRiMA subunits are essential for the assembly of higher order oligomeric forms such as the tetramers and the asymmetric collagen-tailed forms (14, 28, 29), and this occurs through the PRAD-containing N-terminal domain (7–9).

However, the degradation of misfolded and catalytically inactive AChE by the ERAD is unlikely to be mediated by occlusion of an endoplasmic reticulum retention signal sequence as suggested previously (30–32). Although the C-terminal CAEL sequence found in Torpedo AChE partially resembles the KDEL endoplasmic reticulum retention signal (33), it is unlikely to act...
as such (34). Moreover, the C-terminal CSDL residues conserved in most if not all mammalian AChE sequences, including rat (35), mouse (36), bovine (37), and human (38), do not function as endoplasmic reticulum retention signals when tested in eukaryotic cells (34).

This could have practical and functional consequences as well. Recently, Campanari et al. (39) found that although the catalytically active AChE decreased by 40% in the brains of patients with Alzheimer disease, the levels of AChE protein did not. These results suggest the presence of a large pool of catalytically inactive AChE in the CNS of these patients that could arise from a decrease in available PRiMA subunits.

These studies show that synthetic PRAD peptides with the C-terminal KDEL sequence can be taken up by skeletal muscle cells, or even cell lines expressing AChE, and transported back to the ER (Figs. 2 and 3). The capacity of cells to retrieve KDEL-tagged protein extends to the Golgi apparatus (40), and exogenous peptides containing the retrieval sequence can be rapidly taken up and retrogradely transported back to the endoplasmic reticulum (41). In nature, a large number of protein toxins such as the shiga, cholera, and anthrax toxins exploit the exportable protein trafficking pathway of cells to become active and reach their targets by having functional localization sequences (21–24). Cholera toxin, in particular, has a C-terminal KDEL sequence responsible for retrograde transport to the ER, and the heat-labile toxin from *Escherichia coli* has the related sequence RDEL that also binds to KDEL receptors for ER retrieval. In some cases, these are even retrotranslocated back into the cytosol (21, 22). Inside the cells, PRAD peptides organize AChE tetramers (Figs. 2 and 3) using AChE monomers from at least two different sources, pre-existing active monomers and dimers and newly synthesized AChE molecules (Fig. 7). By organizing a larger fraction of stable AChE tetramers using newly synthesized AChE molecules, the synthetic PRAD peptides can increase total cell-associated AChE activity by several hundred percent.

The rapidly turning over pool of AChE molecules in vivo is an important endogenous source of catalytic subunits that could be rescued from intracellular degradation by administration of PRAD peptides. Therapeutic strategies to increase the ratio of active exportable over inactive AChE molecules would provide alternative therapies that could overcome some of the weaknesses of currently available treatments for organophosphate cholinesterase inhibitor poisoning.

**Author Contributions**—C. A. R., S. G. R., and R. L. R. designed the experiments; C. A. R. carried out the experiments presented in Figs 1, 2, and 5; S. G. R. carried out the experiments presented in Figs. 3, 4, and 6; and C. A. R. and R. L. R. carried out the experiments presented in Figs. 7 and 8. C. A. R., S. G. R., and R. L. R. wrote the manuscript and reviewed and rewrote the revised manuscript.

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**References**

1. Rotundo, R. L. (2003) Expression and localization of acetylcholinesterase at the neuromuscular junction. *J. Neurocytol.* **32**, 745–766
2. Legay, C. (2000) Why so many forms of acetylcholinesterase? *Microsc. Res. Tech.* **49**, 56–72
3. Taylor, P., and Radic, Z. (1994) The Cholinesterases: from genes to proteins. *Annu. Rev. Pharmacol. Toxicol.* **34**, 281–320
4. Massoulié, J., Pezzementi, L., Bon, S., Krejci, E., and Vallette, F. M. (1993) Molecular and cellular biology of cholinesterases. *Prog. Neurobiol.* **41**, 31–91
5. Krejci, E., Thomine, S., Boschetti, N., Legay, C., Sketelj, J., and Massoulié, J. (1997) The mammalian gene of acetylcholinesterase-associated collagens. *J. Biol. Chem.* **272**, 22840–22847
6. Perrier, A. L., Massoulié, J., and Krejci, E. (2002) PRiMA: the membrane anchor of acetylcholinesterase in the brain. *Neuron* **33**, 275–285
7. Dvir, H., Harel, M., Bon, S., Liu, W. Q., Vidal, M., Garbay, C., Sussman, J. L., Massoulié, J., and Silman, I. (2004) The synaptic acetylcholinesterase tetramer assembles around a polyproline II helix. *EMBOJ.* **23**, 4394–4405
8. Bon, S., and Massoulié, J. (1997) Quaternary associations of acetylcholinesterase. I. Oligomeric associations of T subunits with and without the amino-terminal domain of the collagen tail. *J. Biol. Chem.* **272**, 3007–3015
9. Bon, S., Cousse, F., and Massoulié, J. (1997) Quaternary associations of acetylcholinesterase. II. The polyproline attachment domain of the collagen tail. *J. Biol. Chem.* **272**, 3016–3021
10. Chitlaru, T., Kronman, C., Velan, B., and Shafferman, A. (2001) Effect of human acetylcholinesterase subunit assembly on its circulatory residence. *Biochem. J.* **354**, 613–625
11. Kronman, C., Chitlaru, T., Elhanany, E., Velan, B., and Shafferman A. (2000) Hierarchy of post-translational modifications involved in the circulatory longevity of glycoproteins. Demonstration of concerted contributions of glycan sialylation and subunit assembly to the pharmacokinetic behavior of bovine acetylcholinesterase. *J. Biol. Chem.* **275**, 29488–29502
12. Rotundo, R. L. (1988) Biogenesis of acetylcholinesterase molecular forms in muscle: evidence for rapidly turning over, catalytically inactive precursor pool. *J. Biol. Chem.* **263**, 19398–19406
13. Rotundo, R. L., Thomas, K., Porter-Jordan, K., Benson, R. J., Fernandez-Valle, C., and Fine, R. E. (1989) Intracellular transport, sorting, and turnover of acetylcholinesterase: evidence for endoglycosidase H-sensitive form in Golgi apparatus, sarcoplasmic reticulum, and clathrin-coated vesicles and its rapid degradation by a non-lysosomal mechanism. *J. Biol. Chem.* **264**, 3146–3152
14. Ruiz, C. A., and Rotundo, R. L. (2009) Dissociation of transcription, translation, and assembly of collagen-tailed acetylcholinesterase in skeletal muscle. *J. Biol. Chem.* **284**, 21488–21495
15. Ohno, K., Brengman, J., Tsujiino, A., and Engel, A. G. (1998) Human end-plate acetylcholinesterase deficiency caused by mutations in the collagen-like tail subunit (ColQ) of the asymmetric enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9654–9659
16. Donger, C., Krejci, E., Serradell, A. P., Eymard, B., Bon, S., Nicole, S., Chateau, D., Gary, F., Fardeau, M., Massoulié, J., and Guicheny, P. (1998) Mutation in the human acetylcholinesterase-associated collagen gene, COLQ, is responsible for congenital myasthenic syndrome with end-plate acetylcholinesterase deficiency (type Ic). *Am. J. Hum. Genet.* **63**, 967–975
17. Snapp, E. L., Sharma, A., Lippincott-Schwartz, J., and Hedgpeth, R. S. (2006) Monitoring chaperone engagement of substrates in the endoplasmic reticulum of live cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 6536–6541
18. Rotundo, R. L. (1984) Asymmetric acetylcholinesterase is assembled in the Golgi apparatus. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 479–483
19. Rotundo, R. L. (1984) Purification and properties of the membrane-bound form of acetylcholinesterase form chicken brain. *J. Biol. Chem.* **259**, 13186–13194
20. Lord, J. M., and Roberts, L. M. (1998) Toxicin entry: retrograde transport through the secretory pathway. *J. Cell Biol.* **140**, 733–736
21. Sandvig, K., and van Deurs, B. (2002) Retrograde transport of protein toxins. *FEBS Lett.* **529**, 49–53
22. Wales, R., Roberts, L. M., and Lord, J. M. (1993) Addition of an endoplasmic reticulum retrieval sequence to ricin A chain significantly increases its
cytotoxicity to mammalian cells. J. Biol. Chem. 268, 23986–23990
23. Simpson, J. C., Dascher, C., Roberts, L. M., Lord, J. M., and Balch, W. E. (1995) Ricin cytotoxicity is sensitive to recycling between the endoplasmic reticulum and the Golgi complex. J. Biol. Chem. 270, 20078–20083
24. Braakman, I., Helenius, J., and Helenius, A. (1992) Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. EMBO J. 11, 1717–1722
25. Tatu, U., Braakman, I., and Helenius, A. (1993) Membrane glycoprotein folding, oligomerization and intracellular transport: effects of dithiothreitol in living cells. EMBO J. 12, 2151–2157
26. Chatel, J. M., Vallette, F. M., Massoulie, J., and Grassi, J. (1993) Existence of an inactive pool of acetylcholinesterase in chicken brain. FEBS Lett. 319, 12–15
27. Chatel, J. M., Grassi, J., Frobert, Y., Massoulie, J., and Vallette, F. M. (1993) A conformation-dependent monoclonal antibody against active chicken acetylcholinesterase. Proc. Natl. Acad. Sci. U.S.A. 90, 2476–2480
28. Chen, V. P., Choi, R. C., Chan, W. K., Leung, K. W., Guo, A. J., Chan, G. K., Luk, W. K., and Tsim, K. W. (2011) The assembly of proline-rich membrane anchor (PRiMA)-linked acetylcholinesterase enzyme: glycosylation is required for enzymatic activity but not for oligomerization. J. Biol. Chem. 286, 32948–32961
29. Chen, V. P., Luk, W. K., Chan, W. K., Leung, K. W., Guo, A. J., Chan, G. K., Xu, S. L., Choi, R. C., and Tsim, K. W. (2011) Molecular assembly and biosynthesis of acetylcholinesterase in brain and muscle: the roles of t-peptide, FHB domain, and N-linked glycosylation. Front. Mol. Neurosci. 4, 36
30. Morel, N., Leroy, J., Ayon, A., Massoulie, J., and Bon, S. (2001) Acetylcholinesterase H and T dimers are associated through the same contact. Mutations at this interface interfere with the C-terminal T peptide, inducing degradation rather than secretion. J. Biol. Chem. 276, 37379–37389
31. Belbeoch, S., Massoulie, J., and Bon, S. (2003) The C-terminal T peptide of acetylcholinesterase enhances degradation of unassembled active subunits through the ERAD pathway. EMBO J. 22, 3536–3545
32. Noureddine, H., Schmitt, C., Liu, W., Garbay, C., Massoulie, J., and Bon, S. (2007) Assembly of acetylcholinesterase tetramers by peptidic motifs from the proline-rich membrane anchor, PRiMA: competition between degradation and secretion pathways of heteromeric complexes. J. Biol. Chem. 282, 3487–3497
33. Schumacher, M., Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S. S., Friedmann, T., and Taylor, P. (1986) Primary structure of Torpedo californica acetylcholinesterase deduced from its cDNA sequence. Nature 319, 407–409
34. Raykhol, I., Alane, H., Sado, K., Jurvansuu, J., Nguyen, V. D., Latva-Ranta, M., and Ruddock, L. (2007) A molecular specificity code for the three mammalian KDEL receptors. J. Cell Biol. 179, 1193–1204
35. Legay, C., Bon, S., Vernier, P., Coussen, F., and Massoulie, J. (1993) Cloning and expression of a rat acetylcholinesterase subunit: generation of multiple molecular forms and complementarity with a Torpedo collagenic subunit. J. Neurochem. 60, 337–346
36. Rachinsky, T. L., Camp, S., Li, Y., Ekström, T. J., Newton, M., and Taylor, P. (1990) Molecular cloning of mouse acetylcholinesterase: tissue distribution of alternatively spliced mRNA species. Neuron 5, 317–327
37. Roberts, W. L., Doctor, B. P., Foster, J. D., and Rosenberry, T. L. (1991) Bovine brain acetylcholinesterase primary sequence involved in intersubunit disulfide linkages. J. Biol. Chem. 266, 7481–7487
38. Soreq, H., Ben-Aziz, R., Prody, C. A., Seidman, S., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginzberg, D., and Lipidot-Lifson, Y. (1990) Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G+C-rich attenuating structure. Proc. Natl. Acad. Sci. U.S.A. 87, 9688–9692
39. Campanari, M. L., García-Ayllón, M. S., Blazquez-Llorca, L., Luk, W. K., Tsim, K., and Sáez-Valero, J. (2014) Acetylcholinesterase is preserved in the Alzheimer’s brain. J. Mol. Neurosci. 53, 446–453
40. Miesenbock, G., and Rothman, J. E., (1995) The capacity to retrieve escaped ER proteins extends to the trans-most cisterna of the Golgi stack. J. Cell Biol. 129, 309–319
41. Wieland, F. T., Gleason, M. L., Serafini, T. A., and Rothman, J. E. (1987) The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell 50, 289–300

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