Assembly of acetylcholinesterase tetramers by peptidic motifs from the proline-rich membrane anchor, PRiMA: competition between degradation and secretion pathways of heteromeric complexes.

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ASSEMBLY OF ACETYLCHOLINESTERASE TETRAMERS BY PEPTIDIC MOTIFS
FROM THE MEMBRANE-ANCHOR, PRiMA: COMPETITION BETWEEN
DEGRADATION AND SECRETION PATHWAYS OF HETEROMERIC COMPLEXES*
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Running title: Assembly of AChE tetramers by PRiMA

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The membrane-bound form of acetylcholinesterase (AChE) constitutes the major component of this enzyme in the
mammalian brain. These molecules are hetero-
oligomers, composed of four AChE catalytic
subunits of type T (AChE_T), associated with a
transmembrane protein of type 1, called
PRiMA (Proline Rich Membrane Anchor).
PRiMA consists of a signal peptide, an
extracellular domain that contains a proline-
rich motif (14 prolines with an intervening
leucine, P_{4LP_{10}}), a transmembrane domain
and a cytoplasmic domain. Expression of
AChE_T subunits in transfected COS cells with a
truncated PRiMA, without its transmembrane
and cytoplasmic domains (P_{stp54} mutant),
produced secreted heteromeric complexes (T_{4-
P_{stp54}}), instead of membrane-bound tetramers.
In this study, we used a series of deletions and
point mutations to analyze the interaction
between the extracellular domain of PRiMA
and AChE_T subunits. We confirmed the
importance of the poly-proline stretches and
defined a peptidic motif (RP_{4LP_{10}}RL), which
induces the assembly and secretion of a heteromeric complex with four AChE_T
subunits, nearly as efficiently as the entire
extracellular domain of PRiMA. It is
noteworthy that deletion of the N-terminal
segment preceding the prolines had little effect.
Interestingly, short PRiMA mutants, truncated
within the proline-rich motif, reduced both
cellular and secreted AChE activity, suggesting
that their interaction with AChE_T subunits
induces their intracellular degradation.

In the nervous tissue and muscles of
mammals, acetylcholinesterase (AChE, EC 3.1.1.7)
controls cholinergic transmission by rapidly
hydrolyzing the neurotransmitter acetylcholine after
its release from nerve terminals. The functional
localization of AChE depends on the association of
its T splice variant with structural proteins (1-4).
Thus, the physiologically active AChE species
correspond essentially to the collagen-tailed forms
at the neuromuscular junctions and to membrane
bound tetramers in the brain.

The AChE_T splice variant is characterized
by its 40-residues C-terminal peptide (t peptide),
which contains a C-terminal cysteine and seven
aromatic residues, including three evenly spaced
tryptophans, and can be organized as an
amphiphilic alpha helix (5). This peptide behaves
as an autonomous interaction domain (the
Tryptophan (W) Amphiphilic Tetramerization
domain, or WAT) (6): it allows oligomerization of
AChE_T subunits into homomeric dimers (T_2)
and tetramers (T_4), as well as heteromeric associations
of tetramers with anchoring proteins (7-9).

In the collagen-tailed forms, AChE_T
tetramers are associated with a specific collagen,
called ColQ (7,10). This interaction has been
extensively studied: it is based on a tight interaction
between four t peptides (6) and a proline-rich motif,
called PRAD ("Proline-Rich Attachment
Domain"), located in the N-terminal non
collagenous region of ColQ (11). Synthetic t and
PRAD peptides (respectively 40 and 15 residues)
spontaneously form a complex, the structure of
which has been determined by crystallography: four
alpha-helical t peptides form a staggered coiled-coil
around the PRAD, organized as an elongated poly-
proline II helix (12). All aromatic residues are
oriented towards the interior of this compact
cylindrical complex, and the tryptophans are
apposed to the rings of the proline residues. From
this interaction, it was possible to deduce models
for the quaternary organization of four AChE T
subunits linked to a ColQ chain (12,13).

The existence of an N-glycosylated 20
kDa hydrophobic protein, associated with
membrane-bound AChE tetramers was originally
discovered by Gennari and Brodbeck and by
Inestrosa et al., in 1987 (14,15); this membrane
anchor was more recently cloned and called
PRiMA (“Proline-Rich Membrane Anchor”),
because it contains a proline-rich motif, like ColQ
(16). This suggests that AChE T subunits may
associate with PRiMA and with ColQ in a similar
manner. However, there are significant differences
in the numbers of prolines (8 in ColQ, 14 in
PRiMA) and in the numbers and positions of
cysteines that could form intercatedary disulfide
bonds with a cysteine located near the C-terminus
each of the four t peptides. In addition, PRiMA
contains a putative N-glycosylation site between
the poly-proline stretches and the transmembrane
domain (17). Alternative splicing produces two
PRiMA variants, which differ in their C-terminal
domains: the intracellular domains of the major
variant (PRiMA I) and of the minor variant
(PRiMA II) contain respectively 40 and 11 residues
(18). The mode of association between PRiMA and
AChET subunits is physiologically important since
the resulting PRiMA-anchored AChE tetramers
(16,18) represent the major enzyme species in the
brain (18) and their level is regulated by exercise in
muscles (19).

We have undertaken an analysis of the
association of AChE T with PRiMA. In this study,
we mostly used truncated PRiMA mutants
containing only the extracellular domain or
fragments of this domain, but not the
transmembrane and cytoplasmic domains, thus
producing soluble heteromeric complexes with
AChET subunits. In previous studies, we have
shown that a significant fraction of AChE T subunits
is degraded intracellularly, through the ERAD
process (“Endoplasmic Reticulum Associated
Degradation”) (20,21), and that this is mostly
induced by exposed aromatic residues (21,22), in
agreement with the fact that the formation of a
complex in which these residues are occluded may
reduce their degradation and increase their
secretion. In the present study, we examine how co-
expression with PRiMA mutants affects the
trafficking, degradation and secretion of AChE T
subunits. We show that some truncated mutants of
PRiMA act as degradation inducers, assembling
AChE T subunits into complexes which are
degraded intracellularly, indicating that they fail to
pass the quality control of the secretory pathway.
Using deletions and mutations, we analyze the
influence of residues flanking the poly-proline
stretches and also of the leucine located between
the prolines, and we define a peptidic motif
(RP4LP10RL), which is nearly as efficient as the
complete extracellular domain of PRiMA for
recruitment of AChET subunits into secreted
erhetomeric complexes.

Experimental Procedures

Vectors and site directed mutagenesis - The AChE T
subunit of rat AChE, as well as intact or mutated
mouse PRiMA were expressed by inserting the
corresponding cDNAs into pEF-Bos vectors (23).
Throughout the present article, the numbering of
PRiMA residues corresponds to the mature protein
(Fig. 1A); the extracellular domain corresponds to
residues 1-53. Mutagenesis was performed by the
method of Kunkel et al. (24), as described
previously (25). For deletions, we used mutagenic
oligonucleotides of about 30 nt containing 15 nt on
each site of the deleted fragment. Truncated
mutants are indicated by the position of stop codons
and by the modified residues: for example, R36E-
Psp37 indicates a mutant in which a stop codon was
introduced at position 37 and arginine R36 was
replaced by a glutamic acid. Fig. 1B shows the
structure of mutants used in this study. We
introduced a flag epitope (DYKDE) after the
cleavage site of the signal peptide in PRiMA, so
that it was recognized by the anti-flag monoclonal
antibody M1 (Sigma). We also used an N-terminal
fragment of Torpedo ColQ, with or without its PRAD domain (Fig. 1C).

**Transfection in COS cells** - Plasmids were transfected in COS cells with the DEAE-dextran method, as described previously (9), using 2 µg of vector DNA encoding the catalytic subunit AChE_T and various amounts of vector DNA encoding PRiMA mutants, as specified, per 60 mm dish. For comparison, we also used an N-terminal fragment of Torpedo marmorata ColQ (Q_{stp69}). In each series of transfections, we completed the amount of vector encoding PRiMA mutants with a vector encoding a non interacting protein, the N-terminal domain of ColQ from which the PRAD interaction motif was deleted (A[28-44]-Q_{stp69}) (11), so that the total amount of vector remained constant, to avoid changes in the synthetic capacity of the cells. After transfection, COS cells were incubated at 37°C, in a medium containing 10% NUSerum (Inotech, Dottikon, Switzerland), which had been pre-treated with 10^5 M soman to inactivate serum cholinesterases. The medium and the cells were collected after 3-4 days.

**Analysis of AChE recovery after irreversible inhibition** - The AChE activity of transfected cells (3 or 4 days after transfection) was irreversibly inhibited by incubation with the membrane-permeant inhibitor soman (pinacolylmethylphosphonofluoridate) at 5 10^{-7} M for 30 min, as described in (22). After extensive washing, the recovery of AChE activity was determined by collecting cells at various times in fresh culture medium at 37 °C. Secretion of newly synthesized AChE only resumed after about 150-180 min and during that period the recovery of cellular activity reflected a balance between neosynthesis and intracellular degradation.

**Cell extracts** - Intracellular and membrane-bound AChE was extracted for 15 min at 20°C in a TMg buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2) containing 25 mM benzamidine, followed by centrifugation for 10 min at 13 000 r.p.m at 4°C. The culture medium containing the secreted enzyme was also centrifuged at 13 000 r.p.m for 10 min to remove cell debris before analysis.

**Enzyme assays** - AChE activity was determined with the colorimetric method of Ellman *et al.* (26) at room temperature. 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. Alkaline phosphatase and β-galactosidase from Escherichia coli were assayed with the chromogenic substrates p-nitrophenyl phosphate and o-nitrophenyl galactoside, respectively.

**Sedimentation and electrophoresis analyses** - Centrifugation in 5-20% sucrose gradients (50 mM Tris-HCl pH 7.5, 20 mM MgCl_2, in the presence of 1% Brij-96 or 0.2% Triton X-100) was performed in a Beckman SW41 rotor, at 36 000 r.p.m, for 17 h 30 at 6°C. Approximately 40 fractions were collected and AChE activity was measured with the Ellman colorimetric assay, allowing the determination of the sedimentation coefficients of the different molecular forms, and of their relative activities. The gradients contained *E. coli* β-galactosidase (16 S) and alkaline phosphatase (6.1 S) as internal sedimentation standards. Amphiphilic molecules are characterized by the fact that they interact with detergent micelles: they generally sediment slower in the presence of Brij-96 than of Triton X-100.

Electrophoresis in non-denaturing polyacrylamide gels was performed as described by Bon *et al.* (27); The gels contained 0.25% Triton X-100 with or without 0.05% deoxycholate; electrophoresis was performed in a refrigerated apparatus under 40 V/cm, for 2 h. Enzymatic activity was revealed by the histochemical method of Karnovsky and Roots (28). This method allows a rapid qualitative comparison of up to 20 samples in a single gel. In charge shift electrophoresis, the electrophoretic migration of amphiphilic molecules was accelerated in the presence of sodium deoxycholate, when compared to migration in the presence of the neutral detergent, Triton X-100.

**Effect of synthetic peptides on oligomerization of AChE_T subunits** - Synthetic peptides corresponding to the interaction motif of PRiMA, (RP4LP[10] and RP4LP[10]RL) were synthesized by the Merrifield solid phase method in an Applied Biosystems (ABI) 431A automated peptide synthesizer, with small-scale 9-fluorenlymethoxycarbonyl (Fmoc)
Formation and secretion of soluble complexes with truncated PRiMA mutants, lacking the transmembrane domain - PRiMA and its different mutants were expressed in COS cells together with AChE_T subunits (also called T subunits). To evaluate the formation of heteromeric complexes, we analyzed the total AChE activity of the cellular extracts and of the medium, as well as their composition in molecular forms, using both sedimentation and non-denaturing electrophoresis. It should be noted that all molecular forms of AChE, which differ in their degree of oligomerization, with or without associated proteins, possess the same catalytic activity per active site, so that the level of AChE activity reflects the number of AChE_T subunits, independently of the composition in molecular forms.

Fig. 2 illustrates the molecular forms obtained by expressing AChE_T subunits alone, with full-length PRiMA and with its extracellular fragment, P_{stp54}. As a control, we used an N-terminal fragment of ColQ which contains the PRAD (residues 28-44), without the collagenous and trimerization domains (Q_{stp69} (Fig. 1C). When expressed alone or in the presence of a non-interacting fragment of ColQ from which the proline-rich motif was deleted (Δ[28-44]-Q_{stp69}), T subunits produced mostly amphiphilic monomers (T_1) and dimers (T_2) (27), with a small proportion of nonamphiphilic tetramers sedimenting at 10.5 S, which are thought to be homomeric (T_4), i.e. with no associated endogenous non-catalytic component (Fig. 2A). Monomers and dimers are not readily resolved in the sedimentation profiles, and we therefore quantified their sum (T_1+T_2). The proportion of T_4 tetramers is markedly higher in the medium than in the cell extract, indicating that they are secreted more efficiently than monomers and dimers, as previously observed (9).

Co-expression of T subunits with full length PRiMA induced the formation of membrane-bound T_4-PRiMA complexes, expressed at the cell surface as demonstrated by immunofluorescence (not shown). These heteromeric complexes were recovered in the cell extract after solubilization in the presence of 1% Triton X-100; unlike homomeric tetramers (T_4), which sediment at 10.5 S regardless of the presence or absence of detergents, the PRiMA-associated tetramers are
amphiphilic and their sedimentation is influenced by detergents: they sediment at 9.8 S in the presence of Triton X-100 and 9 S in the presence of Brij-96. This was also shown by the fact that they migrated more slowly than T4 tetramers in non-denaturing electrophoresis (Fig. 2B). In addition, the medium contained an increased level of soluble molecules sedimenting at 10.5 S, suggesting that they correspond to nonamphiphilic tetramers. When AChE T subunits were co-expressed with an N-flagged PRiMA, these secreted tetrameric molecules were recognized by an anti-flag antibody (M1), as shown by retardation of their migration in non denaturing electrophoresis (Fig. 3), demonstrating that they contain an N-terminal fragment of PRiMA, that might be produced either intracellularly or at the cell surface.

Co-expression of AChE T subunits with the P_{stp54} mutant, corresponding to the extracellular domain of PRiMA, induced the formation of a soluble AChE form, sedimenting at 10.5 S, and migrating as an AChE tetramer in non-denaturing electrophoresis (Fig. 2A and B). This form, which could not be distinguished from homomeric T4 tetramers by sedimentation, corresponds to AChE T tetramers associated with the P_{stp54} protein (T4-P_{stp54}). The fact that the ratio of T4-P_{stp54} to (T1+T2) is higher in the medium than in the cell extract, shows that they are preferentially secreted, as observed for homomeric tetramers T4.

The formation of soluble T4-P_{stp54} complexes demonstrates that the amphiphilic character of T4-PRiMA complexes is due to the transmembrane domain of PRiMA and confirms that AChE T subunits interact with a peptidic motif located in the N-terminal extracellular region of PRiMA, in agreement with a previous study (16).

**Effect of progressive C-terminal deletions in the extracellular domain of PRiMA on the recruitment of AChE T subunits** - We examined the interaction between AChE T subunits and PRiMA mutants in which stop codons were introduced upstream of position 54 in mutants P_{stp46}, P_{stp43}, P_{stp39}, P_{stp38}, P_{stp37}, P_{stp36}, P_{stp33} and P_{stp31} (see Fig. 1B). We studied the AChE activity and molecular forms produced in COS cells expressing a constant amount of AChE T subunits (2 µg of vector DNA per 60 mm culture dish), together with varying amounts of each mutant.

The proportion of AChE T tetramers increased in the cells and in the medium with the amount of associated non catalytic protein, as illustrated for P_{stp33} and P_{stp54} and Q_{stp69} in Fig. 4. This proportion was found to plateau at similar values for P_{stp54} and Q_{stp69}, but at a much lower level for P_{stp33}.

Fig. 5 illustrates the effect of the P_{stp} mutants on the cellular activity and on the rate of secretion, with a fixed amount of plasmid encoding the P_{stp} mutants (2 µg of DNA encoding AChE T and PRiMA mutant, each, per 60 mm culture dish). The level of cellular activity varied very little when AChE T subunits were co-transfected with the various PRiMA mutants. Secretion increased by about 30% when AChE T subunits were co-transfected with Q_{stp69}, indicating that they were partially rescued from intracellular degradation by the formation of T4-Q_{stp69} complexes (not shown), but reduced by about 50% when they were co-transfected with P_{stp31}. The secreted activity gradually increased with the longer mutants P_{stp33} and P_{stp36}, approximately reaching the value obtained with AChE T alone for longer constructs.

The level of secreted (T1+T2) was markedly reduced, when AChE T subunits were co-transfected with any of the truncated P_{stp} mutants, as well as with Q_{stp69}, indicating that they interacted with all mutants, even the shorter ones, such as P_{stp33} or P_{stp31}, which produced only minimal levels of cellular or secreted T4-P_{stp} form. The variations observed in the level of secreted activity appeared correlated with the proportion of heteromeric complex in the medium, as shown in Fig. 5C. Remarkably, this correlation includes all the truncated PRiMA mutants analyzed in this study, and also Q_{stp69}. Fig. 5D shows that the secretion of T4-P_{stp} complexes appears proportional to their cellular activity, except for T4-P_{stp36}, which was more efficiently secreted, and for mutants containing A, D or E residues preceding the proline-rich segment, which are less efficiently secreted.

The fact that AChE activity was reduced, both in the cells and in the medium, when AChE T subunits were co-expressed with short truncated
PRiMA mutants such as P_{stpn}31 suggests that they were more degraded than when expressed alone. This was verified by following the initial rate of recovery of AChE activity, during 2 hours after irreversible inhibition, i.e. before secretion of the newly synthesized enzyme: this rate represents the balance between neosynthesis and intracellular degradation. The rate of neosynthesis must be identical when AChE_{T} subunits are expressed with a non interacting protein and with different PRiMA mutants. We found that the rates of recovery varied in the order AChE_{T} + ∆[28-44]-Q_{stpn}69 > AChE_{T} + P_{stpn}46 > AChE_{T} + P_{stpn}33, indicating that P_{stpn}33, and to a lesser degree P_{stpn}46, induced some degradation of newly synthesized active AChE_{T} subunits.

The ratio of secreted to the cellular activity of each molecular form of AChE can be considered as an index of its secretability: Fig. 6 shows that this ratio remained essentially constant for (T_{1}+T_{2}), which were the same in all cases, but varied markedly for AChE tetramers associated with P_{stpn} proteins. This indicates that the secretion of the complexes depended on the length of the PRiMA fragment, especially between P_{stpn}31 and P_{stpn}36, confirming that complexes formed with the shorter PRiMA mutants were not efficiently secreted. In addition, the fact that this ratio varied in a non monotonic manner as a function of the length of the mutants from 36 to 54, suggests that the C-terminal residues of the P_{stpn} proteins may either facilitate or reduce secretion.

The P_{stpn}38 mutant seemed to produce a maximum of secreted heteromeric complexes, while P_{stpn}36 appeared optimal for the secretability of such complexes. The differences observed between P_{stpn}36, P_{stpn}37, P_{stpn}38 and P_{stpn}39 show that the residues located immediately downstream of the prolines (RLL) influence the formation and the secretion of the T_{4}-P_{stpn} complexes. However, replacement of R by an alanine or a glutamic acid in mutant P_{stpn}37 (R36A-P_{stpn}37 and R36E-P_{stpn}37), or replacement of RLL by alamines in mutant (RLL/AAA-P_{stpn}39) had essentially no effect on recruitment of AChE_{T} subunits, indicating that the charge of these residues is not important.

It is interesting that P_{stpn}46 and P_{stpn}54 produced very similar results, because P_{stpn}54 possesses a putative N-glycosylation site and a cysteine which are removed in P_{stpn}46, suggesting that these two elements do not interfere, positively or negatively, with the interaction between PRiMA and AChE_{T} subunits in transfected COS cells. The lack of influence of these elements was confirmed by mutating cysteine C48 to a serine in P_{stpn}54, and by suppressing the glycosylation site by replacing the threonine T46 by an alanine: these mutations did not affect the production and secretion of T_{4}-P_{stpn} complexes (not shown).

**Influence of the region preceding the poly-proline stretches** - To assess the possible role of the peptidic region that precedes the poly-proline stretches of PRiMA, we deleted it in P_{stpn}46 and P_{stpn}54. To maintain a proper cleavage site between the signal peptide and the mature protein, the prolines stretches were preceded by an arginine R (as in the wild type), or by other charged or neutral residues (K, E, D, A), as shown in Fig. 1B.

With an arginine preceding the prolines, we found that deletion of the N-terminal fragment (residues 1-19) from mutants P_{stpn}46 or P_{stpn}54 (∆N-R-P_{stpn}46 and ∆N-R-P_{stpn}54) slightly reduced the amount of cellular and secreted complexes (Fig. 5B). The fact that the two deleted mutants produced the same effect confirms that residues 45-53 do not influence the assembly or the trafficking of the complexes.

We then examined a possible effect of a charged or neutral residue preceding the prolines, in mutants ∆N-R-P_{stpn}46, ∆N-K-P_{stpn}46, ∆N-A-P_{stpn}46, ∆N-D-P_{stpn}46, and ∆N-E-P_{stpn}46 (Fig. 5B and Fig. 6). We found that the amount of secreted AChE tetramers is higher with a neutral or acidic residue, but that their secretability appears lower, as indicated by the ratio of secreted to cellular activities, suggesting that this residue influences the efficiency of secretion (Fig. 5D).

It was surprising that the secretion of AChE complexes was lower with a positively charged residue upstream of the prolines, since this residue is an arginine (R) in the wild type PRiMA. However, when we replaced this arginine by a glutamic acid (E) in P_{stpn}46, we found no modification of the yield of T_{4}-P_{stpn}46 complexes, indicating that this charge effect is negligible when the N-terminal segment (1-19) is present (not shown).
Mutations in the proline-rich region - PRiMA contains two groups of successive prolines, separated by a leucine residue (P4LP10). To determine whether this leucine is required for the interaction with AChE subunits, we mutated it to a proline, starting from the ΔN-E-Pstp46 mutant. The production and secretion of AChE tetramers remained unchanged with the resulting mutant, noted ΔN-(P15)-Pstp46. However, the production of complexes was significantly reduced by displacement of the leucine to a symmetrical position in the proline stretches (P10LP4) and even more by addition of a second leucine (P4LP5LP4), as shown in Fig. 5B. These mutations had little effect on the secretability of the complexes (Fig. 6). Thus, the recruitment of AChE\textsubscript{T} subunits into heteromeric T\textsubscript{4}-Pstp46 complexes is weakened by an interruption of the proline stretches by a leucine, and this suggests that a succession of 10 prolines is sufficient to ensure a maximal efficiency.

A proline-rich peptidic motif, sufficient for recruitment of AChE\textsubscript{T} subunits - In the preceding sections, we showed that it is possible to maintain an efficient recruitment of AChE\textsubscript{T} subunits into T\textsubscript{4}-Pstp, hetero-oligomers after deletion of the N-terminal region of PRiMA preceding R20 and of the C-terminal region following L37. Therefore, the 20-37 peptidic motif, which contains the polyproline stretches (RP\textsubscript{4}LP\textsubscript{10}RL), may be sufficient for this interaction. To verify this conclusion, we combined N-terminal and C-terminal deletions in the ΔN-R-Pstp38 mutant. This mutant was co-expressed with AChE\textsubscript{T} subunits, and compared with Pstp54, ΔN-R-Pstp38 and Pstp38, all expressed at 2 μg vector/dish (Fig. 5). All mutants induced the production of T\textsubscript{4}-Pstp, confirming the effects observed with separate deletions of the N-terminal and C-terminal regions. Thus, the 18-residue peptide 20-37, derived from PRiMA, possesses the capacity to assemble four AChE\textsubscript{T} subunits into stable T\textsubscript{4}-Pstp complexes in a manner similar to that of the full length extracellular domain of PRiMA (Pstp54).

It is noteworthy that deletion of the N-terminal region, in ΔN-R-Pstp54 and ΔN-R-Pstp38 increased the ratio of secreted to cellular T\textsubscript{4}-Pstp by about 25%, compared to the corresponding mutants Pstp54 and Pstp38, suggesting that secretion was facilitated by the N-terminal deletion. Deletion of the C-terminal region, in Pstp38 and ΔN-R-Pstp38, compared to Pstp54 and ΔN-R-Pstp54, also appeared to increase its secretability.

Assembly of AChE\textsubscript{T} tetramers by synthetic peptides - We synthesized the PRiMA-derived peptides RP\textsubscript{4}LP\textsubscript{10} and RP\textsubscript{4}LP\textsubscript{10}RL, to determine whether they could induce the assembly of AChE\textsubscript{T} monomers and dimers into tetramers. In a first series of experiments, the peptides were added at a final concentration of 10\textsuperscript{4} M to COS cells expressing only AChE\textsubscript{T} subunits, during the transfection process and/or in the culture medium. The cells and the medium were analyzed by non denaturing electrophoresis after 3 days, as indicated above. This showed a clear increase in the proportion of AChE\textsubscript{T} tetramers, most probably associated with the peptides (Fig. 7A). This result indicates that the peptides were able to penetrate the biosynthetic compartments of the living cells.

In a second series of experiments, homogenates of COS cells expressing only AChE\textsubscript{T} subunits were prepared without detergent, and incubated overnight at 37°C with the peptides (10\textsuperscript{4} M). As shown in Fig. 7B, both peptides induced the assembly of tetramers, under these acellular conditions. When a synthetic t peptide (WAT), at 2.10\textsuperscript{4} M, was added to the cell homogenate together with the PRiMA-derived peptides, it reduced markedly the recruitment of AChE\textsubscript{T} monomers and dimers into tetramers, indicating a direct interaction between these peptides.

DISCUSSION

The N-terminal extracellular domain of PRiMA assembles AChE\textsubscript{T} subunits into soluble T\textsubscript{4}-Pstp complexes - The assembly of AChE\textsubscript{T} tetramers with the transmembrane protein PRiMA produces membrane-bound complexes, both in the nervous system (14-18,31) and in muscles (19). In addition, the availability of PRiMA seems to limit the stabilization, as well as the functional localization of AChE in the mammalian brain (18). It is therefore important to analyze the interaction between PRiMA and AChE\textsubscript{T} subunits. In this
study, we attempted to define the peptidic motif of PRiMA which is responsible for its quaternary association with AChE_T subunits, by using deletions upstream, downstream and within the proline-rich segment.

We mostly used truncated PRiMA mutants, lacking the transmembrane and cytoplasmic domains (P_stpn with a stop codon at position n), which were co-expressed with AChE_T subunits in transiently transfected COS cells, producing soluble complexes (T_4-P_stpn), which were secreted instead of remaining attached to the cell membrane.

**Truncated PRiMA mutants recruit AChE_T subunits into heteromeric complexes, following either secretion or degradation pathways** - We have shown in a previous study that a significant fraction of AChE_T subunits is degraded intracellularly through the ERAD process, while another fraction is secreted (20,21). Therefore, the level of intracellular AChE activity reflects a balance between the rate of synthesis and the combined rates of degradation and secretion.

When COS cells were transfected with a fixed amount of plasmid encoding AChE_T subunits, together with various amounts of plasmid encoding PRiMA mutants, a variable fraction of the subunits was integrated in T_4-P_stpn complexes, both in the cells and in the medium. It was surprising to find that, although the proportion of T_4-P_stpn complexes varied widely, it appeared to plateau for similar amounts of plasmid DNA, suggesting that all mutants presented a similar apparent “affinity” for AChE_T subunits. This paradox can be resolved by assuming that even when the observed proportion of complexes was very low, mutants such as P_stp31 did in fact interact with AChE_T subunits, as indicated by a marked reduction in the secretion of (T_1+T_2), but produced rapidly degraded complexes. With longer mutants, the level of secreted T_4-P_stpn complex progressively increased. Thus, the variations observed in the amount of secreted complex appear to reflect a competition between secretion and degradation. Depending on its organization, each type of T_4-P_stpn complex would be oriented with different probabilities towards the secretion pathway or the degradation pathway. This suggests that the rate of secretion depends critically on the structure of the complex.

It is a classical notion that proteins destined to function as a multi-subunit complex may be retained in the endoplasmic reticulum by the quality control system of the cell, unless they are appropriately associated with their partners (32). This can be rationalized by the fact that they expose hydrophobic surfaces in their unassembled state, but not in the complex. This seems to be the case for *Torpedo* AChE_T subunits, which are very little secreted when expressed alone in COS cells, but can be secreted when co-expressed with an N-terminal fragment of ColQ (Q_stp69) (5). We also observed here that rat AChE_T subunits can be rescued by Q_stp69 from degradation through ERAD (20). This is consistent with the fact that their physiological function depends on association with an anchoring protein, ColQ or PRiMA.

The fact that short truncated PRiMA mutants increase the intracellular degradation of AChE_T subunits is more unusual. However, Ulloa-Aguirre and Conn recently suggested that some proteins induce an inappropriate conformation of normally externalized molecules and thus target them towards degradation by the cellular quality control, and proposed to call this hypothetical process “protein shipwrecking” (33). Here, we present evidence that some PRiMA mutants may behave in this manner, and that there is in fact a continuous spectrum between mutants which induce degradation and those which rescue AChE_T subunits from ERAD and promote their secretion as heteromeric complexes.

Since the assembly of a complex between proteins occludes interaction surfaces, rather than exposing hydrophobic zones, how could the association with short PRiMA mutants such as P_stp31 induce the degradation of AChE_T subunits? Although we have presently no experimental evidence, we may propose an hypothetical explanation. We have previously demonstrated that aromatic (but not hydrophobic) residues of the C-terminal t peptide of AChE play a crucial role to induce the degradation of AChE_T subunits by ERAD (22). It is possible that a group of aromatic residues remains exposed in the complexes formed with short PRiMA mutants, engaging their translocation into the cytoplasm and degradation by proteasomes, whereas these residues are masked in
complexes formed with longer mutants, as demonstrated by crystallography in a complex of four t peptides with the PRAD of ColQ (12).

Definition of a sufficient interaction motif (PRAD) in PRiMA - The efficiency of secretion of AChE oligomers increases with their degree of oligomerization (9). The secretability of heteromeric T₄-Pₘₚ₃₁ complexes, indicated by the ratio of secreted to cellular activity, was variable: it is noteworthy that it was increased by deletion of peptidic regions located upstream or downstream of the poly-proline stretches. A maximum was obtained by removing all residues following the prolines (Pstp36); conversely, it was reduced for mutants in which the N-terminal region was deleted (Δ₄₈) and the proline-rich segment was preceded by residues A, E, or D, as opposed to basic residues R or K.

Most of the N-terminal and C-terminal regions flanking the poly-proline stretches could be deleted without suppressing the capacity of P mutants to engage AChET subunits into T₄-Pstp₃₃ complexes. This capacity in fact appeared maximal when most of the C-terminal region was deleted, leaving only two residues (RL) after the prolines, in Pstp73. However, it is not certain that these residues play a specific role in the association with t peptides, because replacement of the three residues following the prolines by alanines had essentially no effect (in RLL/AAA-Pstp39 versus Pstp39). Even though we have no direct evidence that Pstp54 is N-glycosylated under our experimental conditions, we found that the presence of a putative N-glycosylation site, located at position 44, did not influence the association of PRiMA mutants with AChET subunits. Similarly, the cysteine located at position 48 did not affect the formation of T₄-Pstp₃₃ complexes.

The PRAD of ColQ is normally disulfide-linked to two of the t peptides, while the other two are disulfide-linked together (5), and it has been reported that, as in the case of ColQ, two of the AChET subunits are disulfide-linked with the membrane anchor, in brain AChE (14,15). However, we previously found that the formation of heteromeric complexes with the N-terminal region of ColQ could occur without the two adjacent cysteines preceding the PRAD, indicating that disulfide bonds between ColQ and two AChET subunits are not required (5,11). The present results suggest a similar conclusion, since the formation of complexes was only slightly reduced (20% or less) by deletion of most of the N-terminal region, which contains four cysteines, located at positions 6, 13, 17, 19. This does not rule out the possibility that some of these cysteines may be involved in intercatenary disulfide bonds between PRiMA and AChET subunits, as shown in membrane-anchored AChE tetramers from bovine brain (14,15,34), and thus stabilize the complex.

PRiMA contains two poly-proline stretches, separated by a leucine (P₄LP₁₀). We found that this leucine is not required for the interaction, and could be replaced by a proline, resulting in a single suite of 15 prolines (P₁₅). The secretion of T₄-Pstp₃₃ complexes was decreased by displacement of the leucine (P₁₀LP₄) or interruption of the proline stretches by a second leucine (P₄LP₅LP₄) and it was almost totally abolished when 5 prolines were deleted in Pstp₃₁ (P₄LP₅). Thus, the formation of secretable complexes requires the presence of a minimal number of uninterrupted prolines.

Our deletion experiments converged on the idea that the 20-37 segment of PRiMA (RP₄LP₁₀RL) may be able to recruit AChET subunits nearly as efficiently as the entire extracellular domain of PRiMA, and this was confirmed by expressing this 18-residue peptide, as mutant Δ₅₉-R-Pstp₃₈. In fact, addition of the synthetic peptides RP₄LP₁₀ and RP₄LP₁₀RL to COS cells expressing only AChET subunits induced the recruitment of monomers and dimers into stable tetramers. Moreover, we obtained the same result by incubating a cell homogenate with the peptides, and observed that the assembly of AChET tetramers was partially blocked by addition of a synthetic t peptide (WAT). This proves that the PRIMA-derived and t peptides spontaneously interact in vitro, as shown previously for a ColQ-derived PRAD peptide (12), and opens the way to the formation, crystallization and structural analysis of the core complex which forms the basis of the association between PRiMA and AChET. It is noteworthy that the PRAD of ColQ is very efficient with only 8 prolines (P₃MFP₅). This raises the intriguing possibility that the complexes of t peptides with the proline-rich motifs of ColQ and
PRiMA might significantly differ in their quaternary organization. Using short PRiMA-derived peptides, it may also be possible to shed some light on the structural differences between complexes that pass or fail the quality control in the secretory pathway.

REFERENCES

FOOTNOTES

* We thank Dr Noël Perrier and Dr Cinzia Falasca for helpful suggestions. This work was supported by grants from the Centre National de la Recherche Scientifique, the Association Française contre les Myopathies, the Direction des Forces et de la Prospective, and the European Community.

1The abbreviations used are: AChE: acetylcholinesterase (E.C. 3.1.1.7); AChE_T: splice variant T; PRiMA: Proline-Rich Membrane Anchor; ColQ: collagen Q; ERAD: Endoplasmic Reticulum Associated degradation; PRAD: Proline-Rich Attachment Domain; P_{stp}: truncated PRiMA mutant, terminating with a stop codon at position n; ΔN-X-P_{stp}: PRiMA mutant, with a deletion of the N-terminal fragment, starting with residue X before the prolines, and terminating with a stop codon at position n; WAT: Tryptophan (W) Amphiphilic Tetramerization domain.

FIGURE LEGENDS

Fig. 1. Structure of PRiMA and of mutants used in this study. A. Sequence of PRiMA, numbered from the first residue of the mature protein. The putative signal peptide is shown in thin letters (-35 to -1), the extracellular domain in bold letters (1 to 53), the transmembrane domain in bold italics, and the cytoplasmic domain in thin italics (the sequence shown corresponds to the major splice variant PRiMA I, possessing the longer cytoplasmic domain). In the extracellular domain, the putative N-glycosylation site is indicated by an asterisk and the cysteines are underlined. B. Structure of mutants. All mutants possess the signal peptide (not shown), and fragments of the extracellular domain, but not the transmembrane and cytoplasmic domains. They are designated by the position of introduced stop codons (e.g. P_{stp54} contains the entire extracellular domain, 1-53). The deletion of the region preceding the poly-proline stretches is indicated by D; in these mutants, a single residue precedes the prolines, so as to maintain a proper cleavage site after the signal peptide: this residue is indicated, e.g. arginine (R) in ΔN-R-P_{stp54}. Mutants in the proline-rich region are indicated by the distribution of prolines and leucine residues (e.g. P_{4LP10} in the case of the wild type). The interaction motif (RP_{4LP10}RL) corresponds to mutant ΔN-R-P_{stp38}. C. Structure of the N-terminal fragment of Torpedo ColQ (Q_{stp69}) (the signal peptide is not shown). The underlined fragment corresponds to the PRAD interaction domain. It was deleted in Δ[28-44]Q_{stp69}.

Fig. 2. Molecular forms of AChE produced and secreted by transient transfection of COS cells with AChE_T expressed alone, with full length PRiMA and with truncated PRiMA, reduced to its extracellular domain (P_{stp54}). A. Representative sedimentation profiles, obtained from gradients containing 1% Brij-96, normalized to the same total AChE activity, indicating the relative proportions of monomers (T_1), dimers (T_2) and tetramers, associated or not with PRiMA or P_{stp54}. Profiles corresponding to cell extracts are shown in the top panel, and to the medium in the middle panel. (—o—): AChE_T expressed alone; (■): AChE_T expressed with PRiMA; (▲): AChE_T expressed with P_{stp54}. In general, the relative proportion in the medium, compared to cell extracts, increases with the degree of oligomerization, indicating that secretion is facilitated by
oligomerization. Soluble non amphiphilic tetramers (T₄) and heteromeric complexes (T₄-Pₚ₅₄) sediment around 10.5 S, while amphiphilic T₄-PRiMA complexes, containing the transmembrane domain of PRiMA, are retarded by their interaction with the detergent (Brij-96) and sediment around 9 S.

**B. Migration of AChE forms from cell extracts and culture media in non denaturing electrophoresis.** In the profiles corresponding to co-transfected cells (AChE₇ with PRiMA and AChE₇ with the extracellular domain of PRiMA, Pₛₚ₅₄), the simple arrows indicate non amphiphilic AChE tetrameric forms; the arrow with an asterisk indicates the amphiphilic tetramer associated with a complete PRiMA protein.

Fig. 3. Secreted AChE tetramers induced by co-transfection of AChE₇ subunits with PRiMA contain an N-terminal fragment of PRiMA. The electrophoretic migration of AChE tetramers secreted by COS cells expressing AChE₇ subunits with an N-flag-PRiMA was retarded, in non-denaturing gels, after incubation with the monoclonal anti-flag antibody M1. AChE tetramers are indicated by arrows and the retarded component by an asterisk.

Fig. 4. Variation of the proportion of AChE tetramers with the amount of PRiMA mutants. COS cells were co-transfected with a fixed amount of DNA encoding AChE₇ subunits (2 µg per 60 mm culture dish) and the indicated amount of DNA encoding PRiMA mutants, or the N-terminal region of ColQ (Qₛₚ₆₉), complemented with DNA encoding a non interacting protein, so that the total amount of vector was kept constant to avoid modifications in the protein synthesis capacity of the cells. The proportions tetramers, associated or not with Pₛₚ₅₄ proteins (T₄ + T₄-Pₛₚ₅₄) was determined from sedimentation profiles, as in Fig. 2. The figure illustrates the results obtained for Pₛₚ₃₁ and Pₛₚ₅₄ mutants, producing respectively high or low proportions of T₄-Pₛₚ₅₄ complexes. The curves obtained with all other mutants were very similar in shape, differing mostly by the maximal levels of tetramers: co-transfection with 2 µg DNA/dish encoding the various mutants was used in most subsequent experiments.

**Fig. 5. Effect of the PRiMA mutants on cellular and secreted AChE activity and molecular forms.** COS cells were co-transfected with 2 µg of DNA encoding AChE₇ and 2 µg of DNA encoding PRiMA mutants. A. Sequence and numbering of the extracellular domain of PRiMA; the cysteines are underlined and the putative N-glycosylation site is indicated by an asterisk. B. The cellular and secreted AChE activities corresponding to the different mutants were normalized, for each experiment, to those of AChE₇ subunits expressed alone, taken as 100%. The proportions of the molecular forms were determined from sedimentation profiles. The data corresponding to C-terminal truncated mutants (1 to 9) are the means of 4 to 7 independent transfection experiments, ± standard errors; those for other mutants correspond to a representative experiment. The lightly hatched boxes correspond to the activity of monomers and dimers (T₁ + T₂) and the darkly hatched boxes to tetramers (homomeric tetramers T₄ and heteromeric T₄-Pₛₚ₅₄ complexes). If homomeric T₄ tetramers are present in the same proportion to T₁ and T₂, as in the case of AChE₇ subunits alone, they represent a minor species and can be generally neglected. In mutants 16 to 18, the deletion of the N-terminal region is associated with mutations in the proline-rich segment, as indicated in parentheses. Note that a mutant reduced to the RP₄LP₁₀RL motif (expressed as mutant ΔN-R-Pₛ₉₃₈) induced the production and secretion of essentially the same amount of complex as the complete extracellular domain (Pₛₚ₅₄ mutant). C. Correlation between the total secreted AChE activity and the proportion of tetramers in the medium. The numbers identify the mutants, as indicated in the left column of A. D. Correlation between the percentages of T₄ complexes in secreted and cellular AChE.
**Fig. 6.** Ratio of secreted to cellular activity of AChE forms. The secreted activity reflects the rate of secretion, whereas the cellular activity, 3-4 days after transfection, approximately corresponds a steady state value; therefore, these activities are not comparable, but their ratio may be considered as an index of the efficiency of secretion, for each AChE form. These ratios were arbitrarily normalized to 100 for AChE tetramers produced in co-transfection with the complete N-terminal domain of PRiMA, Pstp54. For monomers and dimers (T1+T2), the ratio remained approximately constant, in agreement with the fact that their structure did not change.

**Fig. 7.** Synthetic PRiMA peptides induce oligomerization of AChET subunits *in vitro*. A. COS cells were transfected with AChET and the synthetic peptides RPKLP10 and RPKLP10RL were added with fresh culture medium after 2 days. The cells were extracted 24 hours later and analysed by non denaturing electrophoresis, as well as the medium. B. The peptides were added at a concentration of $10^{-4}$ M to an homogenate of COS cells expressing only AChET subunits, and incubated overnight at 37°C. In some samples, a synthetic t peptide (WAT) was added at 2 $10^{-4}$ M, as indicated. The homogenate was then analyzed by non denaturing electrophoresis in the presence of detergents (see Methods). The peptides induced the formation of stable AChET tetramers (indicated by arrows); this effect was reduced by addition of the t peptide (WAT).

*Keywords:* Acetylcholinesterase; PRiMA; PRAD; degradation; secretion; protein shipwrecking
## Fig. 1

### A  Sequence of mouse PRiMA

<table>
<thead>
<tr>
<th>Signal peptide</th>
<th>MLLRD\text{VPRHG}CC\text{WPSLLLH}\text{CALHPLWGLVQVTHA}</th>
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<tr>
<td>N-term. domain</td>
<td>EPQK\text{SCSKVTD}C\text{QHICQCRPPPLP}PPPPPPPPPP\text{PRLLSAPAP*STSCPAEDS}</td>
</tr>
<tr>
<td>TM domain</td>
<td>WW\text{SGLVIIAVV}CASLVFLTVL\text{VII}</td>
</tr>
<tr>
<td>C-ter. I</td>
<td>CYKAIK\text{RKLKDENGTSVAEYPMSSSQSHKGVDVNAAVV}</td>
</tr>
<tr>
<td>C-ter. II</td>
<td>CYKAI\text{KRQAI}</td>
</tr>
</tbody>
</table>

\* Nflag-PRiMA: DY\text{KDEPQKSCSKVTD}C\text{QHICQCRPPPLP}PPPPPPPPPP\text{PRLLSAPAP*STSCP}...

### B  Sequences of truncated PRiMA mutants

#### C-terminal truncations (Pstpn)

<table>
<thead>
<tr>
<th>Pstpn</th>
<th>Sequence</th>
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<tr>
<td>Pstpn46</td>
<td>EPQK\text{SCSKVTD}C\text{QHICQCRPPPLP}PPPPPPPPPP\text{PRLLSAPAPNS}</td>
</tr>
<tr>
<td>Pstpn31</td>
<td>EPQK\text{SCSKVTD}C\text{QHICQCRPPPLP}PPPP</td>
</tr>
</tbody>
</table>

#### N-terminal deletions

| ΔN-R-Pstpn46 | RPPPPPLPPPPPPPPPP\text{PRLLSAPAPNS} |
| ΔN-E-Pstpn46 | E-------------------PPPPLPPPPPPPP\text{PRLLSAPAPNS} |

#### Interaction motif

| ΔN-R-Pstpn38 | RPPPPPLPPPPPPPPPPRL |

#### Mutations of leucines in the poly-proline stretches

| P15         | E-------------------PPPPLPPPPPPPPPP\text{PRLLSAPAPNS} |
| P4LP5LP4    | E-------------------PPPPLPPPPPPPPPP\text{PRLLSAPAPNS} |
| P10LP4      | E-------------------PPPPLPPPPPPPPPP\text{PRLLSAPAPNS} |

### C  Sequence of the ColQ-derived N-terminal fragment

<table>
<thead>
<tr>
<th>Qstpn69</th>
<th>ESTFDKL\text{AFLQ}A\text{ALLPM}EHKKR\text{SVNK}CC\text{LLTPPPPMFPPPFFTETNIL}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\text{QEVDLNNLPLEIKPTEPS}</td>
</tr>
</tbody>
</table>
Fig. 2

A Sedimentation profiles

- Cell extract
- Medium
- AChE activity (arbitrary units)

B Non-denaturing electrophoresis

- Migration
- Origin

Table:

<table>
<thead>
<tr>
<th></th>
<th>c</th>
<th>m</th>
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<tbody>
<tr>
<td>T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T+PRiMA</td>
<td></td>
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<tr>
<td>T+Pstp54</td>
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</table>
Fig. 4

A

Cellular AChE

Percentage of AChE tetramers

B

Secreted AChE

µg of DNA vector encoding AChE-associated proteins / dish

Q_{stp69}
P_{stp54}

P_{stp33}
A

Pstp54

[EPQKSCSKVTDSCQHIQCCRPPPFLPPPPPPFPPRLLSAPAPNSTSCPAEDS]

B

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<th>T</th>
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<th>medium</th>
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<tr>
<td>1</td>
<td>Pstp31</td>
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<td>9</td>
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<td>19</td>
<td>ΔN-E-(P10LP4) sp54</td>
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<td>20</td>
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<tr>
<td>21</td>
<td>(RP4LP10RL)</td>
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</table>

motif

Cellular and secreted AChE activity

C

Total secreted activity

Percentage of tetramers in secreted AChE

D

Activity of secreted AChE tetramers

Activity of cellular AChE tetramers
Ratio of secreted to cellular tetramers

Ratio of secreted to cellular (T1 + T2)

[Bar chart with data points for various conditions]
Fig. 7

A

migration

origin

cell extract

control RP4LP10 RP4LP10RL

control RP4LP10 RP4LP10RL

B

migration

origin

control -WAT + WAT RP4LP10

control -WAT + WAT RP4LP10RL