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The mongoose acetylcholine receptor α-subunit: analysis of glycosylation and α-bungarotoxin binding

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Abstract The mongoose AChR α-subunit has been cloned and shown to be highly homologous to other AChR α-subunits, with only six differences in amino acid residues at positions that are conserved in animal species that bind α-bungarotoxin (α-BTX). Four of these six substitutions cluster in the ligand binding site, and one of them, Asn-187, forms a consensus N-glycosylation site. The mongoose glycosylated α-subunit has a higher apparent molecular mass than that of the rat glycosylated α-subunit, probably resulting from the additional glycosylation at Asn-187 of the mongoose subunit. The in vitro translated mongoose α-subunit, in a glycosylated or non-glycosylated form, does not bind α-BTX, indicating that lack of α-BTX binding can be achieved also in the absence of glycosylation.

Key words: Acetylcholine receptor; α-Subunit; Glycosylation; α-Bungarotoxin; Mongoose

1. Introduction

The nicotinic acetylcholine receptor (AChR) is a ligand-gated cation channel that mediates synaptic transmission in muscle and neuronal systems. The functional molecule of muscle AChR is a pentamer with the composition of $\alpha_2\beta\gamma\delta$ [1,2]. The α-subunit has been shown to contain the binding sites for acetylcholine (ACh) and for α-bungarotoxin (α-BTX). Affinity labeling experiments demonstrated that the two adjacent cysteine residues at positions 192 and 193 of the AChR α-subunit, are in the ACh binding site [3,4] and other studies indicated that within the α-subunit, the ligand binding site is located in close proximity to these two cysteines [4–10]. Recent studies with ACh site-directed affinity reactions suggest that aromatic residues from other regions of the α-subunit [2], as well as negatively charged amino acid residues in the γ- and δ-subunits [11–13] also participate in the agonist binding site.

For the analysis of the binding site of muscle AChR, and in particular for elucidating the structural requirements for α-BTX binding, we have been studying AChRs from animal species which are resistant to α-BTX, such as the snake [14], mongoose [15] and hedgehog [16]. We have previously cloned, sequenced and expressed a 250 bp fragment corresponding to amino acid residues 122–205 of the AChR α-subunit from these three species as well as from the mouse. The expressed protein fragments from the snake, mongoose and hedgehog AChR did not bind α-BTX, whereas the mouse fragment did bind α-BTX. Sequence comparison [15,16], and analysis by site-directed mutagenesis [17], demonstrated that amino acid substitutions at positions 187, 189, 194 and 197 in the mongoose AChR α-subunit, which are conserved in the α-BTX-sensitive animals, participate in determining the resistance to α-BTX. It is noteworthy that in both the snake and mongoose, one of these substitutions (Asn-189 and Asn-187, in the snake and mongoose, respectively [14,15]) creates a putative N-glycosylation site, which may contribute uniquely to the resistance of these species to α-BTX [14,15,18,19].

In the present study we have cloned and sequenced the entire mongoose AChR α-subunit gene and analyzed the glycosylation potential of the translated protein and its interaction with α-BTX.

2. Materials and methods

2.1. Preparation and amplification of cDNA

Mongoose muscles (*Herpestes ichneumon*) were obtained from the Canadian Center of Ecological Zoology (Tel-Aviv University) and total RNA was prepared as described [20]. cDNA and PCR were performed as previously described [14]. The PCR reaction conditions were as follows: one cycle of 94°C for 3 min, and 35 cycles of 94°C for 2 min, 56°C for 3 min and 72°C for 5 min. Primer A was a sense primer (5′-GGAATTCCACAGCCCATGGAGGCRC-3′ where R = C or T) and included an ATG (underlined) which corresponds to the first methionine of the rat or mouse AChR α-subunit. Primer B was an antisense primer (5′-GGAATTCTGCTCATCCTTGRTG-3′ where R = C or T) and included a stop codon (underlined). Both primers (A and B) had an *Eco*RI adapter and were based on nucleotides −10 to 10 (A) and 1363–1380 (complementary strand) (B) of the rat and mouse α-subunit sequences [21,22]. The PCR fragment was subcloned into *Eco*RI-digested pBluescript (Stratagene). Sequence analysis was performed on double-stranded DNA templates on both strands by Applied Biosystem automatic DNA sequencing facility of the Weizmann Institute of Science. The mouse and rat α-subunit cDNA clones were kindly provided by Dr. S. Heinemann (Salk Institute, La Jolla) and Dr. V. Witzemann (Max-Planck Institut, Heidelberg), respectively.

2.2. In vitro translation and immunoprecipitation

Cloned cDNA encoding the mongoose, mouse and rat AChR α-subunits were in vitro translated using the Promega TnT rabbit reticulocyte lysate kit with ^{35}S methionine (>3000 Ci/mmole) (Amersham). Following translation, samples were immunoprecipitated with α-subunit-specific anti-peptide antibodies, elicited in rabbits against a peptide corresponding to residues 351–368 of the α-subunit of the human AChR [23] and electrophoresed in 10% polyacrylamide gels as described previously [24].

2.3. Binding of in vitro translated α-subunits to a-BTX-Sepharose columns

α-BTX (Sigma, St. Louis, MO), was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Then, 20 μl of the ^{35}S methionine-labeled, in vitro translated products were applied onto α-BTX-Sepharose columns for 45 min at room temperature. Absorption to and elution from the columns were carried out as previously described [24].

2.4. Glycosylation of in vitro translated α-subunits

Cotranslational processing of the ^{35}S Met-labeled proteins was carried out in the presence of canine microsomal membranes (Promega).

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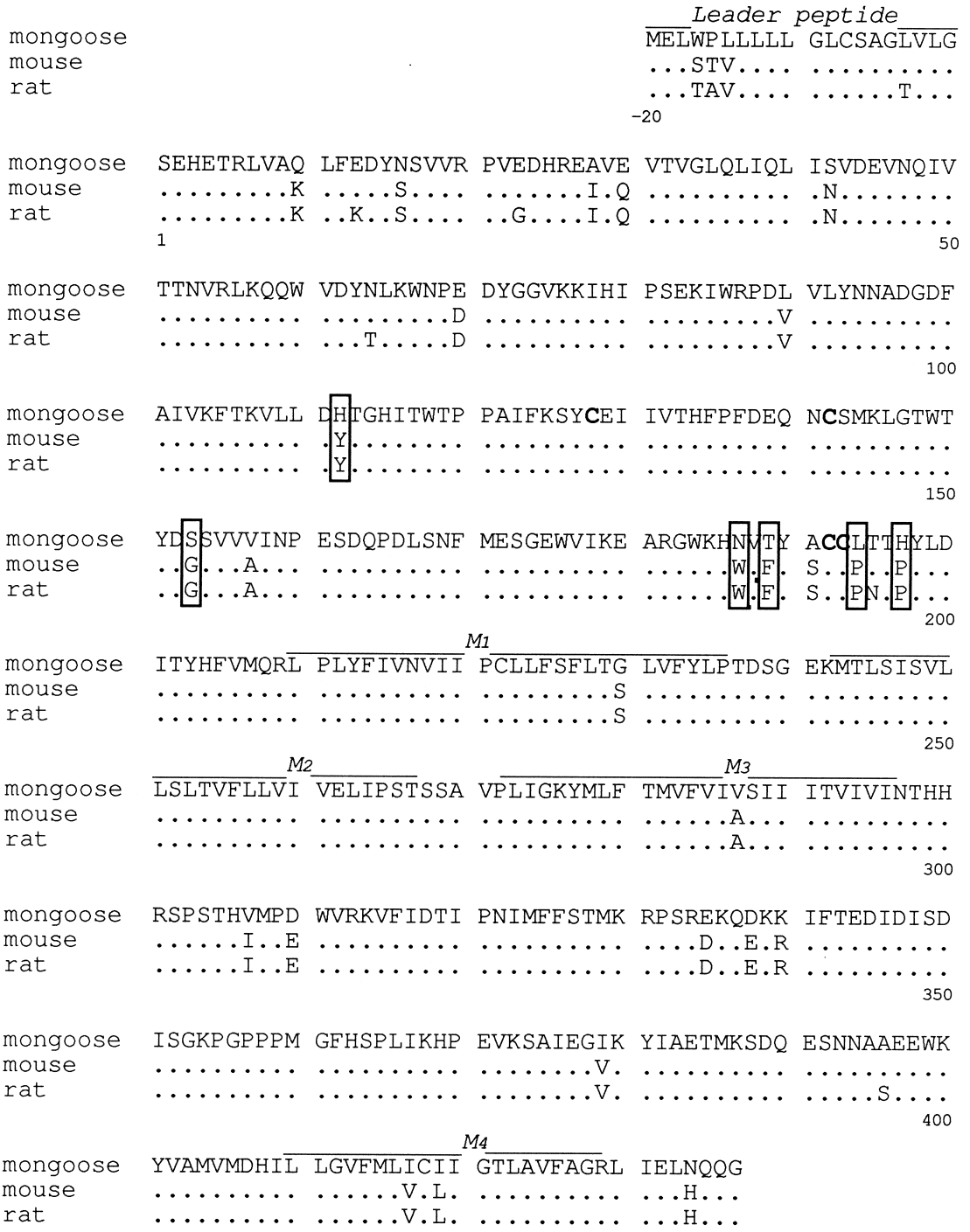

Fig. 1. Alignment of the amino acids sequences for the mongoose, mouse and rat AChR α-subunits. The four putative transmembrane domains (M1–M4) are marked, as well as the leader peptide. Amino acid residues at positions 112, 153, 187, 189, 194 and 197 are framed and Cys residues at positions 128, 142, 192 and 193 are bold.

Treatment with 10 U/μl Endo H (New England Biolabs) was performed on the in vitro glycosylated proteins for 1 h at 37°C in the presence of 50 mM sodium phosphate buffer. The samples were electrophoresed in 10% polyacrylamide gels.

3. Results

3.1. Isolation and characterization of the mongoose AChR α-subunit cDNA clone

In order to isolate the full-length mongoose α-subunit, RT-PCR amplification was performed using primers (see Section 2) based on the rat and the mouse sequences, starting from the untranslated region flanking the coding region, and terminating at the initiator ATG and TGA stop codons, respectively. PCR on mongoose muscle cDNA resulted in amplification of the entire AChR α-subunit, which was cloned into pBluescript and sequenced. This AChR α-subunit clone has an open reading frame of 1374 bp, which translates to form a protein of 437 residues (Fig. 1).

Alignment of the amino acid sequence of the mongoose α-

subunit with those of the mouse and rat α-subunits, shows 93% and 92% homology with these two species, respectively (Fig. 1). Altogether, there are 29 amino acid differences between the α-subunit of the mouse and the mongoose AChR, and 35 amino acid differences between the rat and the mongoose AChR. Only six differences are at positions that are conserved in animal species that are sensitive to α-BTX. These six differences include four residues within the binding site domain at positions 187 (Trp to Asn), 189 (Phe to Thr), 194 (Pro to Leu) and 197 (Pro to His), and two others at positions, 112 (Tyr to His) and 153 (Gly to Ser). Asn-187 in the mongoose α-subunit is a putative N-glycosylation site, in addition to the single N-glycosylation site (Asn-141), which is present in all muscle AChR α-subunits. The other differences are at positions which are not conserved among animal species that are susceptible to α-BTX.

3.2. Characterization of the in vitro translated mongoose α-subunit

The mongoose, mouse and rat α-subunit cDNAs were in vitro translated as described in Section 2. The protein products were immunoprecipitated specifically by rabbit anti-peptide antibodies specific for mammalian AChRs [23]. The mongoose and the mouse α-subunit displayed apparent molecular masses of 40 kDa and the rat α-subunit displayed an apparent molecular mass of 41 kDa in SDS/polyacrylamide gel (data not shown).

We tested the ability of the entire mongoose α-subunit to bind α-BTX by affinity chromatography on toxin columns. The in vitro translation products of the mongoose and rat α-subunit were applied onto α-BTX-Sepharose columns. As depicted in Fig. 2, the mongoose α-subunit protein did not absorb to the α-BTX column and was detected in the effluent, whereas most of the rat α-subunit did absorb to the column and could be eluted, indicating that the in vitro translated α-subunit of the mongoose AChR does not bind α-BTX under these conditions.

The mongoose α-subunit has two putative N-glycosylation sites; Asn-141 that is present in all α-subunits, and Asn-187 that is unique to the mongoose α-subunit and is located within the binding site domain. To follow the glycosylation pattern of the mongoose α-subunit, in vitro translation was carried out in the presence of canine microsomal membranes which allow glycosylation to take place. Indeed, when in vitro translation was carried out in the presence of microsomes, the glycosylated mongoose α-subunit displayed a higher apparent molecular mass of 46 kDa, and an additional band at 44 kDa, whereas the glycosylated form of the rat α-subunit yielded only a band of a molecular mass of 44 kDa (Fig. 3). The higher molecular mass of the glycosylated mongoose α-subunit (46 kDa) is most likely due to an additional glycosylation at position 187. The lower band of 44 kDa may represent incomplete glycosylation. Following deglycosylation of the mongoose and rat glycosylated proteins with Endo H, the molecular weights returned to the original non-glycosylated forms (Fig. 3). The radioactive band at about 50 kDa which is present in the samples of the mongoose α-subunit (Fig. 3a–c) probably represents a non-related protein resulting from the in vitro translation reaction.

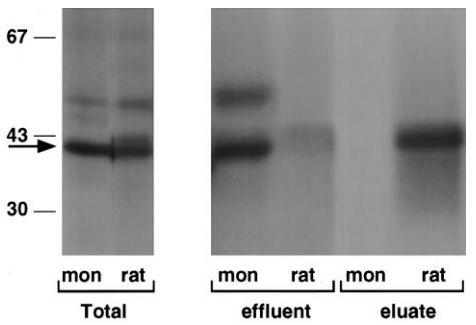

Fig. 2. Binding of in vitro translated mongoose and rat α-subunits to α-BTX-Sepharose columns. In vitro translated ^{35}S-Met-labeled products of the mongoose and rat α-subunit were applied to α-BTX-Sepharose columns. The effluents and the eluates (see Section 2) were electrophoresed on a 10% polyacrylamide gel and autoradiographed.

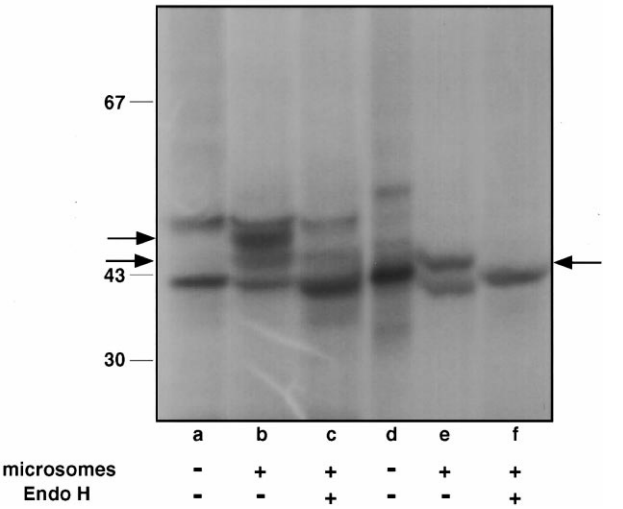

Fig. 3. Glycosylation of the mongoose and the rat α-subunits. In vitro translation of cloned mongoose (a–c) and rat (d–f) α-subunits was carried out in the presence (lanes b, c, e, f) or absence (lanes a, d) of canine microsomal membranes. The glycosylated mongoose and rat α-subunits were treated with Endo H (lanes c, f), and were separated on a 10% polyacrylamide gel and autoradiographed. The arrows on the left indicate the glycosylated forms of the mongoose (44, 46 kDa) and the arrow on the right indicates the glycosylated form of the rat subunit (44 kDa).

4. Discussion

Cloning of the full-length mongoose α-subunit cDNA reveals that there are only six amino acid differences in the α-subunit of this species, at positions that are conserved among animal species that bind α-BTX. Four of these differences cluster in the ligand binding site and include changes at positions 187 and 189 from aromatic to non-aromatic residues, and at positions 194 and 197 from prolines to leucine and histidine, respectively. We have shown previously by site-directed mutagenesis that these four changes in the mongoose AChR α-subunit are important in determining its lack of binding to α-BTX [17]. The only other two substitutions at conserved positions, throughout the entire α-subunit, are at positions 112 (Tyr-112-His) and 153 (Gly-153-Ser). We do not know yet whether these two changes contribute to the ligand binding and/or channel properties of the mongoose AChR. It is interesting to point out that in the snake AChR α-subunit there is a change from a conserved Thr at position 111 to Asn,

creating a potential glycosylation site [14] and suggesting that this region (around positions 111 and 112) could be a hot spot for mutations that may result in modified binding properties. However, it has been demonstrated by Keller et al. [18] that replacing Thr-111 in the mouse α-subunit for Asn, thus introducing a potential glycosylation site, did not result in a notable decrease in α-BTX binding to the receptor. As to the Gly-153-Ser mutation in the mongoose α-subunit, it is interesting to point out that such a mutation has been recently reported in the human AChR α-subunit of patients with a slow-channel congenital myasthenic syndrome and was shown to be associated with increased ACh binding affinity [25]. It would be interesting to find out whether this change in the mongoose AChR affects its channel properties.

Asn-187 in the mongoose α-subunit [16] and Asn-189 in the snake [14] are putative N-glycosylation sites located within the binding domain. Although not shown yet, it is likely that these positions are indeed glycosylated in the intact animal, providing a bulky group at the binding site domain that interferes with the interaction of AChR with α-BTX, without affecting ACh binding [16]. In the present study we have demonstrated by in vitro translation experiments that Asn-187 in the mongoose α-subunit can be glycosylated, as the glycosylated mongoose α-subunit has a higher molecular mass (46 kDa) than that of the rat glycosylated α-subunit (44 kDa) (Fig. 3). This higher molecular mass results most likely from the additional N-glycosylation site at Asn-187 in the mongoose α-subunit.

The in vitro translated non-glycosylated form of the mongoose α-subunit did not bind α-BTX (Fig. 2), as has been also shown by us for a fragment (residues 122–205) of this subunit [16]. It thus seems that even in the absence of a bulky sugar moiety, binding to α-BTX can be markedly hampered, provided that there are other appropriate substitutions. This is also supported by our earlier study on the hedgehog AChR [16]. In the hedgehog binding region there are two non-aromatic residues at positions 187 and 189 (Arg and Ile, respectively), and no putative glycosylation site, like those in the snake and mongoose. Nevertheless, the hedgehog was demonstrated to be resistant to α-BTX administration, in comparison with the mouse, though the degree of resistance was lower than that of the snake or mongoose. In addition, only marginal levels of α-BTX binding to the hedgehog muscle extract were demonstrated, and the expressed hedgehog AChR fragment (residues 122–205 of the α-subunit) did not bind α-BTX [16].

As stated earlier by us, there appear to be several means by which various degrees of resistance to α-BTX have developed during evolution. Such means include replacing aromatic residues at the site by different non-aromatic ones, replacing proline residues at the site, and introducing a glycosylation site within the ligand binding site. It is possible that in the intact animal, glycosylation at the binding site is an important factor that contributes to an additional reduction in the affinity of α-BTX to the mongoose AChR and results in a high level of resistance to α-BTX. This may be supported by previous binding studies on muscle extracts, showing that the hedgehog AChR, which does not have a glycosylation site at the ligand binding domain, exhibits a higher binding to α-BTX than the mongoose AChR [16]. Further support for the importance of such a glycosylation in conferring high α-BTX resistance to the snake and mongoose, comes from site-directed mutagenesis experiments on the mouse AChR [23,25]. These experiments demonstrated that introducing a glycosylation site into the mouse AChR α-subunit at position 187 or 189 (corresponding to the mongoose and snake glycosylation sites) resulted in a marked decrease in α-BTX binding.

It should be noted that all highly conserved amino acid residues in the AChR α-subunit, that were proposed to participate in ACh binding, on the basis of affinity labeling experiments, namely, Tyr-93, Trp-149, Tyr-190, Cys-192, Cys-193 and Tyr-198 [4,26–30], have been conserved in the mongoose α-subunit as well. It seems that the mongoose developed during evolution resistance to α-BTX by substitutions of several amino acid residues in the AChR α-subunit, at positions that are essential for the toxin binding, though not crucial for ACh binding. It would be interesting to study the channel properties of the mongoose AChR in order to find out whether the unique amino acid substitutions, and which of them, have an effect on the binding to ACh.

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