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A four-to-one association between peptide motifs: four C-terminal domains from cholinesterase assemble with one proline-rich attachment domain (PRAD) in the secretory pathway

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The major type of acetylcholinesterase in vertebrates ($AChE_T$) is characterized by the presence of a short C-terminal domain of 40 residues, the 'tryptophan amphiphilic tetramerization' (WAT) domain. The presence of this domain is not necessary for catalytic activity but is responsible for hydrophobic interactions and for the capacity of $AChE_T$ subunits to form quaternary associations with anchoring proteins, thereby conditioning their functional localization. In the collagen tail of asymmetric forms, we characterized a small conserved region that is sufficient for binding an $AChE_T$ tetramer, the proline-rich attachment domain (PRAD). We show that the WAT domain alone is sufficient for association with the PRAD, and that it can attach foreign proteins (alkaline phosphatase, GFP) to a PRAD-containing construct with a glycophosphatidylinositol anchor (GPI), and thus anchor them to the cell surface. Furthermore, we show that isolated WAT domains, or proteins containing a WAT domain, can replace individual $AChE_T$ subunits in PRAD-linked tetramers. This suggests that the four WAT domains interact with the PRAD in a similar manner. These quaternary interactions can form without intercatenary disulfide bonds. The common catalytic domains of AChE are not necessary for tetrameric assembly, although they may contribute to the stability of the tetramer.

Keywords: acetylcholinesterase/collagen/peptide motif/proline-rich attachment domain

Introduction

Vertebrates possess two cholinesterase genes, producing acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The catalytic domains of these enzymes are followed by different types of small C-terminal peptides, encoded by distinct exons. These C-terminal regions determine the post-translational processing, so that the mature molecules may be released in soluble form, anchored in cell membranes, or attached to the extracellular matrix. Whereas alternative choices may exist for AChE in some species, all vertebrate AChE and BChE genes contain an exon encoding a C-terminal peptide which characterizes catalytic subunits of type T ('tailed'). $AChE_T$ subunits are expressed in all cholinergic tissues, e.g. muscles and nervous tissues (Massoulié *et al*., 1993). In transgenic *Xenopus* embryos, the C-terminal domain of these subunits is responsible for their specific expression at neuromuscular junctions (Shapira *et al*., 1994). In transgenic mice, this type of subunit is also expressed in brain (Beeri *et al*., 1995). In cultures of glioma cells, it promotes neurite extension, unlike other forms of the enzyme (Karpel *et al*., 1996).

The $AChE_T$ subunits are extremely versatile in their quaternary organization, since they produce monomers, dimers, tetramers and the complex collagen-tailed and hydrophobic-tailed molecules (from which T subunits derive their name) (Massoulié *et al*., 1993). The 'tailed' heteromeric molecules represent the most physiologically important forms of AChE. Hydrophobic-tailed AChE is the predominant form of the enzyme in mammalian brain (Gennari *et al*., 1987; Inestrosa *et al*., 1987; Boschetti *et al*., 1994; Boschetti and Brodbeck, 1996), and collagen-tailed forms are accumulated at neuromuscular junctions.

In the tailed molecules, tetramers of $AChE_T$ subunits are attached to a structural subunit, e.g. one of the strands of the collagen triple helix: the A_4, A_8 and A_{12} forms contain respectively one, two and three tetramers. In this structure, each collagen strand is associated with four catalytic $AChE_T$ subunits, in a one-to-four complex, as illustrated in Figure 1. Two catalytic subunits are directly disulfide-linked to each other, and two subunits are disulfide-linked to the structural subunit (Anglister and Silman, 1978; Lee *et al*., 1982; Lee and Taylor, 1982; Roberts *et al*., 1991). The cloning of the collagen tail (ColQ) of AChE from *Torpedo* (Krejci *et al*., 1991) and from mammals (Krejci *et al*., 1997) allowed us to demonstrate that catalytic tetramers are bound to the non-collagenous, N-terminal domain (Duval *et al*., 1992a; Bon and Massoulié, 1997; Krejci *et al*., 1997) and to define a small proline-rich attachment domain (PRAD) which is sufficient for this interaction (Bon *et al*., 1997). This 17-residue peptide contains two cysteines, as expected, but its critical feature is the presence of three and five consecutive prolines, so that even synthetic polyproline could replace the natural PRAD: both recruit monomers and dimers of $AChE_T$ or $BChE_T$ to form tetramers. When a secretable construct containing the PRAD was co-expressed with $AChE_T$ subunits, it produced soluble AChE tetramers, with which it was stably associated, even when it did not contain any cysteine (Bon and Massoulié, 1997), showing that it did not act as a scaffold or chaperone for tetramer formation, but rather as a keystone in the heteromeric structure. These interactions require the presence of the C-terminal region of $AChE_T$ subunits, because truncated AChE subunits, in which a stop codon was introduced at the end of the catalytic domain, only produced soluble monomers, showing no interaction with the collagen tail or with any derived construct (Duval *et al*., 1992b; Morel and Massoulié, 1997).

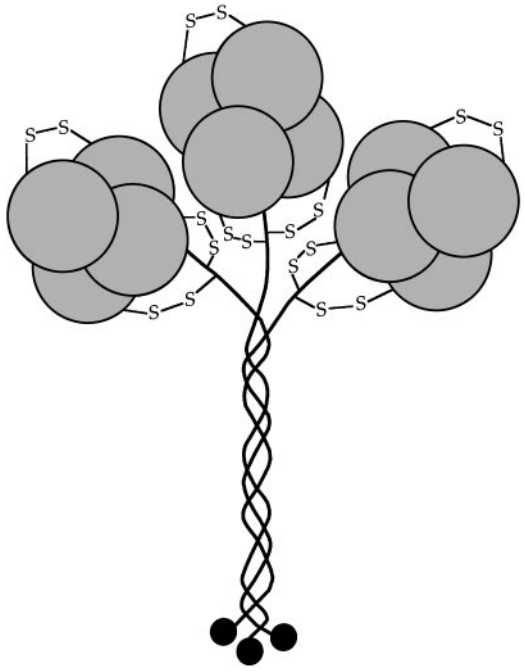

Fig. 1. Schematic organization of an A_{12} collagen-tailed form of AChE. The N-terminal region of each strand, which contains the PRAD, may be associated with a tetramer of $AChE_T$ subunits. Two subunits are disulfide-linked to the PRAD, and the other two are disulfide-linked to each other. The catalytic subunits are shown as lightly shaded spheres and the non-collagenous C-terminal part of the collagen tail as black balls [redrawn from Lee and Taylor (1982)].

Since the binding of $AChE_T$ subunits to their associated collagen, ColQ, is based on the small PRAD motif, we asked whether, similarly, the C-terminal region of AChE might be sufficient for these quaternary interactions. The C-terminal regions of T subunits consist of 40 residues in vertebrate AChEs and 41 residues in mammalian BChEs (Cousin *et al*., 1996, 1998) and are very well conserved throughout vertebrates (75% identity between T peptides of *Torpedo* and mammalian AChEs). The conserved residues comprise a cysteine located at position –4 from the C-terminus and a series of seven aromatic residues, including three equally spaced tryptophans. This region can form an amphiphilic α helix (Massoulié *et al*., 1993). We now show that it constitutes an autonomous interaction domain and therefore propose to name it the 'tryptophan amphiphilic tetramerization' (WAT) domain. According to this nomenclature, $AChE_T$ is equivalent to AChE–WAT.

We analyzed the localization of constructs encoding WAT domains and foreign proteins containing a C-terminal WAT domain, when they were expressed alone or together with a chimeric protein containing a PRAD associated with a C-terminal GPI-addition signal, in transfected COS cells. We had previously shown that this PRAD–GPI chimera recruits $AChE_T$ subunits and targets them to the cell surface as GPI-anchored tetramers (Duval *et al*., 1992a).

We studied further the stoichiometry of the observed interactions in *Xenopus* oocytes, which were injected with defined mixtures of mRNAs. In these competition experiments, the PRAD-containing protein was confronted to $AChE_T$, together with various amounts of WAT domains or of other constructs containing WAT domains. We compared WAT domains of various origins, in order to generalize the validity of our results.

Results

Protein constructs

Figure 2 shows the constructs that we used in this work, as well as the corresponding PRAD and WAT sequences. WAT domains from rat and *Electrophorus* were associated with a 'flag' peptide epitope (DYKDE) which was inserted immediately after the secretion signal peptide.

In the case of human alkaline phosphatase, we replaced the endogenous C-terminal GPI addition signal with the *Electrophorus* WAT domain. For the green fluorescent protein (GFP), we added a secretory signal at the N-terminus, and WAT domains from rat AChE, *Electrophorus* AChE and human BChE at the C-terminus. In some of these constructs, (the rat 'N-flagged' WAT domain and GFP with the *Electrophorus* WAT domain) we also replaced the C-terminal cysteine residue with an alanine.

These constructs were co-expressed with a protein derived from the rat ColQ gene, rQ_R, which contains the N-terminal domain including the PRAD, but not the collagenic domain. We also used a chimeric protein that had been described previously: in Q_N/H_C, the N-terminal domain of the *Torpedo* ColQ gene (Q_N) which contains the PRAD, is fused to the C-terminal domain of the *Torpedo* $AChE_H$ subunit (H_C) which contains a GPI addition signal, allowing its anchoring at the cell surface. For clarity and brevity, these proteins are called here PRAD and PRAD–GPI, respectively.

Membrane anchoring of isolated WAT domains and of proteins containing a C-terminal WAT domain, expressed with the PRAD–GPI construct in COS cells

Figure 3A shows that the N-flagged WAT domain is retained intracellularly, probably in the endoplasmic reticulum (ER) and in the Golgi apparatus, when expressed alone in transfected COS cells, exactly like $AChE_T$ subunits, as reported previously (Bon and Massoulié, 1997). This is consistent with the view that the WAT domain behaves as an ER retention signal (Velan *et al*., 1994). When the same proteins were co-expressed with the PRAD–GPI construct, they were both clearly detected at the cell surface (Figure 3B). Furthermore, they could be removed by the specific phospholipase, PI-PLC (not shown).

This clearly shows that the WAT domain, like the complete $AChE_T$ subunit, is able to combine with the PRAD–GPI protein, which anchors it in the cell membrane. Therefore, the catalytic domain of AChE is not required for this interaction and the WAT domain is sufficient for association with the PRAD. It thus seemed possible to endow other secreted proteins with the capacity to interact with the PRAD, by adding a WAT domain at their C-terminus. Human alkaline phosphatase contains a GPI addition signal and forms GPI-anchored tetramers (Hawrylak and Stinson, 1988). We replaced its C-terminal GPI-addition signal by the WAT domain from *Electrophorus* AChE (hAP–eWAT). Figure 4 shows the localization of alkaline phosphatase in COS cells expressing the wild-type enzyme, the modified hAP–eWAT construct alone and hAP–eWAT with PRAD–GPI.

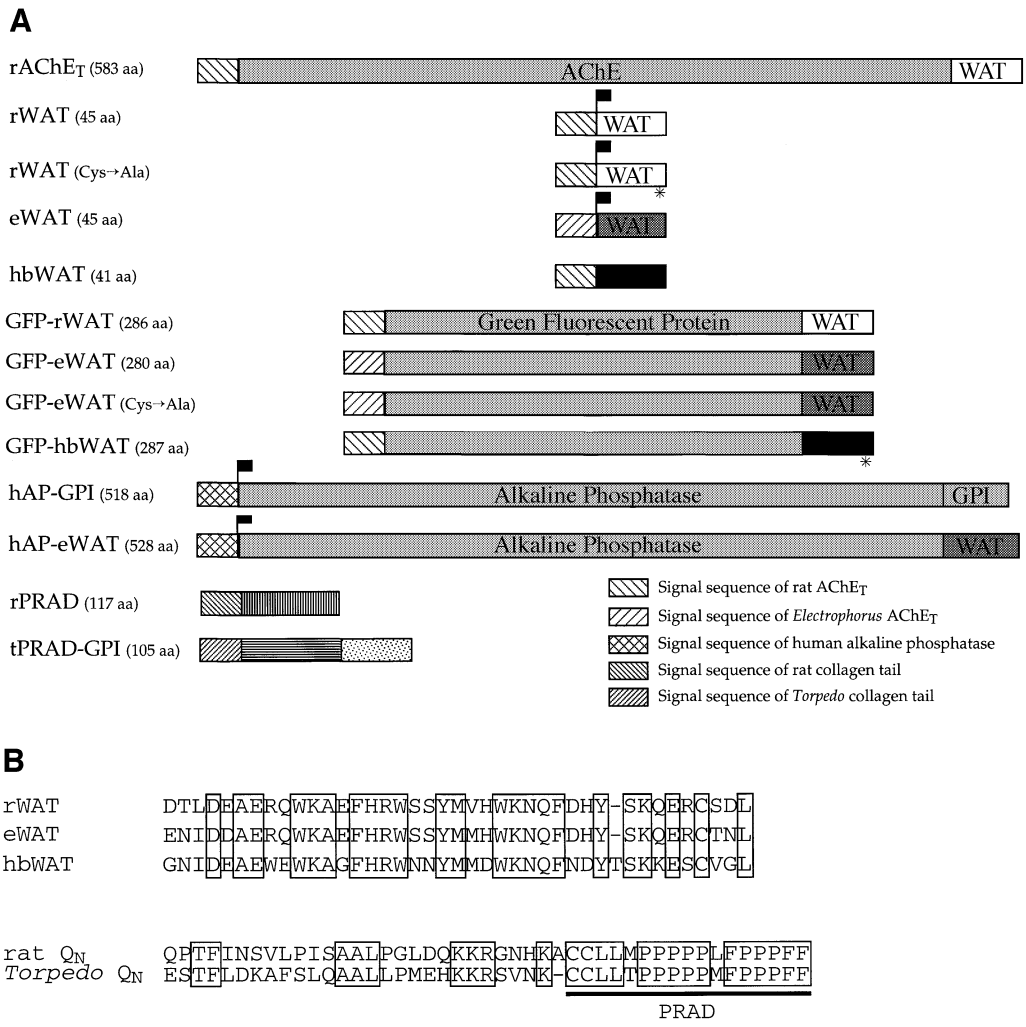

Fig. 2. Schematic representation of protein constructs used in this study; peptide sequences of WAT and PRAD. (**A**) These constructs were inserted in the pCDNA3 vector for expression in COS cells, and/or in the TST7 vector for production of synthetic mRNA by the T7 RNA polymerase, and expression in *Xenopus* oocytes. The AChE and human alkaline phosphatase (hAP) constructs included the endogenous signal peptides of these proteins. In the case of the Green Fluorescent Protein (GFP), we introduced the signal peptide of rat or *Electrophorus* AChE. The 'flag' peptide epitope was added immediately after the cleavage site of the signal peptide, in the case of isolated WAT domains and of alkaline phosphatase, in order to allow their detection by the M1 monoclonal antibody from Eastman Kodak. The WAT domains were added at the C-terminus of GFP and alkaline phosphatase. The letters b, e, h, r and t stand respectively for BChE, *Electrophorus*, human, rat and *Torpedo*. A star indicates that the cysteine was mutated to alanine. A flag symbol indicates the presence of the 'flag' epitope (DYKDE). The rPRAD construct corresponds to the rQ$_R$ transcript (Krejci *et al*., 1997); in the tPRAD–GPI construct, the N-terminal region of *Torpedo* ColQ was combined with the GPI-addition signal of the *Torpedo* AChE$_H$ subunit (Duval *et al*., 1992a; Bon and Massoulié, 1997). (**B**) Sequences of the WAT domains that were used in this study and sequences of the N-terminal regions of rat and *Torpedo* ColQ (Q$_N$), from the N-terminus of the mature protein to the end of the PRAD, which is underlined. Conserved residues are boxed.

Like AChE$_T$ or the N-flagged WAT domain, the hAP–eWAT protein is not visible at the surface of non-permeabilized cells when expressed alone, but only in reticular and vesicular intracellular structures after permeabilization of the cells. When it was co-expressed with PRAD–GPI, it was exposed at the cell surface, exactly like the wild-type GPI-anchored protein.

In a further series of experiments, we targeted GFP to the secretory pathway by adding an N-terminal leader sequence, and introduced a WAT domain at its C-terminus. We observed the expressed protein either directly by its own fluorescence, or with rhodamine-conjugated antibodies (Figure 5). When expressed alone, GFP–WAT remained intracellular, in reticular structures and vesicles, like hAP–eWAT, and was not detected by antibodies in the case of non-permeabilized cells. In the presence of PRAD–GPI, it was clearly present at the cell surface, where it was best visualized with anti-GFP antibodies on non-permeabilized cells, without interference from intracellular compartments, which were seen by endogenous fluorescence or by antibodies in permeabilized cells.

Co-expression experiments in Xenopus oocytes: formation of mixed PRAD-linked tetramers of WAT domains

This series of experiments was designed to test whether WAT domains or GFP–WAT proteins might compete with AChE$_T$ subunits for association with the PRAD. We used *Xenopus* oocytes, because they could be injected with defined proportions of mRNAs encoding these proteins,

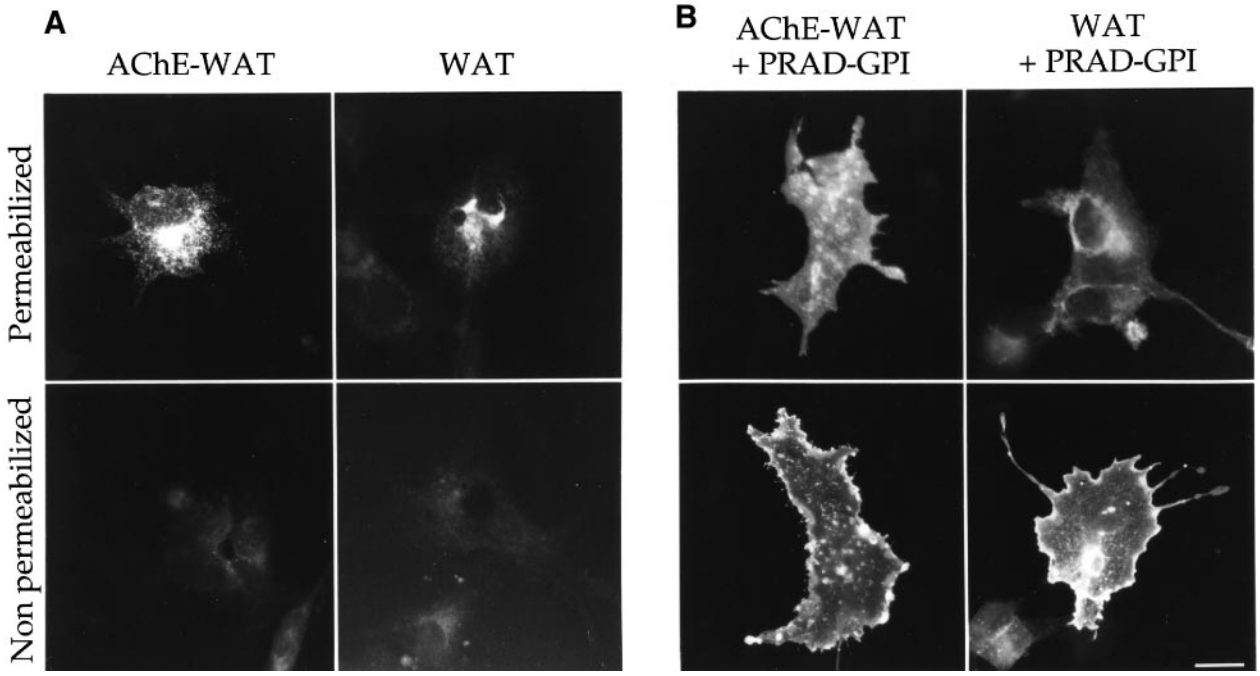

Fig. 3. Cellular localization of $AChE_T$ and an isolated WAT domain, expressed alone or with the PRAD–GPI construct. (**A**) When expressed alone, both $AChE_T$ (AChE–WAT) and the WAT domain are located in an intracellular reticular compartment, probably the ER (permeabilized cells, upper panels), and are not exposed at the cell surface (non-permeabilized cells, lower panels). (**B**) When co-expressed with the PRAD–GPI construct, they are also detected in intracellular vesicular structures (upper panel) and exposed at the cell surface (lower panels), from which they can be removed by PI-PLC (not shown). Rat AChE was detected with the mouse monoclonal antibody ZR3 (Rackonczay and Brimijoin, 1986) and the WAT domain by the anti-'flag' M1 monoclonal antibody, with secondary fluorescent antibodies (see Materials and methods). The scale bar represents 80 μm.

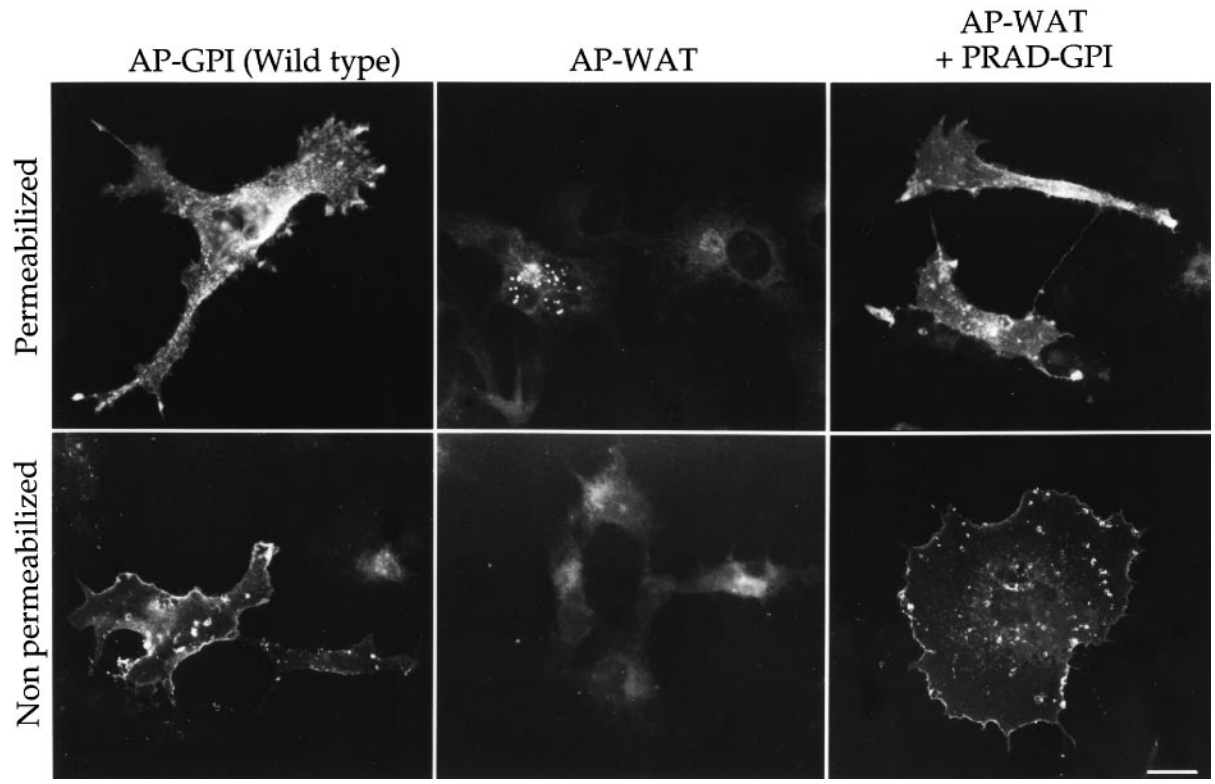

Fig. 4. A C-terminal WAT domain allows alkaline phosphatase to associate with PRAD–GPI. Immunofluorescence of COS cells expressing wild-type alkaline phosphatase, with its C-terminal GPI-addition signal (left panels), a modified alkaline phosphatase in which this signal was replaced by a WAT domain (middle panels) and the same construct together with PRAD–GPI (right panels). We used the anti-'flag' antibody to detect N-terminally flagged alkaline phosphatase. Alkaline phosphatase containing the WAT domain was exposed at the cell surface when co-expressed with PRAD–GPI, exactly like the N-flagged wild-type form, but was not detected at the cell surface when expressed without PRAD–GPI. The scale bar represents 80 μm.

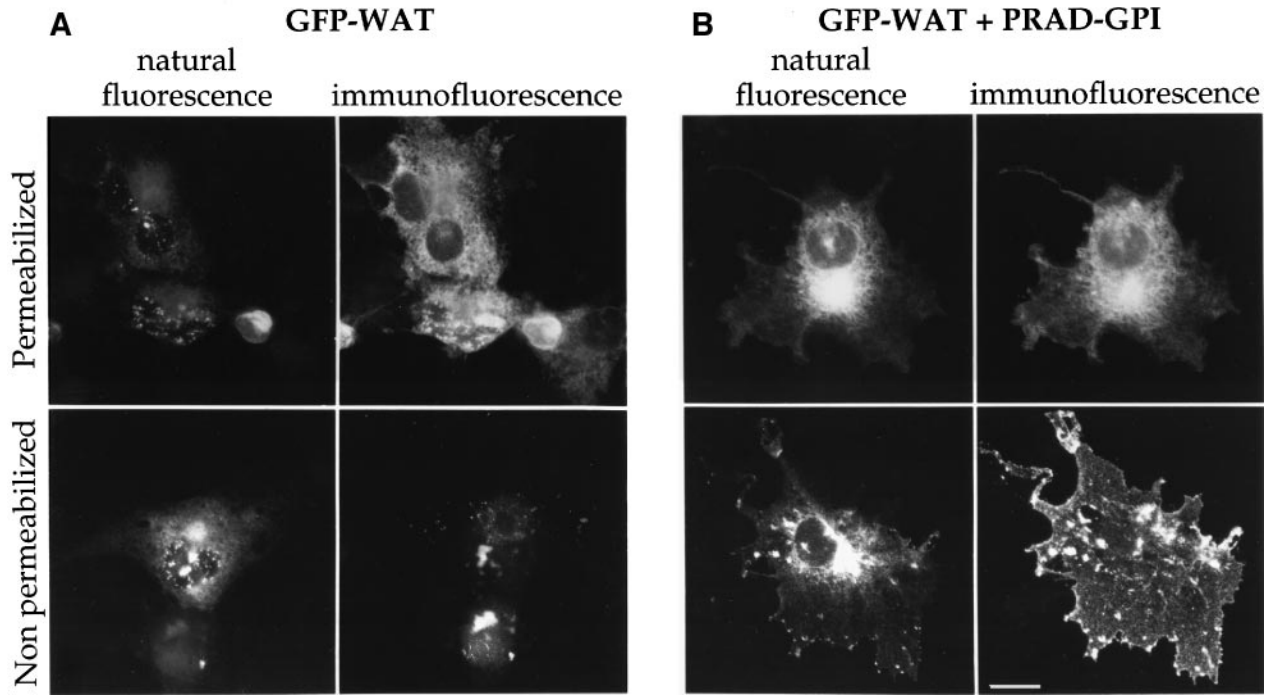

Fig. 5. When synthesized in the ER, GFP possessing a C-terminal WAT domain can be exposed at the cell surface in combination with PRAD–GPI. The GFP–WAT construct was expressed in COS cells, (**A**) alone or (**B**) together with PRAD–GPI, and its localization was examined by its own fluorescence, or with polyclonal anti-GFP antibodies, in permeabilized and non-permeabilized cells, as indicated. In permeabilized cells, the natural and indirect fluorescence patterns were essentially identical, except that small intracellular vesicles were more clearly visible with the GFP endogenous fluorescence, perhaps because they were not fully accessible to the antibodies. In the co-expression with PRAD–GPI, immunofluorescence of non-permeabilized cells clearly showed a membrane-bound component, which was less visible on the background of intracellular GFP, as seen by endogenous fluorescence, or by immunofluorescence of permeabilized cells. The scale bar represents 80 μm.

which is not possible in COS cells. When $AChE_T$ subunits are expressed alone in *Xenopus* oocytes, they produce mainly monomers (G_1^a), with a low proportion of dimers (G_2^a) (Krejci *et al*., 1997), as in COS cells (Bon and Massoulié, 1997). The only difference between the two expression systems is that oocytes did not produce any nonamphiphilic tetramers (G_4^{na}), while COS cells produced a significant proportion of both amphiphilic and nonamphiphilic G_4 forms.

When $AChE_T$ subunits were co-expressed with an N-flagged WAT construct, we observed no modification of the sedimentation profile. In particular, the M1 antibody had no effect on the sedimentation of AChE, indicating the absence of any association of the WAT and $AChE_T$ (not shown).

WAT-containing proteins compete with $AChE_T$ subunits for association with PRAD

When co-expressed with PRAD, the $AChE_T$ subunits were recruited into PRAD-linked tetramers (G_4^{na}). We defined relative quantities of $AChE_T$ and PRAD mRNAs such that nearly all catalytic subunits were included in tetramers, but the amount of PRAD was still limiting. To such a fixed mixture, we added variable amounts of mRNA encoding a WAT domain (Figure 6) or a GFP–WAT construct (Figure 7). We found that this resulted in a decrease in the number of tetramers (G_4^{na}) and an increase in the number of monomers (G_1^a), demonstrating that a WAT domain or a GFP–WAT protein is able to compete with $AChE_T$ subunits for association with the PRAD.

Replacement of individual $AChE_T$ subunits by a WAT-containing element in the complex

Furthermore, we found that these co-expression experiments resulted in the formation of a series of intermediate forms containing $AChE_T$ subunits, sedimenting between the tetramers and the monomers. These molecules were shown to contain the PRAD and the WAT domain or the GFP–WAT, since their sedimentation was shifted by antibodies against a flag epitope that was added to the WAT domain, or against GFP (not shown). Assuming that the masses of these molecules were proportional to $S^{3/2}$, we obtained values that corresponded to mixed PRAD-linked tetramers in which one, two or three catalytic subunits were replaced by the competing element (Table I). Therefore, the PRAD associated with sets of four WAT domains, which could be either isolated (WAT), part of an $AChE_T$ subunit (AChE–WAT) or hooked to the foreign protein GFP (GFP–WAT).

The C-terminal cysteine of the WAT domain is not necessary for association with the PRAD

We compared WAT domains from rat $AChE_T$, from *Electrophorus* $AChE_T$ and from human $BChE_T$ (Figures 6 and 7). In some cases (Figure 2) we mutated the cysteine located at – 4 from the C-terminus to an alanine. We observed identical patterns of mixed complexes with or without a cysteine, in the case of the rat WAT domain (Figure 6A and B) and in the case of the GFP-eWAT construct (not shown). Therefore, intercatenary disulfide bonds are not required for these quaternary interactions.

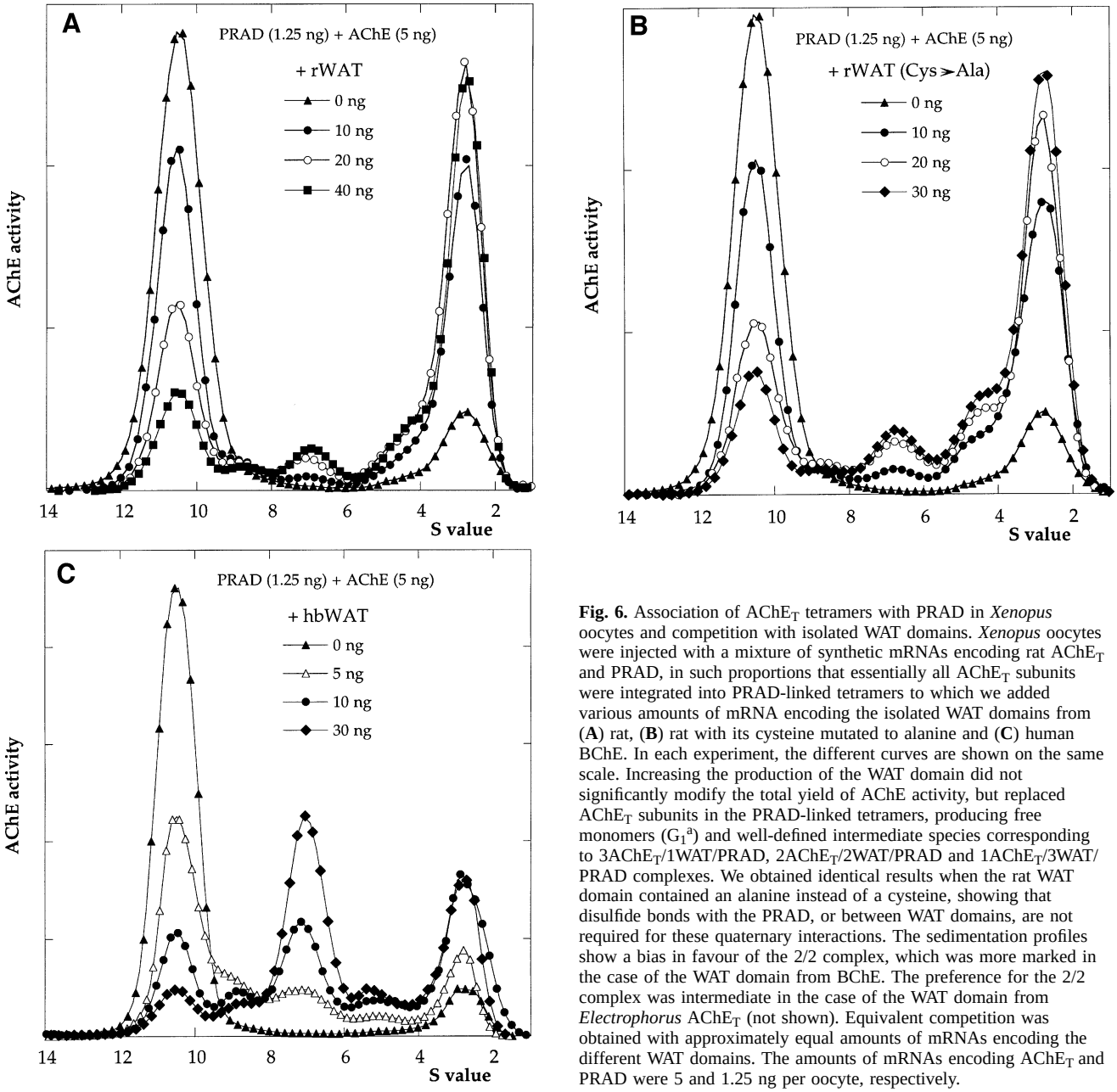

Fig. 6. Association of $AChE_T$ tetramers with PRAD in *Xenopus* oocytes and competition with isolated WAT domains. *Xenopus* oocytes were injected with a mixture of synthetic mRNAs encoding rat $AChE_T$ and PRAD, in such proportions that essentially all $AChE_T$ subunits were integrated into PRAD-linked tetramers to which we added various amounts of mRNA encoding the isolated WAT domains from (**A**) rat, (**B**) rat with its cysteine mutated to alanine and (**C**) human BChE. In each experiment, the different curves are shown on the same scale. Increasing the production of the WAT domain did not significantly modify the total yield of AChE activity, but replaced $AChE_T$ subunits in the PRAD-linked tetramers, producing free monomers (G_1^a) and well-defined intermediate species corresponding to $3AChE_T$/1WAT/PRAD, $2AChE_T$/2WAT/PRAD and $1AChE_T$/3WAT/PRAD complexes. We obtained identical results when the rat WAT domain contained an alanine instead of a cysteine, showing that disulfide bonds with the PRAD, or between WAT domains, are not required for these quaternary interactions. The sedimentation profiles show a bias in favour of the 2/2 complex, which was more marked in the case of the WAT domain from BChE. The preference for the 2/2 complex was intermediate in the case of the WAT domain from *Electrophorus* $AChE_T$ (not shown). Equivalent competition was obtained with approximately equal amounts of mRNAs encoding the different WAT domains. The amounts of mRNAs encoding $AChE_T$ and PRAD were 5 and 1.25 ng per oocyte, respectively.

Quaternary interactions of the catalytic domains introduce a bias in favour of mixed complexes containing two catalytic subunits

The distribution patterns suggested that WAT domains from different origins are not identical, as illustrated in Figure 6 (N-flagged rat WAT, with and without a cysteine, and WAT from human $BChE_T$) and in Figure 7 (GFP with rat WAT and with *Electrophorus* WAT). In all cases, however, we observed a bias in favour of the complex containing two AChE subunits, which was more or less marked depending on the competing molecule. Thus, the relative abundance of the various combinations does not correspond to a random association of PRAD with $AChE_T$, with WAT domains or with GFP–WAT. These structures are extremely stable, since they do not dissociate even at very high dilutions, e.g. during sedimentation in sucrose gradients, so that their distribution does not represent an equilibrium state of dissociation–reassociation, but rather reflects the pathway which led to their assembly; this suggests that $AChE_T$ subunits, WAT domains and GFP–WAT preferentially assemble with the PRAD as dimers of identical elements. The successive binding of two dimers to a PRAD would thus explain the observed distribution of mixed tetramers. It is entirely consistent with previous observations which have shown the existence of PRAD-linked dimers (Bon and Massoulié, 1997). The observed preference for complexes containing two AChE subunits may be due to the contribution of the catalytic domain in the formation of dimers.

Discussion

The WAT domain is sufficient for assembly of a tetramer with a PRAD

Precise targeting and localization of AChE is critical for its physiological function, and this is largely achieved, at

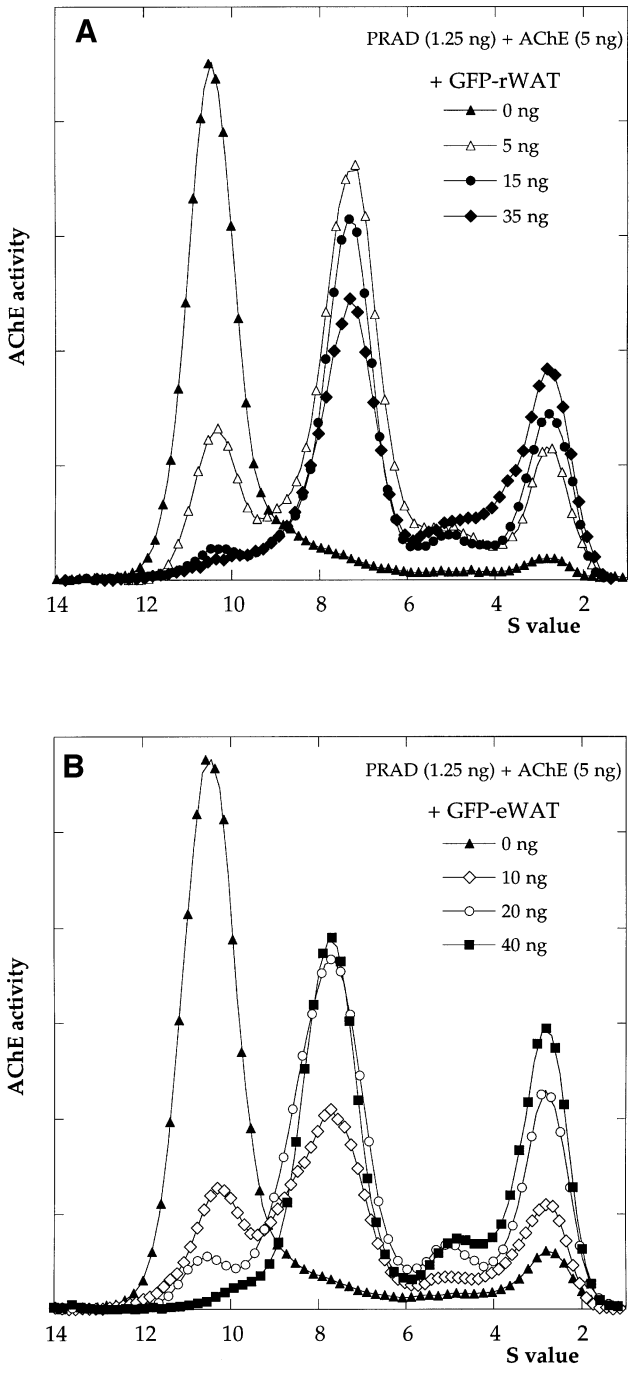

Fig. 7. Competition between $AChE_T$ subunits and GFP containing a WAT domain, for association with PRAD. The experiment is similar to that shown in Figure 6. We illustrate the cases of (**A**) GFP-containing WAT domain from rat AChE and (**B**) WAT domain from *Electrophorus* AChE. Identical results were obtained with the C-terminal cysteine of the *Electrophorus* WAT domain mutated in alanine and with WAT domain of human BChE (not shown). The progressive replacement of $AChE_T$ subunits from their association with the PRAD produced free monomers (and a smaller proportion of dimers), and the mixed complexes $3AChE_T$/1GFP–WAT/PRAD, $2AChE_T$/2GFP–WAT/PRAD and $1AChE_T$/3GFP–WAT/PRAD, which were shifted in the presence of polyclonal anti-GFP antibodies (not shown). As in the case of the isolated WAT domains, the $2AChE_T$/2GFP–WAT/PRAD combination was favoured. The amounts of mRNAs encoding $AChE_T$ and PRAD were the same as in Figure 5.

Table I. Deduction of the masses of the complexes, assuming that their sedimentation coefficients are related to their mass ($M^{2/3}$) with the same proportionality constant

Predicted structure	Predicted mass M_0 (kDa)	Complex S_0 (S)	Deduced mass $[M/M_0 = (S/S_0)^{3/2}]$ (kDa)
4A/PRAD	312	10.5	
3A/1WAT/PRAD	241	8.7–8.9	235–243
2A/2WAT/PRAD	171	6.9–7.2	166–177
1A/3WAT/PRAD	100	4.7–5.2	93–109

These deduced masses show a perfect agreement with theoretical values obtained with 75 kDa for an AChE subunit, 12 kDa for a PRAD and 4.5 kDa for a WAT. In the case of GFP–WAT, the calculated mass values were lower than expected, probably because the frictional ratio of the mixed complex is higher than that of the AChE tetramer.

least in higher vertebrates, by heteromeric associations of $AChE_T$ subunits with anchoring proteins, e.g. the collagen tail. The catalytic subunits are characterized by the presence of a C-terminal domain (WAT). The AChE-associated collagen, ColQ, contains a proline-rich attachment domain, the PRAD. Both WAT and PRAD represent functional domains which are much better conserved throughout vertebrates than the rest of the cholinesterase or ColQ proteins (Figure 2B). This explains that WAT and PRAD domains from species as diverse as *Torpedo* and mammals are able to associate together (Legay *et al*., 1993). We have shown previously that our rat PRAD construct can assemble with four $AChE_T$ subunits, forming a light dimer which does not contain the PRAD and a heavy dimer which does contain it; this shows that one PRAD associates with four catalytic subunits, as in natural collagen-tailed AChE forms (Krejci *et al*., 1997).

The present results demonstrate unambiguously that interactions between the PRAD and WAT domains are sufficient for assembly of a PRAD-linked tetramer. In a tetramer of $AChE_T$ subunits assembled around a PRAD, the quaternary interactions between the catalytic domains therefore appear secondary and even dispensable. This contrasts markedly with the traditional view of such tetramers: biochemical analyses showed that two of the subunits are disulfide-linked to the PRAD, constituting a 'heavy' dimer, while the other two are disulfide-linked to each other, constituting a 'light' dimer. This suggested a possible organization in which the PRAD interacts with only two of the subunits, as illustrated in Figure 8A. We now show that the core of the structure is assembled through direct interactions of the PRAD with four WAT domains, as shown in Figure 8B.

The PRAD assembles with a tetramer of WAT domains: 'stabilizer' or 'organizer'?

The role of the PRAD can be viewed in two ways, depending upon whether it simply stabilizes a tetrameric structure that might also form in its absence, and is therefore only a 'stabilizer', or whether it is essential for the assembly of four WAT domains and therefore acts as an 'organizer'.

Many proteins form homo-tetramers, including alkaline phosphatase. It is currently considered that $AChE_T$ or $BChE_T$ subunits also assemble as homomeric tetramers (Lockridge *et al*., 1987), which may then associate with

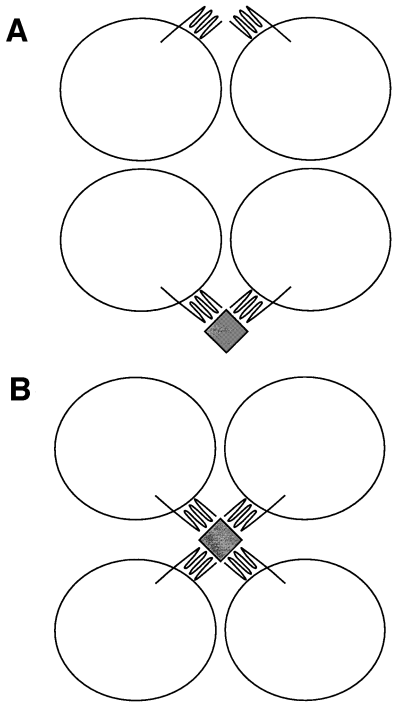

Fig. 8. Models of tetramer assembly. (**A**) Classical view: the PRAD interacts with only one dimer (the heavy dimer) and the association between the light and heavy dimers exclusively reflects quaternary interactions between the catalytic domains. (**B**) Proposed model: a tetramer of WAT domains is organized by the PRAD. Quaternary interactions between the catalytic domains may exist but are not required for this tetrameric assembly. Disulfide bonds may exist between two AChE subunits (light dimer), and between the PRAD and the other two subunits (heavy dimer), but this does not reflect an exclusive interaction of the PRAD with the latter. The PRAD is schematically represented as a shaded square (light grey) and the WAT domains as wavy lines. The intercatenary disulfide bonds that may join two WAT domains to each other or to the PRAD are not shown, because they are dispensable.

anchoring proteins, such as the collagen tail (Rotundo, 1984). In this view, the PRAD would act as a 'stabilizer'.

Our data, however, show that $AChE_T$ subunits are recruited by the PRAD into heteromeric assemblies, which do not form in its absence, as shown here in the case of *Xenopus* oocytes, and as shown previously in mammalian COS and RBL cell lines (Coussen *et al*., 1995; Bon and Massoulié, 1997). Therefore, it rather acts as an 'organizer' for this oligomeric assembly.

In addition, the present results prove unambiguously that the PRAD can organize a tetramer of WAT domains: when added to the C-terminus of a foreign protein such as GFP, WAT domains from various vertebrate AChEs or from human BChE can induce their assembly with a PRAD-containing protein, in a ratio of four to one. Moreover, mixed tetramers could be formed with $AChE_T$ and another protein containing a WAT domain.

Existence of multiple 'organizers'?

In *Xenopus* oocytes, tetramers were not formed under the conditions used in the present experiments. However, at higher levels of expression of rat $AChE_T$, we observed the production of a 13S component in the cells, and of soluble amphiphilic tetramers (G_4^a) in the medium (not shown). A 13S species was also found in COS cells and was easily dissociated into smaller oligomers, including G_4^a (Bon and Massoulié, 1997). This indicates that $AChE_T$ subunits may form homo-oligomers at high concentrations in the biosynthetic compartments.

Thus, $AChE_T$ subunits may produce two types of tetramers, depending on the presence or absence of a PRAD-like organizer. The formation of tetramers without an organizer probably requires a sufficiently high concentration and may not be possible without a C-terminal cysteine (Velan *et al*., 1991) (S.Bon, unpublished result). Mammalian cells, e.g. COS cells, produce both G_4^a and G_4^{na} tetramers (Bon and Massoulié, 1997), possibly corresponding to the two proposed classes, suggesting the presence of an endogenous 'organizer'. This would explain the considerable differences observed in the ratio of G_1 to G_2 and G_4 forms, which seems to exclude a single oligomerization process.

We know that the ColQ gene does not generate the hydrophobic anchor (20 kDa, or P subunit) of mammalian brain $AChE_T$ tetramers (Krejci *et al*., 1997) demonstrating the existence of at least one other 'organizer', distinct from the PRAD. We do not know whether the P subunit contains a proline-rich motif similar to the PRAD, but proline-rich motifs are quite common. It does not seem unlikely that they might exist in the secretory pathway of various types of cell. Considering the present results, such molecules could be assembled without the C-terminal cysteine, in agreement with the fact that mutation of this cysteine prevents the formation of dimers, but not of tetramers (Velan *et al*., 1991; Gough and Randall, 1995).

The presence of an 'organizer' in cholinesterase tetramers could have been missed easily. The organizer might not be disulfide-linked to $AChE_T$ subunits, since cysteines are dispensable both in the PRAD (Bon *et al*., 1997) and in the WAT domain, as shown in the present work. Therefore, electrophoresis in non-reducing conditions would not necessarily reveal the presence of a true dimer and an organizer-linked dimer. In addition, Western blotting under denaturing conditions, or chemical analyses of peptides, might not detect the presence of an organizer, because (i) it would be substoichiometric compared with catalytic subunits, (ii) it might be very small and (iii) it might consist essentially of prolines.

Generality and originality of the PRAD–WAT association?

The PRAD, and possibly other organizers, may interact with several proteins carrying WAT domains or similar motifs. We have shown previously that mammals contain a single ColQ gene, which generates the collagen tails of both AChE and BChE (Krejci *et al*., 1997). Tsim and colleagues have shown that muscles of chick embryos contain mixed collagen-tailed forms containing both AChE and BChE subunits (Tsim *et al*., 1988). The existence of such structures is in perfect agreement with the formation of mixed tetramers in our competition experiments. The possible existence of a variety of proteins containing WAT domains and associating with the PRAD would explain that expression of ColQ is not strictly related to the production of collagen-tailed cholinesterase but is also abundant in the heart, where these molecular forms are scarce, and in a non-cholinergic tissue such as the lung (Krejci *et al*., 1997); it may serve as an extracellular

matrix anchor for other proteins that it will be interesting to identify.

The interaction between the PRAD organizer and the WAT domain could represent the prototype of a very general mode of assembly of protein subunits in the secretory pathway. Although this interaction involves proline-rich domains, it is quite distinct from that of SH3 and WW motifs with their proline-rich ligands (Lim *et al*., 1994; Yu *et al*., 1994; Chan *et al*., 1996) because (i) it occurs in the secretory pathway; (ii) it produces stable, permanent associations; and (iii) it presents an original four-to-one stoichiometry.

The assembly of PRAD and WAT domains may be used for constructing multi-protein assemblies, and targeting and anchoring them in various ways, *in vitro* or *in vivo*. In this report, we have shown only that it was possible to use this system for anchoring proteins at the cell membrane via a GPI anchor. Other possibilities are wide open, including associations with lectins, toxins, antigens or antibodies, creation of multi-enzymatic complexes, etc. This original type of protein assembly thus offers a wide range of potential applications in biotechnology, in addition to its considerable physiological significance.

Materials and methods

Standard methods in molecular biology were used to construct the fusion proteins (Sambrook *et al*., 1989). PCR amplification was performed with the Taq Expand High Fidelity PCR system (Boehringer Mannheim) to limit errors. Primers were synthesized by Genset (Paris, France) or Eurobio (Paris, France). Point mutagenesis and the introduction of the 'flag' epitope (DYKDE) were carried out with specific oligonucleotides using the method of Kunkel *et al*. (1987), using T7 DNA polymerase (New England Biolabs, Ozyme France).

Competitor peptide constructs

For the rat WAT domain, we used the rat $AChE_T$ coding sequence (Legay *et al*., 1993), flanked by the 5′ and 3′ α-globin (Krieg and Melton, 1987), in the pCDNA3 vector (InVitrogen), and deleted the common part of the AChE by PCR with an antisense primer that hybridized onto the nucleotides encoding the first four amino acids of the mature protein (EGRE) and the last nucleotides encoding the signal peptide and that contained the *Nhe*I site at its 3′ end, and with a sense primer that hybridized with the 5′ extremity of the WAT domain and contained the *Nhe*I site at its 5′ end. The amplified fragment was then cut with *Nhe*I, ligated and transformed in *Escherichia coli*. The residues EGREAS connect the leader sequence and the WAT domain.

WAT domain from human BChE (McTiernan *et al*., 1987): the WAT domain was amplified by PCR, using a sense oligonucleotide containing a *Nhe*I restriction site at its 5′ end, and an antisense primer in the vector. The PCR product ends were blunt-ended with the Klenow fragment (USB Amersham) then phosphorylated with T_4 polynucleotide kinase (Biolabs), and digested with *Nhe*I restriction enzyme. The final product was ligated in pCDNA3 in place of rat WAT in-frame with the remaining signal peptide, using the *Nhe*I and *Eco*RV restriction sites, giving the construct hbWAT (Figure 2). This construct possesses six amino acids connecting the signal peptide and the WAT domain from human BChE (EGREAS).

For construction of GFP–WAT, the cDNA encoding a mutant of GFP (phGFP-S65T, ref. 6088-1, Clontech) was subcloned in the pCDNA3 vector at *Hin*dIII and *Xba*I restriction sites.

For construction of GFP–rWAT (with the rat WAT domain), GFP was amplified by PCR with oligonucleotides containing the *Nhe*I site at their extremities. The PCR product was cut with *Nhe*I and ligated at the *Nhe*I site in the rat WAT construct. The residues EGREAS connect the leader sequence and the GFP, and AS connect GFP and the rat WAT domain.

For construction of GFP–hbWAT (with the human butyrylcholinesterase WAT domain), the construct hbWAT was used to make GFP–hbWAT by insertion of the GFP flanked by the *Nhe*I sites from GFP–rWAT. The six amino acids, EGREAS, connect the signal peptide and GFP, and the two amino acids AS (containing the *Nhe*I site in the nucleotidic sequence) connect GFP and BChE WAT domain.

For construction of GFP–eWAT (with the *Electrophorus* WAT domain), using PCR, we added the signal peptide sequence and the first two amino acids of *Electrophorus electricus* AChE to the N-terminus of GFP, and the WAT domain of *E.electricus* AChE (eWAT) to the C-terminus (see Figure 2).

For construction of hAP–eWAT, the WAT domain of *E.electricus* AChE (eWAT) was added by PCR at the C-terminus of human alkaline phosphatase (hAP) in place of the hydrophobic sequence responsible for the GPI anchoring.

Using directed mutagenesis (Kunkel *et al*., 1987) (Figure 2), we fused the signal sequence of *Electrophorus* AChE with its WAT domain (eWAT). We deleted added residues and replaced them with a 'flag' epitope sequence (DYKDE) (Knappik and Plückthun, 1994) between the signal peptide and the rat or *Electrophorus* WAT domain (rWAT and eWAT) or between the signal peptide and hAP. We also replaced the cysteine of the WAT domain by an alanine in the GFP–eWAT [GFP–eWAT(Cys→Ala)] and in the rat WAT domain [rWAT(Cys→Ala)] constructs.

Expression in Xenopus oocytes

TST7 plasmids (Krieg and Melton, 1987) containing rat $AChE_T$ ($rAChE_T$, Figure 2) and PRAD (rQ_R) (Krejci *et al*., 1997) coding sequences between the 5′ and 3′ untranslated sequences of *Xenopus* globin, as well as competitors in pCDNA3, were used for expression in oocytes. Synthetic transcripts were prepared with the 'Ambion mMESSAGE mMACHINE^{TM} *In vitro* Transcription kit'. *Xenopus* oocytes were injected with samples of ~50 nl (5 ng of $rAChE_T$ mRNA, 1.25 ng of PRAD mRNA and varying amounts of competitors, from 5 to 40 ng). Analysis of molecular forms was performed 1 day after injection.

Extraction and purification of AChE

The injected *Xenopus* oocytes were homogenized by repeated pipetting in 50 mM Tris–HCl pH 7.5, 50 mM $MgCl_2$, 0.5% Triton X-100, 0.01 mg/ml bacitracin (10 µl per oocyte) at 4°C. The homogenate was centrifuged for 10 min at 4°C at 13 000 *g*. AChE activity of the supernatant was assayed by the colorimetric method of Ellman *et al*. (1961).

Sedimentation analyses

If necessary, injected *Xenopus* oocyte extracts were incubated with 1:25 of anti-'flag' M1 antibody (Eastman-Kodak) with 5 mM $CaCl_2$ or with 1:25 of polyclonal anti-GFP antibodies (Clontech), for 3 h at 4°C, prior to sedimentation analyses.

Centrifugation in 5–20% sucrose gradients, in 50 mM Tris–HCl pH 7.5, 50 mM $MgCl_2$, 1% Brij-96, 0.01 mg/ml bacitracin (and 5 mM $CaCl_2$ when incubated with M1 antibody) was performed in a Beckman SW41 rotor at 40 000 r.p.m. for 16 h at 5°C. β-galactosidase (16S) and alkaline phosphatase (6.1S), or rat $AChE_T$ nonamphiphilic tetramers (10.5S) and amphiphilic monomers (2.8S), were used as internal sedimentation standards. Gradients were collected from the bottoms of the tubes and distributed in microtitration plates into about 96 fractions of ~150 µl. AChE activity was assayed using 50 µl of each fraction.

Transfection of COS cells and immunofluorescence

Plasmidic DNA was prepared with the Nucleobond plasmid purification kit (Macherey-Nagel). Cells were plated onto polyornithine-coated coverslips (15 mg/ml) 1 day before transfections, which were performed with 1 µg of each DNA per 3 cm dish containing about 10^5 cells, using the DEAE–dextran method as described previously (Duval *et al*., 1992b). After 3 h of incubation with DNA–DEAE–dextran diluted in Nu-serum, cells were rinsed with phosphate-buffered saline (PBS), incubated in 10% fetal calf serum (FCS)-decomplemented serum for 72 h at 37°C, and then maintained for 18 h at 30°C (to achieve the folding of GFP). After fixation of cells with 4% paraformaldehyde for 10 min at room temperature, cells were incubated for 15 min at room temperature in Tris-buffered saline (TBS) buffer containing 1 mM $CaCl_2$, 0.2% BSA, with or without 0.05% saponin. Cells transfected with rat $AChE_T$, or constructs flag–eWAT or GFP–eWAT, with or without PRAD–GPI (Duval *et al*., 1992a; Bon and Massoulié, 1997), were incubated for 1 h at room temperature with monoclonal anti-rat AChE antibody (ZR3) (Rackonczay and Brimijoin, 1986), monoclonal M1 antibody or polyclonal anti-GFP antibodies, respectively. Antibodies were diluted at 1:400 in the preceding buffer, with or without saponin. Cells were then washed three times in the buffer without saponin and incubated for 45 min at room temperature in the dark with rhodamine-conjugated donkey anti-rabbit antibody for anti-GFP, rhodamine and fluoresceine-conjugated donkey anti-mouse

antibody for ZR3 and M1, respectively. Cells were washed three times in TBS buffer and rinsed in water, and dried coverslips were fixed onto slides with Mowiol.

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