Soluble, Oligomeric, and Ligand-binding Extracellular Domain of the Human α7 Acetylcholine Receptor Expressed in Yeast

REPLACEMENT OF THE HYDROPHOBIC CYSTEINE LOOP BY THE HYDROPHILIC LOOP OF THE ACh-BINDING PROTEIN ENHANCES PROTEIN SOLUBILITY*

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The N-terminal extracellular domain (ECD; amino acids 1–208) of the neuronal nicotinic acetylcholine receptor (AChR) α7 subunit, the only human AChR subunit known to assemble as a homopentamer, was expressed as a glycosylated form in the yeast Pichia pastoris in order to obtain a native-like model of the extracellular part of an intact pentameric nicotinic AChR. This molecule, α7-ECD, although able to bind the specific ligand α-bungarotoxin, existed mainly in the form of microaggregates. Substitution of Cys-116 in the α7-ECD with serine led to a decrease in microaggregate size. A second mutant form, α7-ECD(C116S,Cys-loop), was generated in which, in addition to the C116S mutation, the hydrophobic Cys-loop (Cys128–Cys142) was replaced by the corresponding hydrophilic Cys-loop from the snail glial cell acetylcholine-binding protein. This second mutant protein was water-soluble, expressed at a moderate level (0.5 ± 0.1 mg/liter), and had a size corresponding approximately to a pentamer as judged by gel filtration and electron microscopy studies. It also bound 125I-α-bungarotoxin with relatively high affinity (Kd = 57 nM), the binding being inhibited by unlabeled α-bungarotoxin, d-tubocurarine, or nicotine (Kᵢ = 0.8 × 10⁻⁷ M, Kᵢ = 1 × 10⁻⁵ M, and Kᵢ = 0.9 × 10⁻² M, respectively). All three constructs were expressed as glycosylated forms, but in vitro deglycosylation reduced the heterogeneity without affecting their ligand binding properties. These results show that α7-ECD(C116S,Cys-loop) was expressed in P. pastoris as an oligomer (probably a pentamer) with a near native conformation and that its deglycosylated form seems to be suitable starting material for structural studies on the ligand-binding domain of a neurotransmitter receptor.

Nicotinic acetylcholine receptors (AChRs)¹ are the prototypic members of the Cys-loop LGIC superfamily, which also includes serotonin (5-hydroxytryptamine 3), glycine, γ-aminobutyric acid type A, and γ-aminobutyric acid type C receptors (1).

These membrane glycoproteins, which mediate rapid chemical synaptic transmission, form either homo- or heteropentamers made up of homologous subunits that show significant similarities in amino acid sequence, transmembrane topology, and overall secondary, tertiary, and quaternary structure, implying a common evolutionary origin (2, 3).

AChRs, which are classified into muscle and neuronal types, are the best characterized members of the LGIC superfamily. Muscle-type AChRs, found at the vertebrate neuromuscular junction and in fish electric organ, have a fixed stoichiometry ((α1)₂β1δ in Torpedo and embryonic mammalian AChR or (α1)₂β1δ in adult mammalian AChR), mediate neuromuscular transmission, and are implicated in the autoimmune disease myasthenia gravis. Neuronal AChRs, widely distributed in the pre-, post-, and peri-synaptic nerve terminals of the central and peripheral nervous system (4, 5), exist either as heteropentamers containing two or three α-subunits (α2–6) or 2 or 3 β-subunits (β2–4) or as homopentamers (α7–9), with α7 being the only human subunit known to form a homopentamer (5). Neuronal AChRs play key roles in various neuron-neuron interactions and are therefore involved in many functions (reviewed in Ref. 5) such as ganglionic transmission, modulation of the release of various neurotransmitters (γ-aminobutyric acid, acetylcholine, serotonin, glutamate, dopamine, and noradrenaline) (6), attention, learning, memory consolidation, arousal, sensory perception, the control of locomotor activity, pain perception, and body temperature regulation (7). They are thus implicated in a number of serious neurological disorders including Alzheimer’s disease, Parkinson’s disease, schizophrenia, depression, autism, and forms of epilepsy as well as nicotine addiction (8). Because of this, neuronal AChRs have attracted much recent attention.

The thorough understanding of AChR function and its manipulation for therapeutic approaches requires the elucidation of its structure at high resolution. Currently available structural information on AChRs has been mainly derived from elegant cryo-electron microscopy studies on two-dimensional crystals of Torpedo AChR, which can be purified in large amounts. Such studies on membrane-bound Torpedo AChRs have recently provided the 4.6- and 4-Å resolution structures, respectively, of the extracellular and transmembrane domains of the AChR (9–11). However, no x-ray structure of any AChR or any other LGIC has yet been obtained, although very small, non-diffracting three-dimensional crystals of Torpedo AChR have been reported (12, 13). Attempts by several investigators to obtain diffraction quality three-dimensional crystals of this large membrane protein have met with little success.

The use of hydrophilic AChR polypeptide fragments, rather than full-length subunits, seems to be a more realistic ap-
proach to the expression of and structural studies on the AChR. The ECDs of AChR subunits, corresponding to the N-terminal −210 amino acids of the subunits (14), are involved in ligand binding and are therefore of high importance. The ECD of α-subunits bears the major loops contributing to the binding sites for agonists and competitive antagonists (15), whereas loops from adjacent subunits also contribute to ligand binding (reviewed in Ref. 16). A functional α7 5-hydroxytryptamine 3 receptor chimera consisting of the α7 AChR ECD fused to the 5-hydroxytryptamine 3 receptor transmembrane and cytoplasmic domains (17) displayed typical α7 AChR pharmacology, showing the following: (a) that the N-terminal ECD and a complementary C-terminal domain can fold autonomously; and (b) that the α7-ECD contains all of the structural elements contributing to the neurotransmitter binding site. High-resolution structural analysis has been performed on the mulluscain glial cell AChBP (18), a structural and functional homologue of the ECD of LGIC subunits. The AChBP forms a stable homopentamer and has 24% sequence similarity with human α7-ECD and less similarity with ECDs from all the subunits of other nicotinic receptors (20–24%) or other LGICs (15–18%). However, the high-resolution structure of a LGIC ECD is not yet available. The ECD of the α7 subunit, which is known to form homopentamers (19, 20), seems to offer the most promising approach to the problem because it could provide a model of the whole (pentameric) ECD of an AChR. Several investigators have expressed α7-ECDs (21–24), either alone or fused to another (soluble) protein; however, the recombinant proteins, although soluble, were produced either in the form of microaggregates or in minute amounts and were thus unsuitable for x-ray studies.

In this study we expressed the human α7-ECD as a glycosylated form in the yeast P. pastoris (25, 26), which has previously been used successfully in our laboratory to produce a glycosylated, soluble, and ligand- and antibody-binding human α1 ECD (27). The recombinant α7-ECD was found to be mainly in the form of microaggregates. By replacing the normally unpaired Cys-116 with serine and exchanging the hydrophobic Cys<sup>249</sup>–Cys<sup>302</sup> loop with the corresponding hydrophilic Cys-loop of the AChBP, we obtained a protein, α7-ECD/C116S,Cys-loop), which was produced in quantities of ~0.5 mg per liter of culture, was water-soluble, and existed as an oligomer in the form of microaggregates. By replacing the normally unpaired Cys-116 with serine and exchanging the hydrophobic Cys<sup>249</sup>–Cys<sup>302</sup> loop with the corresponding hydrophilic Cys-loop of the AChBP, we obtained a protein, α7-ECD/C116S,Cys-loop), which was produced in quantities of ~0.5 mg per liter of culture, was water-soluble, and existed as an oligomer in the form of microaggregates. By replacing the normally unpaired Cys-116 with serine and exchanging the hydrophobic Cys<sup>249</sup>–Cys<sup>302</sup> loop with the corresponding hydrophilic Cys-loop of the AChBP, we obtained a protein, α7-ECD/C116S,Cys-loop), which was produced in quantities of ~0.5 mg per liter of culture, was water-soluble, and existed as an oligomer in the form of microaggregates. By replacing the normally unpaired Cys-116 with serine and exchanging the hydrophobic Cys<sup>249</sup>–Cys<sup>302</sup> loop with the corresponding hydrophilic Cys-loop of the AChBP, we obtained a protein, α7-ECD/C116S,Cys-loop), which was produced in quantities of ~0.5 mg per liter of culture, was water-soluble, and existed as an oligomer in the form of microaggregates. By replacing the normally unpaired Cys-116 with serine and exchanging the hydrophobic Cys<sup>249</sup>–Cys<sup>302</sup> loop with the corresponding hydrophilic Cys-loop of the AChBP, we obtained a protein, α7-ECD/C116S,Cys-loop), which was produced in quantities of ~0.5 mg per liter of culture, was water-soluble, and existed as an oligomer in the form of microaggregates.

EXPERIMENTAL PROCEDURES

**Constructions for Soluble Expression in the Yeast P. pastoris**

pPICZαA-α7-ECD—DNA coding for the N-terminal ECD of the human nicotinic AChR α7 subunit (amino acid residues 1–208) was enzymatically amplified by PCR from a full-length α7 cDNA clone (kindly provided by Dr. J. Lindstrom) (Fig. 1A). The upstream (5′-TTTATTC-CCCGGGGCGAGTCCCG-3′) and downstream (5′-GCCATAGTCCGTCCGCGG-3′) primers were constructed to contain SmaI and XbaI restriction sites (underlined), respectively. Using appropriate restriction endonucleases, the purified PCR product of 648 bp was subcloned into the EcoRI/XbaI sites of the expression vector pPICZαA (Invitrogen) (Fig. 1B, section a).

pPICZαA-α7-ECD/C116S—The 59-base 5′-AATTCTTCTGCGGCGACCTCGCCAGTCATTACCTGGTCTCAGAT-3′ and 5′-CAGTTGAAGGCTACGGGGTCACTGGAATGATATACCATGCTGAGTGGAGC-3′ primers were designed to contain Smal and XbaI restriction sites (underlined), respectively. Using appropriate restriction endonucleases, the purified PCR product of 648 bp was subcloned into the EcoRI/XbaI sites of the expression vector pPICZαA (Invitrogen) (Fig. 1B, section a).

**Protein Expression and Purification**

**Protein Expression in P. pastoris**—In pilot experiments, single colonies of transformed cells were used to inoculate 2.5 ml of BMGY medium (1%/v/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 7.0, 1.34% (w/v) yeast nitrogen base, 4 × 10<sup>−5</sup> % (w/v) thiamine hydrochloride, 1% (w/v) glucose, and 1% (w/v) glycerol. After 16–20 h of incubation at 30 °C, the cells were resuspended in 5 ml of BMGY medium (BMGY medium in which the glycerol was replaced by 0.5% (v/v) methanol) to induce expression. Over the next 4 days, induction was maintained with the daily addition of 0.5% (v/v) methanol, and the culture supernatants were tested for expression of the recombinant proteins by dot-blot analysis using the anti-Myc mAb 9E.10 (American Type Culture Collection). The clone with the highest protein yield was selected for medium scale protein expression in 2-liter flasks. In all subsequent preparations the cells were harvested at ~24 h of methanol induction, as very little increase in the yield for any of the three products was seen after longer induction periods.

**First Stage Protein Purification**—The culture supernatant was concentrated 10-fold by the addition of solid NH<sub>4</sub>SO<sub>4</sub> to 60% saturation in the presence of 0.05% (w/v) Na<sub>2</sub>SO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaF followed by overnight precipitation at 4 °C and dissolution of the pellet in the appropriate volume of distilled H<sub>2</sub>O. The concentrated protein solution was dialyzed against 50 mM PB, pH 8, and the recombinant protein was purified by metal affinity chromatography on Ni<sup>2+</sup>-NTA (Qiagen) according to the manufacturer’s instructions. Elution was performed stepwise under native conditions with 50 mM PB, pH 8, containing 0.5 mM NaCl and 50, 100, 150, or 1000 mM imidazole. The eluates were analyzed by 12% SDS-PAGE followed by Coomassie Brilliant Blue staining or Western blot analysis using either the anti-Myc mAb 9E.10 or rabbit polyclonal anti-α7 antibodies. Anti-α7 antibodies were produced by Dr. Leslie Jacobson in our laboratory by immunizing rabbits with either α7 synthetic peptide 176–212 (anti-α7 peptide polyclonal antibody) or Escherichia coli-expressed α7-ECD—(1–209) (anti-bacterial α7-ECD polyclonal antibody). The 100 mM imidazole eluate was used for in vitro glycosylation as described.

**Gel Filtration Chromatographic Analysis and Second Stage Purification**—After high speed centrifugation (1 min at 13,000 rpm) and filtration through a 2.2-μm filter (Millipore), 1 ml samples of the Ni<sup>2+</sup>-purified α7-ECDs were analyzed on an AKTA 90 purifier system (Amersham Biosciences) using a 24-ml Superose 12 column (Amersham Pharmacia) and an isocratic elution with 100 mM Na<sub>2</sub>SO<sub>4</sub>, 50 mM PB, pH 8, and a flow rate of 0.5 ml/min. 0.5 ml fractions were collected and analyzed for 125I-Bgt binding. The protein concentration of the gel-filtered material was determined spectrophotometrically based on the absorbance at 280 nm (A<sub>280</sub>) and the protein content in aromatic residues (Trp and Tyr) and

It appears that the text is cut off and not entirely legible. The content seems to be about the expression and characterization of the α7 nicotinic acetylcholine receptor (nAChR) and its extracellular domain (ECD) in Saccharomyces cerevisiae (S. cerevisiae). The text discusses the expression of α7-ECD and its purification using yeast and mammalian expression systems. The purification process involves steps such as protein expression, affinity chromatography, and gel filtration. The protein is then analyzed for its biochemical properties and concentration. The text seems to be a scientific report, likely from a scientific journal or a research conference.
domains (M4 cysteine residues, and the approximate ligands such as nicotine, carbamylcholine, shown on the (40 acetylcholine, methyllycaconitine, or gallamine, only gel-filtered samples were incubated with 50 ng of recombinant protein at 4 °C with 500 units of 

The C-terminal it was fused to a c-Myc epitope and a His6 residue tag.

dimerase (transcriptional control of the alcohol oxidase (AOX) promoter, which is cleaved by host enzymes (inverted triangle (W) demonstrates the cleavage site), whereas at the C-terminal it was fused to a c-Myc epitope and a His6 residue tag.

Filter Assays for Ligand Binding to a7-ECDs

Various amounts of Ni2+-purified or gel filter-purified protein were incubated with 50,000 cpm 125I-a-Bgt at 4 °C, either for 1.5 h (a7-ECD(C116S,Cys-loop)) or overnight (a7-ECD or a7-ECD(C116S)) in a final volume of 50 μl of PB-BSA (10 mM phosphate buffer, pH 7.4, containing 0.2% BSA). The samples were then diluted in 1 ml of Tris-Triton X-100 buffer and filtered immediately through two Whatman DE81 filters presoaked with Tris-Triton X-100 buffer; they were then washed twice with 1 ml of Tris-Triton X-100 buffer, and the bound radioactivity was measured on a γ-counter. Samples without recombinant ECDs were used to measure non-specific binding.

In Vitro Deglycosylation—Approximately 100 μg of Ni2+-NTA purified a7-ECDs was deglycosylated by the addition of 500 units of N-glycosidase F (peptide:N-glycosidase F; New England Biolabs) under native conditions without heating, denaturation, or the addition of any detergent. The reaction was performed in 50 mM PB, pH 7.5 (final volume 400 μl), at 4 °C (a much lower temperature than that suggested by the manufacturer) and incubated overnight (although the reaction was completed within 1–2 h). The resulting deglycosylated protein was detected on Western blots using anti-Myc mAb 9E.10.

Electron Microscopic Analysis—A colloidin film bearing copper grids (400 mesh; TAAB Laboratories Equipment Ltd.) was coated with a thin carbon layer and glow-discharged for 2 min prior to use. Reombinant protein gel-filtered samples (1 μg/μl) were added to the carbon-coated grids and embedded in 2% (w/v) uranyl acetate as negative stain. Images were taken at 40,000× magnification using a Philips 208 electron microscope operating at 80 kV (30).

RESULTS

Expression and Study of the a7-ECD—Reombinant a7-ECD was expressed as a soluble protein in the P. pastoris culture supernatant. This protein was concentrated by ammonium sulfate precipitation and dialyzed against 50 mM PB, pH 8.0; then a7-ECDs was purified by affinity chromatography on a Ni2+-NTA column using the His6 tag fused to its C terminus (Fig. 1C). Approximately 60% of the recombinant protein was eluted in the 100 mM imidazole fraction, and another 30% was eluted in the 150 mM imidazole fraction as judged by SDS-PAGE followed by staining with Coomassie Brilliant Blue (Fig. 2A, lanes 1 and 2) and Western blot using the anti-Myc mAb (Fig. 2B, lanes 1 and 2) and confirmed by 125I-a-Bgt binding experiments (data not shown). The eluted protein was not pure, with the main contaminants being two bands of ~40 and ~70 kDa (see Fig. 2A, lanes 1 and 2). The protein was further identified in Western blots by the anti-α7 peptide polyclonal antibody and the anti-bacterial α7-ECD polyclonal antibody. The purified a7-ECD was able to bind 125I-a-Bgt in solution in filter assay experiments and was blocked by unlabeled a-Bgt or d-tubocurarine (data not shown).

The molecular mass of the product estimated by SDS-PAGE (Fig. 2, A and B, lanes 1 and 2) was ~36–37 kDa, much higher
than the value of 27.3 kDa predicted from its amino acid sequence. This difference was shown to be due to glycosylation, because in vitro deglycosylation of the Ni\(^{2+}\)-purified protein with N-glycosidase (peptide-N-glycanase) reduced the apparent molecular mass on SDS-polyacrylamide gels to approximately that expected as shown by Western blot analysis (Fig. 3A, left pair of lanes). This shift indicates that, like the native \(\alpha7\) AChR, the recombinant protein was produced as a glycosylated form. The deglycosylated bands also appeared to be sharper, probably due to elimination of various glycosylated forms with different molecular masses. When the glycosylated and in vitro deglycosylated forms were tested for 125I-7-ECD-Bgt binding, no significant difference was seen in their binding efficiencies (Fig. 3B, left pair of bars).

The oligomeric state and size of the recombinant protein in detergent-free solution (10 mM PB, pH 7.5) were studied by gel filtration analysis on a Superose 12 column, the fractions being assayed for 125I-\(\alpha7\)-Bgt binding. The bulk of the protein was eluted close to the column void volume, indicating the presence of high molecular mass aggregates (Fig. 4A). In fact, precipitation of the protein usually occurred after 1–2 days of storage at 4 °C in imidazole-containing 50 mM PB and 0.5 mM NaCl. This shift indicates that, like the native \(\alpha7\) AChR, the recombinant protein was produced as a glycosylated form. The deglycosylated bands also appeared to be sharper, probably due to elimination of various glycosylated forms with different molecular masses. When the glycosylated and in vitro deglycosylated forms were tested for 125I-\(\alpha7\)-Bgt binding, no significant difference was seen in their binding efficiencies (Fig. 3B, left pair of bars).

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The elution pattern was identical at concentrations of the α7-ECD(C116S,Cys-loop) up to at least 7 mg/ml. No monomeric form was detected.

Binding of 125I-α-Bgt and Cholinergic Ligands to Purified α7-ECD(C116S,Cys-loop)—The ability of the three gel filtration-purified α7-ECDs to bind 125I-α-Bgt in solution was tested by filter assay experiments as an indication of correct protein folding (Fig. 5). Although all were able to bind this specific ligand, the α7-ECD(C116S,Cys-loop) showed a much higher binding capacity. Moreover, 125I-α-Bgt binding to α7-ECD(C116S,Cys-loop) reached saturation at 45 min of incubation at 4 °C, whereas for the other two constructs overnight incubation with 125I-α-Bgt was required to achieve saturation (not shown). The Kᵢ for the gel-filtered double mutant, estimated by Scatchard analysis, was 57 nM (Fig. 6A).

The specificity of 125I-α-Bgt binding to gel-filtered α7-ECDs was demonstrated by competition experiments with various concentrations (1 nM–10 μM) of unlabeled α-Bgt (Fig. 6B). In the case of the α7-ECD(C116S,Cys-loop) for which Kᵢ was estimated, the results of competition experiments (Kᵢ = 1.5 × 10⁻⁷ M) were consistent with the Kᵢ estimated from Scatchard plot (Kᵢ = 57 nM).

To further test the native-like conformation of the α7-ECD(C116S,Cys-loop), several known cholinergic ligands were tested in competition experiments for inhibition of 125I-α-Bgt binding to the gel-filtered protein. As shown in Fig. 6B, the competitive antagonist d-tubocurarine had an IC₅₀ of 3 × 10⁻⁵ M (Kᵢ = 3 × 10⁻⁵ M). Interestingly, the agonist nicotine also inhibited 125I-α-Bgt binding, although with a much lower affinity (Kᵢ = 1 × 10⁻² M). The muscle AchR-specific antagonist gallamine had no effect on 125I-α-Bgt binding. The lack of effect of this ligand, even at high concentrations, shows that the inhibition caused by the other ligands was specific and that the ligand-binding site retains, at least to some extent, its α7 subtype specificity. However, no inhibition of 125I-α-Bgt binding was seen using the AchR ligands carbachol, epibatidine, acetylcholine, or methyllycaconitine (data not shown).

Electron Microscopic Evidence for Oligomeric Assembly of ECD Complexes—Electron microscopy of samples in negative stain was used to monitor the presence of complexes formed by the ECDs (kindly performed by Dr. N. Unwin, Cambridge, UK). The glycosylated and deglycosylated α7-ECD(C116S) was rather in the form of aggregates much larger than pentamers. The deglycosylated α7-ECD(C116S,Cys-loop) sample was quite homogeneous, consisting of oligomeric complexes resembling in size and shape the negative stained images of the native AchR (31) and the assembly of four muscle AchR subunit ECDs heterologously expressed in a baculovirus system (30). The characteristic donut shape could be seen, although not very clearly. The complexes showed a preferential "end-on" orientation, although side views of the ECD pentamers could also be seen (Fig. 7).

DISCUSSION

Nicotinic AchRs are the best studied members in the LGIC superfamily. In depth knowledge of their structure would facilitate the understanding of their function and the design of subtype-specific drugs. Despite numerous biochemical, biophysical, and cryo-electron microscopy studies, no high-resolution structure of these proteins is available. Even when sufficient protein amounts can be isolated, as in the case of the Torpedo AchR, crystallization of such large membrane proteins seems to be a difficult task mainly because of the large transmembrane (hydrophobic) region. The production of their N-terminal domains in heterologous expression systems appears to be a more
realistic approach toward crystallization and high-resolution crystallographic studies, because these domains are extracellular and are therefore expected to be hydrophilic and, thus, water-soluble. Recombinant α7-ECD production would be appropriate for obtaining an oligomeric AChR ECD form.

Several attempts have been made to express α7-ECDs from different species in both prokaryotic and eukaryotic expression systems. Expression of chick α7-ECD in Xenopus oocytes resulted in a water-soluble (at least at the low concentrations tested) ligand-binding protein of a size probably corresponding to pentamers, but the protein was produced in only minute amounts (21). When sufficient protein is produced, as in the case of rat α7-ECD expressed in bacteria as a fusion protein with maltose-binding protein (22) or glutathione S-transferase (24), the main problem seems to be the formation of high molecular mass aggregates rather than monomers or small oligomers, probably due to incomplete folding of the protein and exposed hydrophobic regions.

In the present study, we used the P. pastoris eukaryotic system to express human α7-ECD. This system has been successfully used in our laboratory to express the human muscle AChR α1-ECD in a soluble, ligand-binding monomeric form (27). However, expression of human α7-ECD in this same yeast system resulted in the recombinant protein being produced mainly in the form of microaggregates, which, however, could bind 125I-α-Bgt somewhat better than did unmodified α7-ECD (Fig. 5), and gel filtration chromatography showed a shift of the elution peak to fractions corresponding to lower molecular masses, which were, however, still mainly larger than pentamers. However, a small fragment eluted in fractions corresponding approximately to the size expected for the pentamer.

Construction of the second mutation involved the amino acids of the Cys-loop (Cys128–Cys142) in addition to the C116S point mutation. In pentameric LGICs, this Cys-loop is well conserved and mainly hydrophobic (CYIDVRWFPPDVHQHC in human α7), whereas in AChBP (the only LGIC-related protein yet crystallized) it is more hydrophilic (CDVSQVDTESGATC). The crystal structure of the AChBP suggests that the Cys loop is located at the bottom of the ECD, close to the C terminus of this domain. This implies that the corresponding hydrophobic region in the LGICs could interact with the membrane or the transmembrane region of these receptors. Thus, it may be mainly buried in intact proteins but exposed in free ECDs and be a source of microaggregate formation. In fact, the Torpedo

![Fig. 6. α7-ECD(C116S,Cys-loop) affinity for 125I-α-Bgt and unlabeled ligands. A, Scatchard plot analysis of the binding to 125I-α-Bgt. The Kd for 125I-α-Bgt binding to the α7-ECD(C116S,Cys-loop) was estimated to be 57 nM. B, inhibition of 125I-α-Bgt-binding by unlabeled ligands. The inhibition of 125I-α-Bgt binding to the α7-ECD(C116S,Cys-loop) was studied in competition experiments by adding various concentrations of the unlabeled ligands α-Bgt (*), d-tubocurarine (●), nicotine (▲), or the muscle AChR-specific ligand gallamine (□) to 50 ng of α7-ECD(C116S,Cys-loop) as described under "Experimental Procedures." The %cpm bound in the absence of ligand was taken as 100% binding. C, inhibition of initial rate of 125I-α-Bgt-binding by unlabeled ligands, i.e. a 10-min incubation with 125I-α-Bgt. Other conditions and ligand symbols are the same as those described for panel B.](https://www.jbc.org/content/283/11/38292/F6)

![Fig. 7. Negative-stain electron microscopic image of deglycosylated α7-ECD(C116S,Cys-loop). The vertical arrowhead indicates an end-on view, and the horizontal arrowhead points to a side view of the ECD oligomers.](https://www.jbc.org/content/283/11/38292/F7)
Soluble and Oligomeric Human AChR α7 Extragludomain

In conclusion, we here present evidence that the conserved hydrophobic amino acids in the C125–C145 loop prevent α7-ECD from being water-soluble when expressed alone as a recombinant protein in heterologous expression systems. The replacement of this hydrophobic loop by the corresponding hydrophilic loop of the AChBP dramatically increases the solubility of the recombinant molecule and results in a molecule existing mainly in the form of an oligomer, probably a pentamer. This mutated recombinant human ECD is expressed in *P. pastoris* in satisfactory amounts and seems suitable as a model for structural studies of a functional LGIC, preferably after deglycosylation.

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...the Cys-loop in ECD solubility. Hoping to obtain a more soluble protein, we therefore constructed a mutant in which, in addition to the C116S mutation, the α7 hydrophobic Cys loop (15 amino acids) was replaced by the corresponding hydrophilic AChBP loop (14 amino acids). These amino acid residues do not seem to contribute to the α-Bgt-binding site (34).

This double mutant was expressed at higher levels than were the previous two molecules and was able to bind α-Bgt with higher efficiency. Its *Kd* for α-Bgt binding was estimated to be 57 nM (Fig. 6). This *Kd* value corresponds to an affinity ~44 times higher than that of the rat α7-ECD expressed in *E. coli* as a soluble fusion protein (*Kd* = 2.5 μM) (22) and only 26 times lower than that of the native α7 AChR (*Kd* = 1.9 nM) (35). The fact that the α7-ECD(C116S,Cys-loop) was able to bind the agonist nicotine, even if with low affinity, is a strong indication for the presence of adjacent subunits, possibly generating the correct form of an AChR, i.e. a pentamer. Gel filtration chromatography showed that its size corresponded approximately to that of a pentamer. Further confirmation was obtained from electron microscopy analysis of deglycosylated gel-filtered α7-ECD(C116S,Cys-loop) by Dr. N. Unwin (Cambridge, UK), which showed that the molecule had the size and shape expected for a pentamer complex and that, in several cases, structures that look like donuts seen from the side, as well as end-on images viewed down the central pseudo-5-fold axis, could be observed.

In vitro deglycosylation of any of the three proteins did not affect their ability to bind 125I-α-Bgt. This lack of a significant difference in 125I-α-Bgt binding between the glycosylated and deglycosylated forms suggests that glycosylation is not crucial for α7 ligand binding. This contrasts with our earlier observation that deglycosylation of α1 ECD expressed in the same eukaryotic system dramatically reduced α-Bgt binding (27). The solved structure for the AChBP provides an explanation for this difference. Assuming that the three-dimensional structures of the two ECDs (α1 and α7) are similar to that of the AChBP (36), the α1-subunit glycosylation site (Asn111) would be in close proximity to the ligand-binding site, explaining the dramatic effect of deglycosylation on α-Bgt binding. However, in the case of α7-ECD the three putative glycosylation sites (Asn22, Asn88, and Asn111) would be quite far away from where the ligand-binding site resides and would be less likely to be involved in ligand binding. This assumption is further reinforced by our previous observations on human α1 and α7-ECDs expressed in *E. coli* as a non-glycosylated form. *E. coli* α1-ECD was not able to bind 125I-α-Bgt (37) whereas *E. coli* expressed α7-ECD was able to bind 125I-α-Bgt,2 although with low affinity (34).

The observation that the deglycosylated form binds ligands to the same extent as the glycosylated form may be very useful for crystallization studies, as it means that either form could be used for structural studies on the α7 ligand-binding domain. Deglycosylated α7-ECD(C116S,Cys-loop) seems more promising as a starting material for structural studies because glycosylation is a source of heterogeneity, which is a very undesirable feature in terms of crystallization. However, the use of a eukaryotic system for the initial production of the glycosylated form, followed by its *in vitro* deglycosylation, may be required to produce the molecule in a native-like form, because AChR subunit glycosylation has been reported to be crucial for the correct folding of the proteins during expression (38).

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2 L. Jacobson and S. Tzartos, unpublished results.
Soluble, Oligomeric, and Ligand-binding Extracellular Domain of the Human α7 Acetylcholine Receptor Expressed in Yeast: REPLACEMENT OF THE HYDROPHOBIC CYSTEINE LOOP BY THE HYDROPHILIC LOOP OF THE ACh-BINDING PROTEIN ENHANCES PROTEIN SOLUBILITY

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