Purification and Characterization of a Nicotinic Acetylcholine Receptor from Chick Brain†

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ABSTRACT: Immunohistochemical studies have previously shown that both the chick brain and chick ciliary ganglion neurons contain a component which shares antigenic determinants with the main immunogenic region of the nicotinic acetylcholine receptor from electric organ and skeletal muscle. Here we describe the purification and initial characterization of this putative neuronal acetylcholine receptor. The component was purified by monoclonal antibody affinity chromatography. The solubilized component sediments on sucrose gradients as a species slightly larger than *Torpedo* acetylcholine receptor monomers. It was affinity labeled with bromo[^{3}H]acetylcholine. Labeling was prevented by carbachol, but not by α-bungarotoxin. Two subunits could be detected in the affinity-purified component, apparent molecular weights 48 000 and 59 000. The 48 000 molecular weight subunit was bound both by a monoclonal antibody directed against the main immunogenic region of electric organ and skeletal muscle acetylcholine receptor and by antisera raised against the α subunit of *Torpedo* receptor. Evidence suggests that there are two α subunits in the brain component. Antisera from rats immunized with the purified brain component exhibited little or no cross-reactivity with *Torpedo* electric organ or chick muscle acetylcholine receptor. One antiserum did, however, specifically bind to all four subunits of *Torpedo* receptor. Experiments to be described elsewhere (J. Stollberg et al., unpublished results) show that antisera to the purified brain component specifically inhibit the electrophysiological function of acetylcholine receptors in chick ciliary ganglion neurons without inhibiting the function of acetylcholine receptors in chick muscle cells. All of these properties suggest that this component is a neuronal nicotinic acetylcholine receptor with limited structural homology to muscle nicotinic acetylcholine receptor.

The nicotinic acetylcholine receptor from both electric organ and skeletal muscle has been extensively investigated by using both immunochemical [see Lindstrom et al. (1983) for a review] and, more recently, molecular genetic approaches [see Popot & Changeux (1984) for a review]; however, little is known about the neuronal nicotinic acetylcholine receptor. The main reason for this is the lack of a suitable probe.

α-Bungarotoxin (αBgt)[1] has been an invaluable probe for studying receptor from skeletal muscle and electric organ. This toxin binds with both high affinity and great specificity to the acetylcholine binding sites of these receptors, thereby preventing acetylcholine from triggering opening of the cation channel through the receptor. Lower affinity binding sites for αBgt are found in nervous systems, but their significance is uncertain. Cholinergic retinotectal transmission can be blocked by αBgt in the toad (Freeman, 1977), goldfish (Freeman et al., 1980), and frog sympathetic neurons (Marshall, 1981). However, αBgt has no effect upon transmission in the rat retinotectal system (Schmidt & Freeman, 1980), rat cervical ganglion (Brown & Fumigalli, 1977), or chick ciliary ganglion (Carbonetto et al., 1978; Kouvelas et al., 1979; Ravdin & Berg, 1979). To further complicate matters, some neurons contain both αBgt binding sites which are not functional acetylcholine receptors and acetylcholine receptors which are triggered to open their cation channels by acetylcholine, but which do not bind αBgt (Patrick & Stallcup, 1977; Jacob & Berg, 1983).

A minor component of the same venom from which αBgt is purified binds with moderate affinity to acetylcholine receptors of both neurons and muscle. In the past, this has been variously referred to as 3.1 toxin (Ravdin & Berg, 1979), or toxin F (Loring et al., 1984), or κ Bgt (Grant & Chiappinelli, 1985). Use of this toxin for purification of neuronal receptors has been hampered by the small amounts available, difficulties in retaining biological activity after labeling it with ^{125}I, and its cross-reaction with αBgt binding sites.

Immunological approaches have been used to examine the nature of neuronal toxin binding sites and acetylcholine receptors. Several studies have used antisera to receptors from *Torpedo* electric organ to demonstrate a limited cross-reactivity with the toxin binding component of rat brain (Block & Billiar, 1979; Wonnacott et al., 1982; Mills & Wonnacott, 1984). Additionally, antisera to receptors from denervated cat muscle and two monoclonal antibodies (mAbs) to receptors from chick muscle have been shown to bind to an αBgt binding component from chick brain (Norman et al., 1982; Mehraban et al., 1984). This toxin binding protein has been purified and is composed of at least three subunits (Conti-Tronconi et al., 1985), one of which has amino-terminal amino acid sequence homology with the α subunit of electric organ and muscle receptors. However, it is not yet clear if this molecule is a functional acetylcholine receptor.

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[1] Abbreviations: αBgt, α-bungarotoxin; ^{125}I-αBgt, ^{125}I-labeled α-bungarotoxin; BSA, bovine serum albumin; DPT, diazophenyl thioether; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; mAb, monoclonal antibody; ^{125}I-mAb, ^{125}I-labeled monoclonal antibody; MIR, main immunogenic region; PBS, 10 mM sodium phosphate, pH 7.5, and 100 mM sodium chloride; Tris, tris(hydroxymethyl)aminomethane; [^{3}H]BAC, bromoacetyl[^{3}H]choline; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol.

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Antibodies have been similarly utilized to identify in neurons functional acetylcholine receptors which do not bind αBgt. Patrick and Stallcup (1977) demonstrated that an antiserum to receptors from *Electrophorus* electric organ blocked the function of acetylcholine receptors in the pheochromocytoma cell line PC12 but did not recognize the αBgt binding component from these cells. Ultrastructural studies of chick ciliary ganglion neurons revealed that αBgt does not bind at synapses where receptors are presumably located (Jacob & Berg, 1983) but that a mAb to receptors from electric organ and muscle cross-reacted with a synaptic component distinct from the αBgt binding component (Jacob et al., 1984). This mAb is directed at the main immunogenic region (MIR), a region on the extracellular surface of receptor α subunits against which a majority of antibodies to native receptors are directed (Tzartos et al., 1980). Biochemical studies have demonstrated that this anti-MIR binding component of chick ciliary ganglia has properties expected of a neuronal acetylcholine receptor; it is an integral membrane glycoprotein which sediments as a 10S species and does not bind αBgt (Smith et al., 1985a) and which is modulated by cholinergic ligands (Smith et al., 1985b). Swanson and co-workers (Swanson et al., 1983) used immunohistochemical techniques to show that mAbs specific for the MIR and for each of the subunits of receptors from *Torpedo* electric organ cross-reacted with determinants located in the lateral spiriform nucleus of chick brain and its projections to the optic tectum, but no αBgt binding sites were found in this region of the brain. Lindstrom et al. (1983) demonstrated that ^{125}I-labeled mAbs to the MIR, unlike ^{125}I-Bgt, bound to these brain regions and that ^{125}I-labeled Fab fragments of a mAb to the MIR bound to a component solubilized from chick brains which sedimented on sucrose gradients similarly to monomers of receptors from *Torpedo* electric organ.

In this study, we report the purification and biochemical characterization of the anti-MIR binding component from chick brain and present evidence that it is a neuronal acetylcholine receptor.

Materials and Methods

Antisera and Monoclonal Antibodies. Hybridoma cell lines were obtained by fusing mouse myeloma cells with spleen cells from rats immunized with purified receptor. mAb 35 was raised against receptors from *Electrophorus* electric organ (Tzartos et al., 1981), and mAb 210 was raised against receptors purified from fetal bovine muscle and the mouse neuroblastoma cell line BC3H1 and then partially denatured with $NaDodSO_4$ (S. Hochschwender, unpublished results). Both mAbs bind to the MIR; mAb 35 binds only to native receptors, while mAb 210 also binds denatured subunits. Stocks were prepared by ammonium sulfate precipitation of hybridoma culture supernatants. mAb 35 was further purified by chromatography on DEAE-Affigel Blue (Bio-Rad) (S. Hochschwender, unpublished results) and radioiodinated by a modified chloramine-T method as previously described for αBgt (Lindstrom et al., 1981a) to specific activities of (2–3) × 10^{18} cpm/mol and stored at 4 °C in phosphate-buffered saline (PBS) containing 10 mg/mL bovine serum albumin (BSA).

Fab 35 was prepared by papain cleavage as described previously (Wan & Lindstrom, 1985) and radioiodinated as above to specific activities of (2–3) × 10^{18} cpm/mol.

Antisera to the subunits of *Torpedo* receptors were prepared as described previously (Lindstrom et al., 1979b).

Goat anti-rat IgG was affinity purified by using a rat IgG–Sepharose C14B column and radioiodinated as above to specific activities of (2–3) × 10^{18} cpm/mol.

Purification of the mAb 35 Binding Component. (A) Extraction. Chick brains were obtained from Pel-Freez Biologicals and stored at −70 °C. Frozen chick brains (300 g) were mixed with 1.5 volumes of 50 mM Tris, pH 8.8, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 5 mM iodoacetamide, 5 mM benzamidine, and 2 mM phenylmethanesulfonyl fluoride (buffer A) containing 300 mM sucrose and homogenized for 1 min at high speed in a Waring blender. After centrifugation for 50 min at 82000*g* in a Beckman 19 rotor, the pellet was resuspended by Polytron homogenization in 3 volumes of buffer A and recentrifuged at 82000*g* for 50 min. This was repeated a second time. The pellet was then resuspended in 0.75 volume of buffer A, pH 7.2, containing 2% Triton X-100 and extracted with moderate agitation for 2 h at 4 °C. The extract was centrifuged at 82000*g* for 90 min and the supernatant recentrifuged at 140000*g* for 50 min in a Beckman Ti50.2 rotor. The clear supernatant was then collected.

(B) Affinity Purification. Purified mAb 35 was coupled to Sepharose C14B at 8–10 mg of protein/mL of gel by cyanogen bromide activation, as previously described for preparation of toxin–Sepharose C14B (Lindstrom et al., 1981a). The affinity gel (5–7 mL) was mixed with 400–500 mL of brain extract and incubated for 12–14 h at 4 °C with gentle agitation. The gel was then loaded into a 10-mL column and successively washed with 200 mL of PBS containing 0.5% Triton X-100 and 25 mL of the same buffer containing 500 mM NaCl. Bound protein was then eluted with 20 mL of 50 mM sodium citrate, pH 3.0, 1 M NaCl, and 0.1% Triton X-100. The eluate was collected into 1 M Tris, pH 8.0 (5 mL), and dialyzed against 10 mM phosphate, pH 7.5, containing 0.05% Triton X-100. For application to a second mAb 35–Sepharose column, the eluate was dialyzed for 4 h against 4 L of PBS containing 0.5% Triton X-100 and recirculated for 12–14 h through a 0.5-mL column of mAb 35–Sepharose. After the column was washed with 20 mL of the same buffer, bound protein was eluted with 4 mL of citrate buffer, pH 3.0, as above, neutralized with 1 M Tris, pH 8.0, and dialyzed against 4 L of 1 mM phosphate, pH 7.5, and 0.05% Triton X-100.

For radioiodination of $NaDodSO_4$-denatured protein, the first column eluate was applied to the second affinity column as before, washed with 20 volumes of PBS, and eluted with 4 mL of 50 mM sodium citrate, pH 3.0, and 1 M NaCl. The eluate was neutralized with 1 M Tris, pH 8, and 0.05% $NaDodSO_4$, dialyzed against 4 L of 1 mM phosphate, pH 7.5, and 0.05% $NaDodSO_4$, and lyophilized. It was then radioiodinated by the lactoperoxidase–glucose oxidase method (Bio-Rad). ^{125}I-labeled subunits of the mAb 35 binding component were obtained by preparative electrophoresis of radiolabeled protein and subsequent elution of the polypeptides, as has been described previously (Lindstrom et al., 1979a).

For radioiodination of the purified protein in a nondissociated form, the first column eluate was applied to the second affinity column as before, washed with 20 volumes of PBS and 0.05% Tween 20, and eluted with 4 mL of 50 mM sodium citrate, pH 3.0, 1 M NaCl, and 0.05% Tween 20 into 1 mL of 1 M Tris, pH 8.0. The eluate was desalted and concentrated to 150 μL final volume by using a Centricon 10 microconcentrator (Amicon) and radioiodinated by the lactoperoxidase–glucose oxidase method (Bio-Rad). The ^{125}I-labeled protein was further purified by adsorption to concanavalin A–Sepharose (Pharmacia) and subsequent elution with 1 M methyl α-mannoside in 10 mM Tris, pH 7.4, 10 mM EDTA,

10 mM EGTA, and 0.1% Triton X-100.

Determination of mAb 35 Binding Sites. (A) Detergent Extracts. mAb 35 binding sites in the detergent extract of chick brain were assayed by using DE52 ion-exchange resin (Whatman). Aliquots (10 μL) of the extract were incubated with 2 nM ^{125}I-mAb 35 for 1–2 h. The incubation mixture was diluted with 100 μL of 10 mM phosphate, pH 7.5, 50 mM sodium chloride, and 0.5% Triton X-100 and applied to 150 μL of DE52 resin in a 1-mL micropipet tip. After being washed with 5 mL of the above buffer to remove free ^{125}I-mAb 35, bound antigen–^{125}I-mAb 35 was quantitated on a γ counter. Nonspecific binding of ^{125}I-mAb 35 was determined by preincubation with a 100-fold excess of nonradioactive mAb 35.

(B) Affinity-Purified mAb 35 Binding Component. Aliquots of the affinity-purified component, in 30-μL final volume in PBS, were applied to Millipore millititer 96-well nitrocellulose plates and incubated overnight at 4 °C. The plates were quenched for 30 min of room temperature with 50 μL of PBS containing 1% bovine serum albumin (BSA) and 0.2% Tween 20, and 30 μL of 10 nM ^{125}I-mAb 35, diluted in the same buffer, was added. The plates were incubated for 2 h at room temperature and then washed twice with 300 μL of the buffer. The nitrocellulose disks were punched out, and bound radioactivity was determined by γ counting. The nonspecific binding was determined by incubation in the presence of a 100-fold excess of nonradioactive mAb 35 or by overnight incubation with PBS alone, rather than purified protein.

Determination of Toxin Binding Sites in Brain. αBgt binding sites were assayed by using DE52 ion-exchange resin, as described for the determination of mAb 35 binding sites. Aliquots (10 μL) of brain detergent extract were incubated with 10 nM ^{125}I-αBgt for 1–2 h, diluted in 100 μL of 10 mM sodium phosphate, pH 7.5, and 0.5% Triton X-100, and applied to 150 μL of DE52 resin. After the DE52 resin was washed with 2 mL of buffer, bound radioactivity was determined by γ counting. Nonspecific binding was determined by incubation in the presence of 1 μM nonradioactive αBgt.

Preparation of Receptors from Torpedo Electric Organ and Chick Muscle. Torpedo receptor was purified by affinity chromatography on *Naja-naja siamensis* toxin conjugated to Sepharose, as previously described (Lindstrom et al., 1981a). Individual subunits were isolated by preparative electrophoresis (Lindstrom et al., 1978, 1979a) and labeled with ^{125}I by using the lactoperoxidase–glucose oxidase method (Bio-Rad) to a specific activity of (1–1.5) × 10^{18} cpm/mol.

Chick muscle receptor was prepared from 13-day-old eggs as described previously for the preparation of fetal bovine receptor (Einarson et al., 1982), with the difference that bound receptor was eluted from the toxin–Sepharose column with 2% sodium dodecyl sulfate ($NaDodSO_4$).

Protein concentrations were determined by using the method of Lowry (1951).

Sucrose Gradient Centrifugation. Aliquots (150 μL) of chick brain detergent extract or affinity-purified *Torpedo* receptor were incubated overnight at 4 °C with 0.5 nM ^{125}I-Fab 35, layered onto 4.9-mL (5–20% w/w in 20 mM phosphate, pH 7.5, 10 mM sodium azide, and 0.1% Triton X-100) sucrose gradients, and centrifuged for 70 min at 65 000 rpm, 4 °C, in a Beckman VTi65 rotor. Fourteen-drop fractions were subsequently collected from the bottom of the tubes, and radioactivity was determined by γ counting.

Electrophoresis. Electrophoresis on acrylamide slab gels in $NaDodSO_4$ using the Laemmli discontinuous buffer system (Laemmli, 1970) was conducted as described previously (Lindstrom et al., 1979a,b). Polyacrylamide gels were silver stained for protein according to the method of Oakley et al. (1980). Polyacrylamide gels of radioiodinated protein were autoradiographed for 4–24 h at −70 °C using preflashed Kodak X-Omat-AR film and an intensifying screen. Autoradiograms were standardized for molecular weight by using Bio-Rad low molecular weight standards resolved on the same gel and stained with Coomassie Blue.

Electrophoretic transfer of proteins from gels to diazophenyl thioether (DPT) paper and subsequent probing with antibodies were as described previously (Gullick & Lindstrom, 1982). After being probed, bound antibodies were detected by incubation with 0.5 nM ^{125}I-labeled goat anti-rat IgG [(2–3) × 10^{18} cpm/mol] and subsequent autoradiography (for 4–48 h) as described previously.

Peptide Mapping. Peptide mapping of radioiodinated polypeptides was performed essentially as described by Cleveland et al. (1977) using V8 protease (Miles), trypsin (Worthington), papain (Sigma), and chymotrypsin (Calbiochem). ^{125}I-Labeled subunit (25 000–50 000 cpm) was digested with various amounts of protease for 1 h at 37 °C in a final volume of 25 μL of 10 mM phosphate, pH 7.5. After addition of 2.5 μL of 0.1 M phenylmethanesulfonyl fluoride, and 25 μL of 125 mM Tris-HCl, pH 6.8, containing 2.3% (w/v) $NaDodSO_4$, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.005% (w/v) bromophenol blue, the resulting fragments were resolved on 15% acrylamide gels and detected by autoradiography.

Treatment of ^{125}I-Labeled Subunits with Endoglycosidase H. Radioiodinated *Torpedo* α subunit or mAb 35 binding component polypeptide was digested with 10–0.01 milliunits of endoglycosidase H (Miles) in 25 μL of 50 mM sodium citrate, pH 5.5, containing 0.5% $NaDodSO_4$, 50 mM dithiothreitol, and 10 mM phenylmethanesulfonyl fluoride for 15 h at 37 °C (Van Oriel & Goding, 1985). A control tube contained no enzyme. Following incubation, the proteins were analyzed by $NaDodSO_4$–acrylamide gel electrophoresis and autoradiography as described previously.

Affinity Labeling with Bromoacetyl[^{3}H]choline. Bromoacetyl[^{3}H]choline ([^{3}H]BAC) was prepared by Dr. Kee Wan using [^{3}H]choline and bromoacetyl bromide according to Damle and co-workers (Damle et al., 1978) to a specific activity of 7.6 × 10^{15} cpm/mol. The mAb 35 binding component was immobilized on mAb 35–Sepharose C14B by recirculating a Triton X-100 extract of chick brain for 15 h through a 300-μL antibody column. After the column was washed with 20 mL of PBS containing 0.5% Triton X-100, the affinity column was divided into 25-μL aliquots. The aliquots were reduced for 20 min with 1 mM dithiothreitol in 10 mM Tris, pH 8.0, 50 mM NaCl, and 0.1% Triton X-100, washed with 2 mL of the same buffer, pH 7.0, without DTT, and then affinity labeled for 1 min at room temperature with 1 μM [^{3}H]BAC. The reaction was stopped by dilution with 1 mL of pH 7.0 buffer and pelleting the affinity gel in a microfuge. The affinity gel was washed 3 times in the same buffer and bound protein eluted by addition of 100 μL of 2% $NaDodSO_4$. Triplicate 25-μL aliquots were taken, and radioactivity was determined by liquid scintillation counting. The specificity of the affinity labeling was determined by performing the incubation in the presence of carbachol and αBgt. Additionally, nonspecific labeling of the affinity resin alone was determined.

Production of Antisera. (A) Immunization. Two-month-old female Lewis rats were immunized 3 times at 2–3-weekly intervals subdermally with 10–30 pmol of affinity-purified mAb 35 binding component in Freund's complete adjuvant.

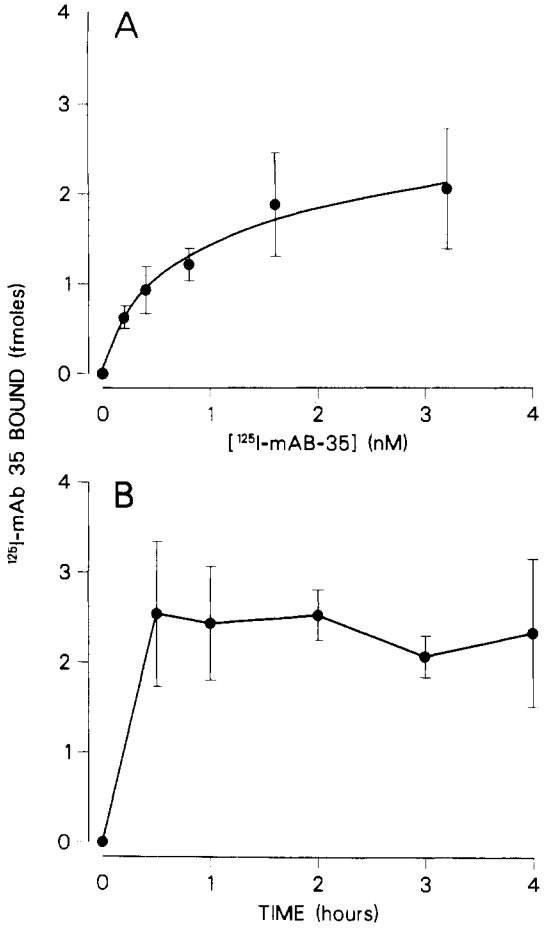

FIGURE 1: Determination of mAb 35 sites in detergent extract of chick brain by DEAE assay. (A) Concentration dependence of ^{125}I-mAb 35 binding. Extract (10 μL) was incubated for 2 h at room temperature with the indicated concentrations of ^{125}I-mAb 35 and then applied to small DEAE columns as described under Materials and Methods. Each point represents the mean ± standard deviation of quadruplicate determinations. (B) Time dependence of ^{125}I-mAb 35 binding. Extract (10 μL) was incubated at room temperature with 2 nM ^{125}I-mAb 35 for the indicated times before assay. Each point represents the mean ± standard deviation of quadruplicate determinations.

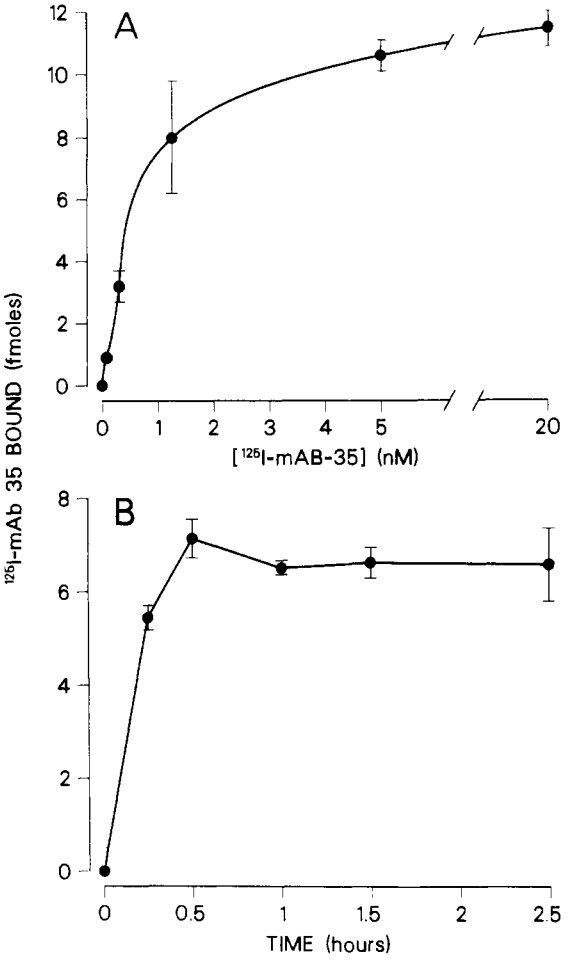

FIGURE 2: Determination of mAb 35 sites in affinity-purified mAb 35 binding component. (A) Concentration dependence of ^{125}I-mAb 35 binding. Purified mAb 35 binding component (10 fmol) was applied to wells of Millipore Millititer plates as described under Materials and Methods. After being quenched, the plates were incubated with the indicated concentrations of ^{125}I-mAb 35 for 2 h at room temperature and washed, and bound radioactivity was determined. Nonspecific binding, determined by application of PBS rather than purified mAb 35 binding component, was subsequently subtracted. Each point represents the mean ± standard deviation of triplicate determinations. (B) Time dependence of ^{125}I-mAb 35 binding. Affinity-purified mAb 35 binding component (7 fmol) immobilized on the wells of a Millititer plates was incubated at room temperature with 10 nM ^{125}I-mAb 35 for the indicated times and bound radioactivity subsequently determined. Nonspecific binding, determined as in (A), has been subtracted. Each point represents the mean ± standard deviation of triplicate determinations.

Serum was subsequently collected.

(B) Antibody Assays. (1) Millititer Assay. Ten to twenty femtomoles of affinity-purified mAb 35 binding sites was bound overnight at 4 °C to the wells of Millipore nitrocellulose plates. After being quenched, as described above, 30 μL of antisera diluted in PBS, 1.0% BSA, and 0.2% Tween 20 was added and incubated for 4 h. The plates were then washed twice in the same buffer before incubation for 2 h with 10 nM ^{125}I-labeled goat anti-rat IgG. After two further washes, bound radioactivity was determined as above. Nonspecific binding was determined by using normal rat serum. Titers were calculated in terms of moles of IgG bound by using a standard curve of immobilized, affinity-purified rat IgG.

(2) Immunoprecipitation Assays. Binding of the antibodies to ^{125}I-αBgt-labeled receptors from *Torpedo* or chick muscle, or ^{125}I-labeled mAb 35 binding component, was investigated by indirect immunoprecipitation using goat anti-rat IgG (Lindstrom et al., 1981a).

RESULTS

mAb 35 Binding. Binding of ^{125}I-mAb 35 to mAb 35 binding component in chick brain extracts was measured by using a DEAE-cellulose column to bind the complexes of mAb and receptor while allowing free mAb to pass through the column. This indicates that the brain component has a relatively low p*I*, in common with receptors from electric organ, which can be assayed by a similar approach (Schmidt & Raftery, 1973). Near saturation of the antigen occurred rapidly at about 2 nM ^{125}I-mAb 35 (Figure 1a,b). The binding of ^{125}I-mAb 35 to the affinity-purified mAb binding component was determined by immobilizing the purified protein on Millititer nitrocellulose plates. Preliminary experiments using affinity-purified muscle acetylcholine receptor demonstrated that at least 400 fmol of receptor could be bound to each nitrocellulose well (data not shown). Optimal quenching of the nitrocellulose was achieved by using PBS containing 1.0% BSA and 0.2% Tween 20. ^{125}I-mAb 35 bound to the immobilized antigen in a rapid and saturable manner under the conditions used (Figure 2A,B).

Purification of the mAb 35 Binding Component. The mAb 35 binding component was purified by affinity chromatography on a mAb 35–Sepharose gel. To minimize proteolysis during

Table I: Purification of mAb 35 Binding Component from 300 g of Chick Brains

	volume (mL)	protein (mg)	mAb 35 binding sites nmol	mAb 35 binding sites %	sp act.[a] (nmol/mg of protein)	purification (x-fold)
detergent extract	480	4032	0.114	100	2.83×10^{-5}	1
extract after passage over affinity column	480	4032	0.016	14		
first affinity column eluate	30	0.165	0.008	7.0	0.59	21 000
second affinity column effluent	30		0.001	0.9		
second affinity column eluate	7		0.005	4.4		

[a] Specific activity of the first affinity column eluate was calculated by assuming all the mAb 35 binding component bound was eluted.

the procedure, the tissues were homogenized and membranes subsequently washed in a pH 8.8 buffer in an attempt to inhibit lysosomal proteases with acidic pH optima. Additionally, the membranes were washed twice before detergent solubilization so as to remove the majority of the soluble proteins.

The amounts of putative neuronal receptor were measured by the DEAE-cellulose assay in terms of moles of ^{125}I-mAb 35 binding sites. The concentration in the detergent extract was 0.26 ± 0.04 nM ^{125}I-mAb 35 binding sites (15 preparations). This represents 0.4 ± 0.07 pmol/g of brain. A typical purification is shown in Table I. When the mAb 35 binding component was purified for subsequent Western blotting or immunization of animals, a single round of affinity chromatography was utilized. However, when highly purified protein was required for analysis by polyacrylamide gel electrophoresis and silver staining, or radioiodination, very small amounts were prepared by a second round of affinity chromatography. In fact, after the second affinity purification, the band pattern on acrylamide gels was essentially unaltered except for the elimination of some faint background bands. The small amount of antibody affinity resin used efficiently bound the mAb 35 binding component. After the column was washed, bound protein was eluted. Various elution conditions were investigated, including acid and alkaline pH, chaotropic salts, and ethylene glycol. The most efficient method in terms of both elution of bound antigen and recovery of nondenatured antigen (that is, still able to bind mAb 35) was found to be pH 3.0 citrate buffer containing 1 M NaCl. Under these conditions, greater than 90% of bound antigen could be eluted (data not shown). However, only a small percent (7–15%) retained the ability to bind mAb 35 after elution, even though the eluate was immediately neutralized with 1 M Tris, pH 8.0. Thus, the brain component, like detergent-solubilized receptor from electric organs, is readily denatured at low pH. This resulted in the second affinity column step being very inefficient, binding only the small, nondenatured fraction of the mAb 35 binding component. The single-step affinity chromatography gave a 21 000-fold purification and a specific activity of 0.59 nmol/mg. Since the exact stoichiometry of mAb 35 binding to the brain component is not known (as is discussed later), we cannot yet calculate the theoretical specific activity of the protein when purified to homogeneity.

Distinction of the mAb 35 Binding Component from the Brain αBgt Binding Protein. To demonstrate that the mAb 35 binding component was distinct from the αBgt binding protein from brain, the ability of *Naja-naja siamensis* toxin–Sepharose to bind the mAb 35 binding sites from chick brain extract was investigated. Figure 3A clearly demonstrates that, while mAb 35–Sepharose bound the majority of the ^{125}I-mAb 35 binding sites, the α-toxin–Sepharose failed to deplete any of those sites from the chick brain extract.

Conversely, the ability of mAb 35–Sepharose to deplete αBgt binding sites from chick brain extracts was also investigated. Toxin–Sepharose was able to remove the majority of the ^{125}I-αBgt binding sites from the extract while, by contrast, the mAb 35–Sepharose failed to deplete any αBgt binding sites (Figure 3B). These two experiments demonstrate that the mAb 35 binding component and the toxin binding protein are different molecules.

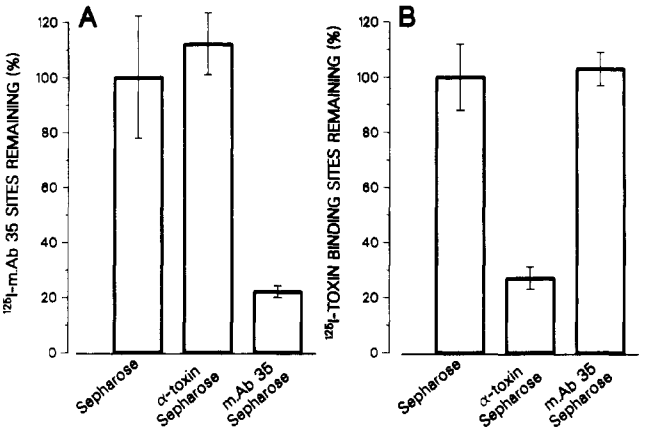

FIGURE 3: Demonstration that ^{125}I-mAb 35 and ^{125}I-αBgt bind to different components. Chick brain extract (200 μL, 0.26 nM in mAb 35 binding sites and 0.66 nM in αBgt binding sites) was incubated with 20 μL of Sepharose, Sepharose–*N. naja siamensis* α-toxin (0.5 mg/mL gel), or Sepharose–mAb 35 (10 mg/mL gel) overnight at 4 °C with gentle shaking and then assayed for (A) ^{125}I-mAb 35 binding sites and for (B) ^{125}I-αBgt binding sites by DEAE assay. Results are given assuming (A) mAb 35 sites or (B) αBgt binding sites in the extract incubated with unconjugated Sepharose were 100%. Each column represents the mean ± standard deviation of triplicate determinations.

Affinity Labeling of the mAb 35 Binding Component. Bromoacetylcholine (BAC) is an affinity labeling reagent initially developed by Karlin and co-workers (Damle et al., 1978) which reacts covalently with the acetylcholine receptor only after reduction of a disulfide bond near the acetylcholine binding site. BAC is much more reactive with one of the two acetylcholine binding sites of receptors from both electric organ and muscle (Wolosin et al., 1980). On chick ciliary ganglion neurons, as on fish electric organs, BAC is a reversible agonist, unless the cells are first treated with dithiothreitol, in which case BAC irreversibly inhibits activation of the receptor by acetylcholine (Stollberg et al., 1984).

The small amounts of the mAb 35 binding component available for affinity labeling necessitated its immobilization upon an affinity column. Other workers have utilized a lectin column to immobilize and affinity label purified receptor (Lydiatt et al., 1979). This approach was not taken here both because the mAb 35 binding component was partially denatured after acid elution from the antibody affinity column and also because lectins have been demonstrated to block acetylcholine-induced ion flux into chick ciliary ganglia (Messing et al., 1984). Antibodies to the MIR bind to the receptor without altering its function (Lindstrom et al., 1981b; Blatt et al., 1986). Thus, the brain component was immobilized directly from detergent extract onto mAb 35–Sepharose and subsequently affinity labeled with [^{3}H]BAC in the presence

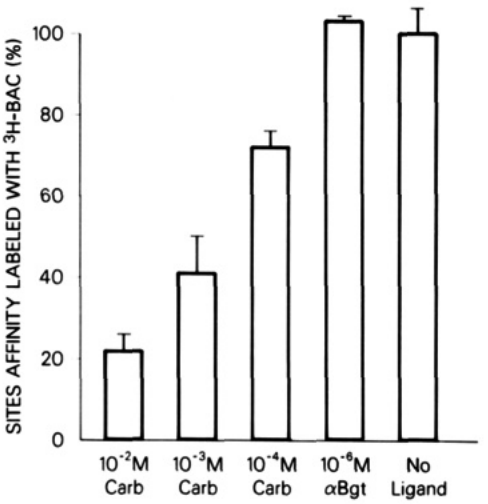

FIGURE 4: Affinity labeling of the mAb 35 binding component by [^{3}H]BAC. The mAb 35 binding component (4–5 pmol) immobilized on 25 μL of mAb 35–Sepharose was affinity labeled with 1 μM [^{3}H]BAC and subsequently eluted with 2% $NaDodSO_4$. Triplicate 25-μL aliquots were taken for scintillation counting. Results are given assuming labeling in the absence of competing ligand was 100%. Nonspecific labeling, determined in the presence of mAb 35–Sepharose alone, was always less than 12% of the specific labeling and has been subtracted. Each column represents the mean ± standard deviation of three separate incubations.

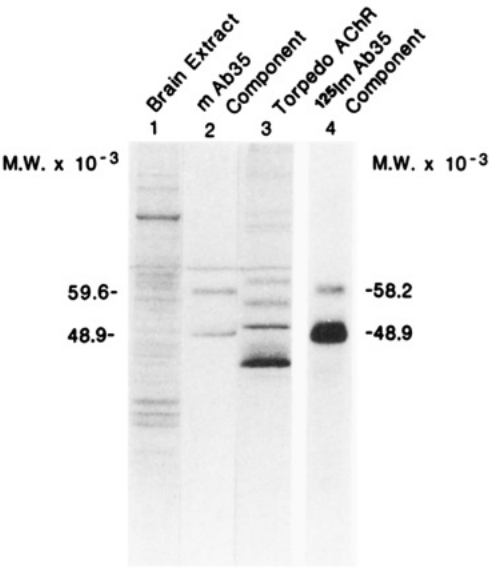

FIGURE 5: Subunit composition of the affinity-purified mAb 35 binding component. $NaDodSO_4$ gel electrophoresis was carried out in 10% acrylamide gels. Lanes 1–3 were stained with silver: lane 1, 2.5 μg of chick brain extract; lane 2, 0.9 pmol of mAb 35 binding component; lane 3, 250 ng of *Torpedo* receptor. Lane 4, autoradiography of ^{125}I-labeled affinity-purified mAb 35 binding component.

and absence of cholinergic ligands (Figure 4). The amount of mAb 35 binding component immobilized was determined by measuring the ^{125}I-mAb 35 sites depleted from the detergent extract. The total binding in the absence of any cholinergic ligands was 2.45 ± 0.16 pmol of [^{3}H]BAC per 4.5 ± 1.05 pmol of mAb 35 binding sites. Nonspecific binding of [^{3}H]BAC, in the absence of immobilized mAb 35 binding component, was always less than 12% of the total binding. Figure 4 clearly demonstrates that the mAb 35 binding component has a [^{3}H]BAC binding site which is protected by carbachol but not at all by a high (1 μM) concentration of αBgt. From this, we conclude that the mAb 35 binding component has an acetylcholine binding site. Further, these results suggest that, as in the case of receptors from electric organs, muscle, and ciliary ganglia, there is a disulfide bond near this site. The stoichiometry of mAb 35 binding to [^{3}H]BAC labeling sites obtained was approximately 1:0.54, a ratio that would be found for *Torpedo* receptor (which binds two mAb 35 molecules per receptor monomer; Conti-Tronconi et al., 1981) at the concentration of affinity ligand used, due to the half-site reactivity mentioned above. However, apart from concluding that the stoichiometry of mAb 35 and BAC is the same order of magnitude, we do not want to interpret these data further due to the inherent difficulties in determining absolute numbers.

Several attempts were made to visualize the polypeptide labeled by [^{3}H]BAC by electrophoresis and fluorography. However, in each case, no specifically labeled band was detected. The reason for this is not known, but it may well be that the labile choline ester bond in the [^{3}H]BAC hydrolyzes, releasing [^{3}H]choline and leaving the protein unlabeled.

As further evidence of the presence of an acetylcholine binding site on this molecule and of its nicotinic pharmacological properties, we have found that this component bound to mAb 35–Sepharose binds [^{3}H]nicotine with high affinity ($K_D \sim 6$ nM). This binding was specifically inhibited by nicotine agonists and antagonists (cytisine, acetylcholine, decamethonium, and curare) but not by αBgt or the muscarinic antagonist atropine (P. J. Whiting and J. M. Lindstrom, unpublished results).

Detection of Polypeptides. To allow acrylamide gel analysis and silver staining, highly purified preparations of the mAb 35 binding component were required. This was achieved by two rounds of affinity chromatography on mAb 35–Sepharose. Figure 5 shows that the purified protein contained two detectable polypeptides of apparent molecular weights 48 300 ± 400 and 58 700 ± 800 (mean ± standard deviation of five determinations). The silver staining technique also revealed a band of apparent molecular weight 66 000 which ran across the entire gel and thus is considered an artifact. There was no detectable contamination with any of the major proteins present in the crude detergent extract of chick brain. The constituent polypeptides of the purified protein were also visualized by radioiodination and subsequent autoradiography. Again two subunits could be detected, apparent molecular weights 48 900 and 58 200 (Figure 5). That these two polypeptides were part of the same macromolecular complex was indicated by the observation that both could be removed from solution by mAb 210 bound indirectly to goat anti-rat IgG–Sepharose, even though mAb 210 binds only to the 48 000 molecular weight component on Western blots, as shown later. In addition, both the 48 000 and 59 000 molecular weight components were removed from solution by either concanavalin A–Sepharose or mAb 35–Sepharose.

Peptide Mapping of Constituent Polypeptides. To compare the two subunits both with each other and with the four subunits of receptor from *Torpedo* electric organ, peptide maps were obtained of each ^{125}I-labeled subunit by using four different proteases. Pure prearations of each subunit were obtained (Figure 6A); the *Torpedo* subunits were isolated by preparative electrophoresis of unlabeled receptor and then individually radioiodinated, while the mAb 35 binding component subunits were isolated by preparative electrophoresis of $NaDodSO_4$-denatured, radiolabeled protein. The peptide maps were obtained by pretitrating the concentration of protease to determine optimal conditions for limited digestion of each polypeptide before resolving the digested polypeptides on the same polyacrylamide gel. The peptide maps suggest a lack of homology between the *Torpedo* subunits and the mAb 35 binding component subunits (Figure 6B–E). How-

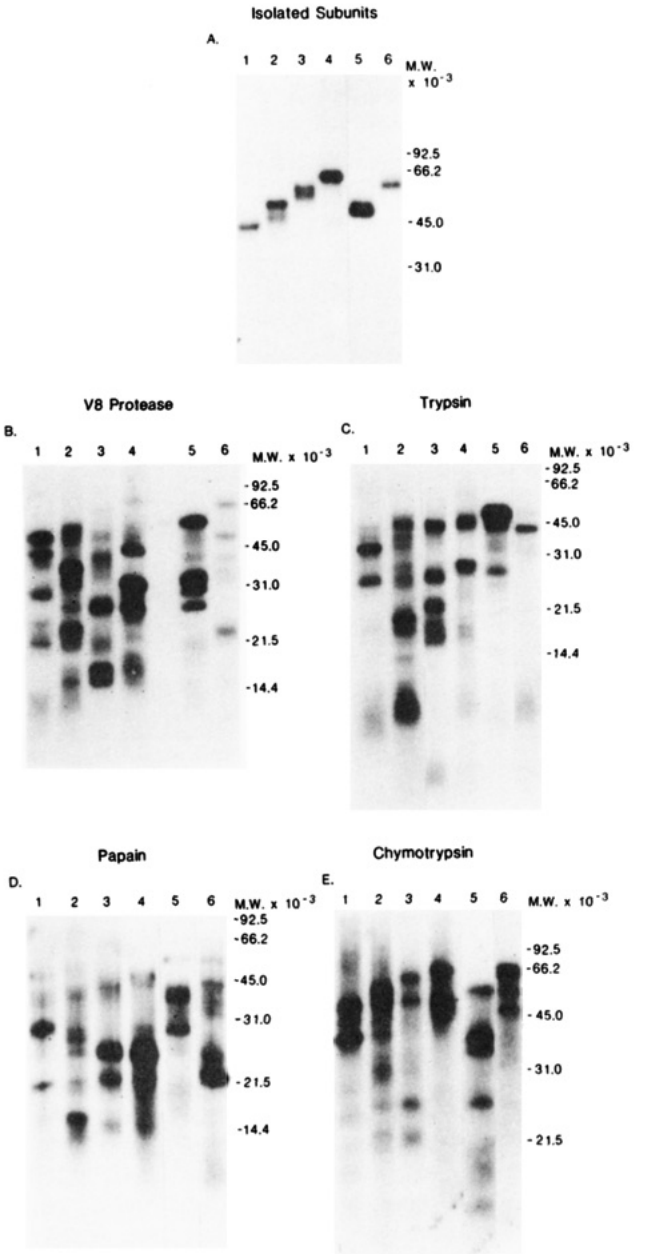

FIGURE 6: Peptide mapping on $NaDodSO_4$–15% acrylamide gels of ^{125}I-labeled subunits of *Torpedo* receptor and mAb 35 binding component. (A) Reelectrophoresis of the isolated subunits. (B) Peptide mapping after limited digestion with V8 protease. (C) Peptide mapping after limited digestion with trypsin. (D) Peptide mapping after limited digestion with papain. (E) Peptide mapping after limited digestion with chymotrypsin. Lane 1, *Torpedo* α subunit; lane 2, *Torpedo* β subunit; lane 3, *Torpedo* γ subunit; lane 4, *Torpedo* δ subunit; lane 5, mAb 35 binding component 48 000 molecular weight subunit; lane 6, mAb 35 binding component 59 000 molecular weight subunit.

ever, this may be misleading; peptide maps of the *Torpedo* subunits are clearly very different even though the polypeptides show extensive homology at the level of the primary amino acid sequence (Noda et al., 1983). In addition, the differences in the peptide maps of the two polypeptides demonstrate that the smaller of the two subunits is not a proteolytic artifact derived from the larger subunit.

Western Blot Analysis. Western blots of affinity-purified receptor from *Torpedo* electric organ and chick muscle, together with the purified mAb 35 binding component, were probed with anti-receptor antibodies in an attempt to identify homologous subunits. Antiserum to receptor α subunits and

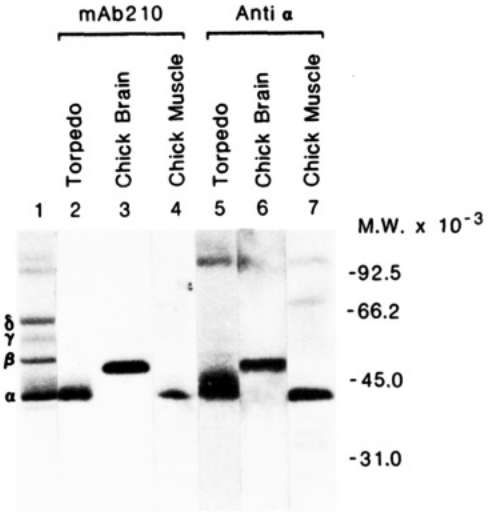

FIGURE 7: Western blots of *Torpedo* receptor, chick muscle receptor, and mAb 35 binding component probed with mAb 210 and anti-*Torpedo* α-subunit serum. Receptor subunits were separated by electrophoresis on a 10% acrylamide gel in $NaDodSO_4$ and then transferred to DPT paper. Lane 1, 50 μg of *Torpedo* receptor excised from the gel before transfer to DPT and stained for protein with Coomassie Blue; lanes 2 and 5, *Torpedo* receptor (50 ng); lanes 3 and 6, mAb 35 binding component (0.9 pmol); lanes 4 and 7, chick muscle receptor (43 ng). Lanes 2–4 were probed with a 10 nM concentration of mAb 210. Lanes 5–7 were probed with a 1.3 nM concentration of antiserum to *Torpedo* α subunits. Bound antibodies were visualized by incubation with ^{125}I-labeled goat anti-rat IgG and subsequent autoradiography.

the α subunit specific mAb 210 bound to a single subunit in all three preparations (Figure 7). The α subunits in the *Torpedo* and chick muscle preparations were of identical apparent molecular weight (41 200 ± 1000, mean ± standard deviation of five determinations) and differed from the single subunit revealed in the mAb 35 binding component preparation (47 800 ± 1300, mean ± standard deviation of five determinations).

Western blot analyses of the mAb 35 binding component using antisera to *Torpedo* β, γ, and δ subunits did not reveal any binding to subunits. Specific binding to the region corresponding to the dye front of the polyacrylamide gel was noted, however, suggesting that there may be binding to small proteolytic fragments of subunits yet to be identified. Control blots probed with normal rat serum showed no such binding.

To further investigate the binding of antisera to α subunits and mAb 210, Western blots of *Torpedo* receptor and the mAb 35 binding component which had been digested with V8 protease were similarly probed (Figure 8). Antiserum to the α subunit bound to fragments of apparent molecular weights 30 300 and 20 200. mAb 210 also bound to two fragments, apparent molecular weights 24 600 and 20 200. Thus, the α subunit of *Torpedo* electric organ acetylcholine receptor shares at least two antigenic determinants with the 48 000 molecular weight α subunit of the mAb 35 binding component.

Endoglycosidase H Digestion. It has been previously noted that the α subunits of receptors from electric organ and muscle have a high-mannose oligosaccharide moiety which can be removed by digestion with endoglycosidase H (Merlie et al., 1982; Gershoni et al., 1983). To investigate whether the 48 000 molecular weight subunit of the mAb 35 binding component is similarly glycosylated, it was digested with various concentrations of endoglycosidase H and the mobility of the subunit upon polyacrylamide gel electrophoresis subsequently investigated. Figure 9 confirms that *Torpedo* α subunit shows a decrease of about 1700 in apparent molecular weight upon

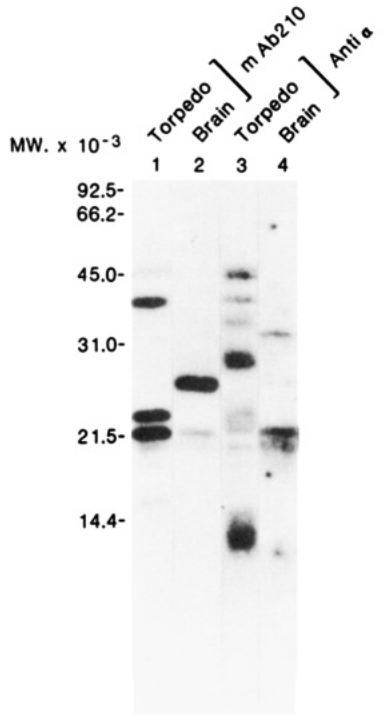

FIGURE 8: Western blots of peptide maps of *Torpedo* receptor and mAb 35 binding component. After proteolysis of the receptors with V8 protease, as in Figure 6, peptide fragments were separated by electrophoresis on a 15% acrylamide gel in $NaDodSO_4$ and then transferred to DPT paper. Lanes 1 and 3, *Torpedo* receptor (250 ng); lanes 2 and 4, mAb 35 binding component (0.6 pmol). Lanes 1 and 2 were probed with 10 nM mAb 210. Lanes 3 and 4 were probed with 1.3 nM antiserum to *Torpedo* α subunits.

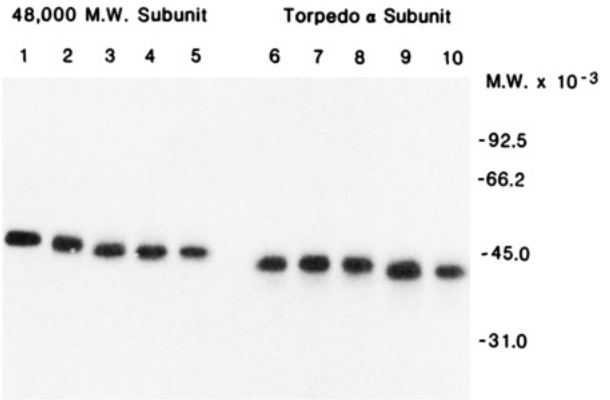

FIGURE 9: Digestion of ^{125}I-labeled *Torpedo* receptor α subunit and mAb 35 binding component 48 000 molecular weight subunit with endoglycosidase H. Lanes 1–5, mAb 35 binding component subunit; lanes 6–10, *Torpedo* α subunit; lanes 1 and 6, no enzyme; lanes 2 and 7, 0.01 milliunit of enzyme; lanes 3 and 8, 0.1 milliunit of enzyme; lanes 4 and 9, 1.0 milliunit of enzyme; lanes 5 and 10, 10 milliunits of enzyme. Digests were electrophoresed on 10% acrylamide gels in $NaDodSO_4$ and autoradiographed.

treatment with endoglycosidase H. The 48 000 molecular weight subunit of the mAb 35 binding component underwent a similar decrease in apparent molecular weight of about 2100. Thus, these two subunits are similar in having high-mannose oligosaccharide side chains.

Sucrose Gradient Analysis. ^{125}I-Fab fragments of mAb 35 were used to specifically label the mAb 35 binding component for density gradient centrifugation (Figure 10). The Triton X-100 solubilized mAb 35 binding component sedimented as a single peak slightly larger than *Torpedo* receptor monomers. The same peak could also be detected when the mAb 35

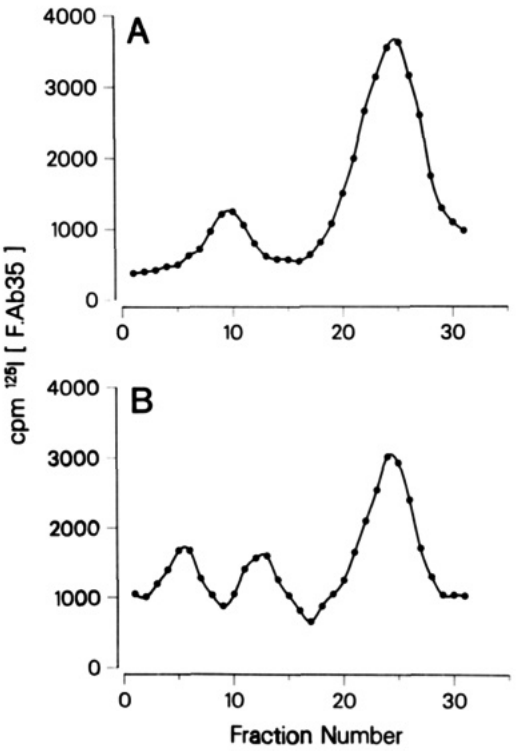

FIGURE 10: Sucrose gradient sedimentation of the mAb 35 binding component. (A) Chick brain extract (0.25 nM mAb 35 binding sites) labeled with 0.5 nM ^{125}I-Fab 35. (B) *Torpedo* receptor (50 nM) labeled with 0.5 nM ^{125}I-Fab 35. The mAb 35 binding component peaked at fraction 10, *Torpedo* dimers peaked at fractions 5–6, and monomers peaked at fraction 13. The peak at fractions 24–25 was unbound ^{125}I-Fab 35.

affinity column eluate was similarly analyzed (data not shown).

Number of mAb 35 Binding Sites. The number of mAb 35 binding sites per brain component molecule was investigated by using an antibody capture assay. mAb 35, immobilized indirectly on Millititer plates by affinity-purified goat anti-rat IgG, was able to specifically bind ^{125}I-Fab 35 labeled component from chick brain extract (data not shown). Thus, there are at least two mAb 35 binding sites per molecule of mAb 35 binding component.

The anti-MIR antibodies mAb 35 and mAb 210 compete for binding to receptors from both electric organ and muscle (Lindstrom et al., 1983; S. Hochschwender and J. Lindstrom, unpublished results). This is also true for the mAb 35 binding component; the two mAbs compete for binding to the affinity-purified mAb 35 binding component (data not shown). Thus, mAb 35 probably binds to the 48 000 molecular weight subunit, of which there must be at least two per mAb 35 binding component molecule.

Antisera Studies. Lewis rats were immunized with affinity-purified mAb 35 binding component. The serum antibody titers were measured by solid-phase immunoassay using the affinity-purified mAb 35 binding component immobilized upon Millititer plates and ^{125}I-labeled goat anti-rat IgG to detect bound antibodies. Purified normal rat IgG was bound to the plates in known amounts to calibrate the ^{125}I-labeled goat anti-rat IgG binding. The antibody concentration is reported as the moles of IgG per liter capable of binding to the purified brain component. All rats developed a high titer against the immunogen (Table II). None of the rats exhibited any signs of experimental autoimmune myasthenia gravis. This probably reflects the denaturation of the receptor (both by acid treatment and by lyophilization), preventing formation of antibodies to the MIR, which is the primary pathogenic specificity in

Table II: Cross-Reactivity of Antisera Raised against the mAb 35 Binding Component

	titer (nM)			
antibody	mAb 35[a] binding component	brain[b] toxin binding protein	muscle[c] receptor	*Torpedo*[c] receptor
serum 1	66 000	0	0	33
serum 2	5 100	0	0	0.19
serum 3	4 900	0	0	0
serum 4	3 300	0.26	0.95	1.90
serum 5	11 000	0	0	0
mAb 35	16 800	0.38	280	83000

[a] Determined by Millititer solid-phase immunoassay. [b] Determined by immunoprecipitation of ^{125}I-αBgt binding sites. Chick brain extract (100 μL of ~0.7 nM αBgt binding sites, depending upon the preparation, as measured by DEAE assay), labeled with 10 nM ^{125}I-αBgt, was incubated overnight at 4 °C with antisera or mAb 35 (5 μL) and subsequently immunoprecipitated with goat anti-rat IgG. The precipitates were pelletted and washed twice with PBS and 0.5% Triton X-100, and bound radioactivity was determined by γ counting. Nonspecific binding was determined in the presence of normal rat serum. Titers are expressed in moles of αBgt binding sites precipitated per liter of antibody. [c] Chick muscle extract (100 μL, 1.5 nM αBgt binding sites) or *Torpedo* receptor (2 nM αBgt binding sites) was labeled with 5 nM ^{125}I-αBgt and incubated overnight at 4 °C with antisera (0.1–5 μL) or mAb 35 (0.001–0.1 μL) and then immunoprecipitated described previously.

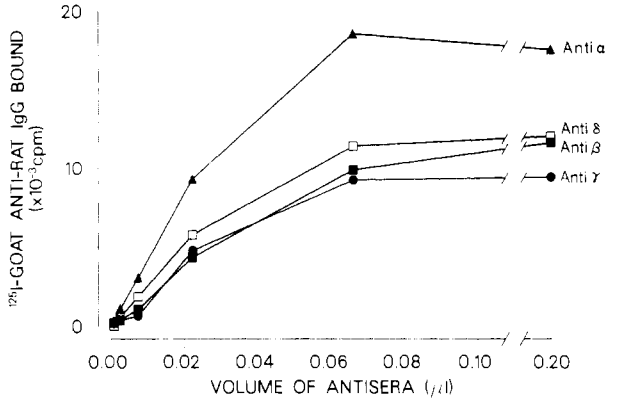

FIGURE 11: Binding of anti-*Torpedo* receptor subunit sera to immobilized mAb 35 binding component. Affinity-purified mAb 35 binding component (10 fmol) was immobilized overnight on wells of a Millititer plate and then probed with the indicated amounts of antisera (titer approximately 2×10^{-6} M; Lindstrom et al., 1979b) diluted in 30 μL of quenching buffer. Bound antibody was detected by subsequent addition of ^{125}I-labeled goat anti-rat IgG as described under Materials and Methods. Nonspecific binding, determined by using equivalent amounts of normal rat serum, has been subtracted. (▲) Antiserum to α subunits; (■) β subunits; (●) γ subunits; (□) δ subunits.

myasthenic sera (Tzartos & Lindstrom, 1980; Tzartos et al., 1982), but may also indicate a lack of cross-reactivity between the mAb 35 binding component and rat muscle receptor. Titers of the antisera against ^{125}I-αBgt-labeled receptor from *Torpedo* or chick muscle, as measured by immunoprecipitation, were found to be minimal, suggesting that there is very limited immunological cross-reactivity between those receptors and the mAb 35 binding component. Additionally, these antisera failed to precipitate any ^{125}I-αBgt binding sites from detergent-solubilized chick brain; mAb 35 similarly demonstrated essentially no binding to the αBgt binding component.

Three additional approaches were taken to investigate whether *Torpedo* acetylcholine receptor subunits share antigenic determinants with the mAb binding component: (1) binding of anti-*Torpedo* subunit sera to immobilized mAb 35 binding component; (2) immunoprecipitation of ^{125}I-labeled

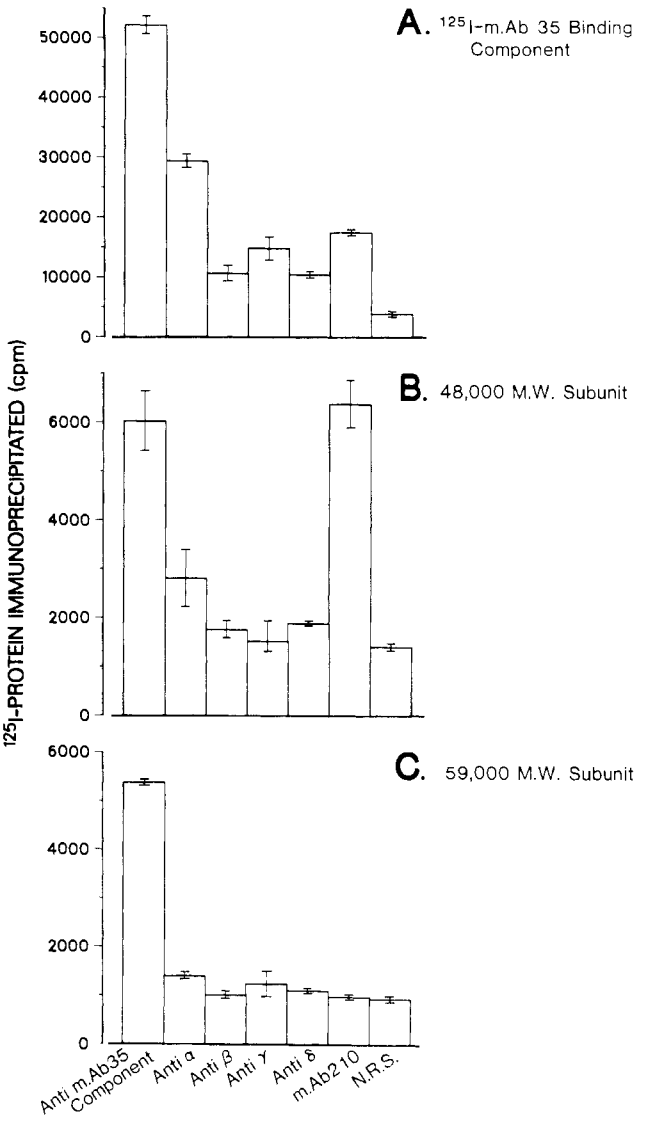

FIGURE 12: Immunoprecipitation of the mAb 35 binding component and its subunits. Serum or mAb (5 μL) was incubated overnight at 4 °C with (A) 10^5 cpm of ^{125}I-mAb 35 binding component, (B) 25 000 cpm of ^{125}I-labeled 48 000 molecular weight subunit, or (C) 15 000 cpm of ^{125}I-labeled 59 000 molecular weight subunit in a final volume of 100 μL of PBS and 0.5% Triton X-100. The antibody–antigen complexes were precipitated with goat anti-rat IgG, pelletted, and washed twice with PBS and 0.5% Triton X-100, and radioactivity was quantitated by γ counting. The antisera to *Torpedo* receptor subunits had titers of approximately 2.5×10^{-6} M, and mAb 210 had a titer against *Torpedo* receptor of 28×10^{-6} M. Each column is the mean ± standard deviation of triplicate determinations. (1) Antisera to mAb 35 binding component; (2) *Torpedo* receptor α subunits; (3) *Torpedo* β subunits; (4) *Torpedo* γ subunits; (5) *Torpedo* δ subunits; (6) mAb 210; and (7) normal rat serum (NRS).

mAb 35 binding component by anti-*Torpedo* subunit sera; and (3) binding of anti-mAb 35 binding component sera to Western blots of *Torpedo* receptor. Probing of affinity-purified mAb 35 binding component immobilized on Millititer plates clearly demonstrated binding to this component of antisera to the α, β, γ, and δ subunits of receptor from *Torpedo* (Figure 11). Antisera to α subunits exhibited the greatest binding, as might be expected if there were two α subunits in each receptor. Antisera to both α subunits and mAb 210 were able to immunoprecipitate the ^{125}I-mAb 35 binding component and the 48 000 molecular weight subunit (Figure 12A,B), which is consistent with data from Western blot analysis (see Figure 7) in suggesting that this component is an α-subunit analogue. Antisera to β, γ, and δ subunits of *Torpedo* precipitated only

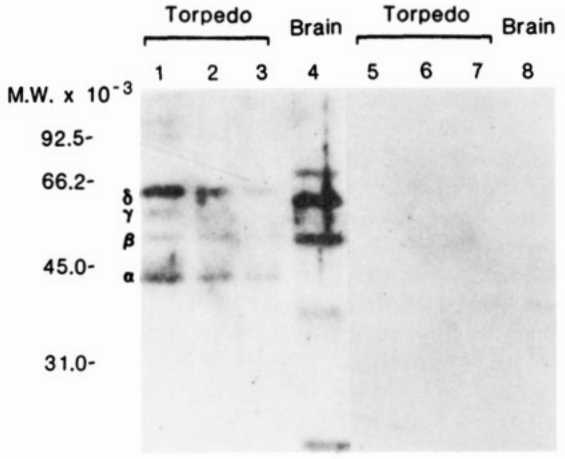

FIGURE 13: Western blots of *Torpedo* receptor and mAb 35 binding component probed with antiserum to the mAb 35 binding component. Lanes 1 and 5, 2500 ng of *Torpedo* receptor; lanes 2 and 6, 250 ng of *Torpedo* receptor; lanes 3 and 7, 25 ng of *Torpedo* receptor; lanes 4 and 8, 0.1 pmol of mAb 35 binding component. Lanes 1–4 were probed with a 1:1000 dilution of antiserum 1 to the mAb 35 binding component (Table II). Lanes 5–8 were probed with a 1:1000 dilution of normal rat serum as a control for nonspecific binding.

about 10% of the total ^{125}I-mAb 35 binding component; this did, however, represent greater than 2.5-fold the immunoprecipitation by normal rat serum. None of the antisera immunoprecipitated the radiolabeled 59 000 molecular weight subunit (Figure 12C), suggesting that it does not contain detectable cross-reacting determinants. This again is consistent with results from Western blot analysis.

The third attempt to identify homology between *Torpedo* receptor subunits and the mAb 35 binding component utilized a reciprocal approach of probing Western blots of *Torpedo* receptor with antisera to the mAb 35 binding component (Figure 13). The antisera bound all four subunits of *Torpedo*, which is further evidence that there are antigenic determinants shared by all four subunits of *Torpedo* receptor and the brain component. Probing Western blots of the affinity-purified mAb 35 binding component with its cognate antisera (Figure 13, lanes 4 and 8) demonstrated that the 48 000 and 59 000 molecular weight polypeptides are the most immunogenic components of the antigen preparation. The 30 000 molecular weight polypeptide was probably a proteolytic fragment of the 48 000 molecular weight subunit, since mAb 210 occasionally bound to a fragment of this molecular weight on immunoblots. The additional polypeptide of molecular weight 66 000 occasionally appeared in both silver-stained and ^{125}I-labeled preparations. Since it never coprecipitated with the 48 000 and 59 000 molecular weight subunits upon incubation with either concanavalin A Sepharose, mAb 35 Sepharose or mAb 210 bound to goat anti-rat IgG–Sepharose, it was confirmed as a contaminant.

Discussion

We report here the purification and initial characterization of a neuronal component bound by mAb 35, a mAb directed against the MIR of acetylcholine receptors from electric organs and muscle. The neuronal component has several properties in common with receptor from electric organ or mammalian skeletal muscle. Both are integral membrane proteins, negatively charged at physiological pH, which behave similarly on sucrose gradients, although the mAb 35 binding component is slightly larger. Unlike muscle or electric organ acetylcholine receptors, the mAb 35 binding component does not bind α-toxins. Both can be affinity labeled by [^{3}H]BAC, demonstrating the existence of an acetylcholine binding site with an adjacent disulfide bond. α-Toxins inhibit the affinity labeling of muscle or electric organ receptor but not of the mAb 35 binding component. The acetylcholine binding site has nicotinic pharmacological properties. Electric organ and skeletal muscle acetylcholine receptors have two mAb 35 binding sites per receptor monomer. The brain component also has more than one mAb 35 binding site per molecule, although the exact stoichiometry was not determined. Electric organ and skeletal muscle receptor have four types of subunits, one of which (41 000 molecular weight) binds antisera to α subunits and mAb 210. The mAb 35 binding component has two identified subunits, the smaller of which (48 000 molecular weight) also binds antisera to α subunits and mAb 210. This clearly suggests that the 48 000 molecular weight subunit is homologous to α subunits of receptors from muscle. The α subunits from the receptors of both electric organ and muscle have a mannose-rich oligosaccharide chain which can be removed by digestion with endoglycosidase H. This was also found for the analogous subunit of the brain component. The increase in mobility of the glycosidase-treated *Torpedo* α subunit and its mAb 35 binding component analogue was in both cases about 2000 daltons, suggesting that the carbohydrate moieties were of similar size.

There are several lines of evidence which suggest that subunits additional to the two so far identified exist which have antigenic determinants shared with *Torpedo* β, γ, and δ subunits: (1) antisera to β, γ, and δ subunits bound to the mAb 35 binding component immobilized on Millititer plates; (2) antisera to β, γ, and δ subunits bound to Western blots of the brain component at a mobility corresponding to the dye front, which suggests they bind to very small proteolytic fragments; (3) antisera to β, γ, and δ subunits precipitated a small yet significant proportion of ^{125}I-mAb 35 binding component; (4) antisera raised against the purified mAb 35 binding component specifically bound to all four subunits of receptor from electric organ on Western blots; and (5) Swanson and co-workers have previously demonstrated by immunohistochemical techniques the binding of mAbs specific for the α, β, γ, and δ subunits of receptor from *Torpedo* to the lateral spiriform nucleus of chick brain (Swanson et al., 1983). One likely possibility is that additional subunits bearing these antigenic determinants have been proteolytically cleaved during the purification procedure such that when dissociated by $NaDodSO_4$ and resolved on polyacrylamide gels they are only present as a heterogenous population of very small fragments running at the dye front. This phenomenon has been observed during the purification of other acetylcholine receptors. For example, receptor from *Torpedo* can be proteolytically degraded so as to appear to contain only α subunits, or even no subunits, yet still retain antigenic determinants corresponding to each subunit, retain its characteristic size and shape, and retain its function (Lindstrom et al., 1980). Similarly, bovine receptor preparations can appear to consist of only the α subunit upon polyacrylamide gel electrophoresis if proteolysis is not carefully controlled during purification (Einarson et al., 1982). Finally, only the α and β subunits of the receptor from the mouse neuroblastoma cell line BC3H-1 are usually observed (Merlie & Lindstrom, 1983) although it is certain than the γ and δ subunits of this molecule exist.

Acetylcholine receptors from chick muscle clearly differed considerably from the brain component, both in immunological cross-reactivity (Table II) and in the molecular weight of the subunits bound by antisera to α subunits and mAb 210 (Figure

7). This strongly suggests that the α-subunit analogues of these proteins are encoded by different genes.

Several lines of evidence support the idea that the molecule characterized here is a physiologically relevant neuronal acetylcholine receptor. Previous studies have demonstrated that the mAb 35 binding component chick brain is distinct from the αBgt binding protein; they are localized in different areas of the brain (Swanson et al., 1983), and on chick ciliary ganglia, the mAb 35 binding component was clearly found at the synapse (as would be expected for a neuronal receptor), whereas the αBgt binding protein was nonsynaptic (Jacob et al., 1984). Distinguishing the mAb 35 binding component from the αBgt binding protein in ciliary ganglia is important because there is a great deal of evidence showing that in ciliary ganglia αBgt does not inhibit cholinergic synaptic transmission (Brown & Fumigalli, 1977; Kouvelas et al., 1978; Carbonetto et al., 1978; Ravdin & Berg, 1979; Ascher et al., 1979). The present study demonstrates biochemically that the mAb 35 binding component does not bind α-toxin: mAb 35 and antisera to the purified mAb 35 binding component failed to significantly immunoprecipitate ^{125}I-αBgt binding sites from detergent extracts of chick brain (Table II), α-toxin–Sepharose failed to deplete any mAb 35 binding sites from chick brain extract (Figure 3A), and mAb 35–Sepharose failed to deplete any αBgt binding sites from brain extract (Figure 3B). Furthermore, the subunit structure of the mAb 35 binding component differs from that reported for the toxin binding protein of chick optic lobe. Betz et al. (1982) identified a single component of apparent molecular weight 57 000, while Barnard and co-workers first identified a similar component (Norman et al., 1982) and more recently have reported two additional components of apparent molecular weights 48 000 and 69 000 (Conti-Tronconi et al., 1985). The amino-terminal amino acid sequence of the 48 000 molecular weight component was reported to resemble that of the α subunit of both *Torpedo* and muscle receptors (Conti-Tronconi et al., 1985). This is a surprising result because it was previously reported that the 57 000 molecular weight component could be specifically labeled with [^{3}H]BAC (Norman et al., 1982). Since the 48 000 molecular weight subunit of the mAb 35 binding component was also found to be homologous (by immunological techniques) to the α subunit, one may propose that they are in fact the same polypeptide. This is, however, highly unlikely, not only because we have demonstrated that the mAb 35 binding component does not bind α neurotoxins but also because the two polypeptides apparently differ in their sensitivity to proteases; the 48 000 molecular weight polypeptide of the toxin binding protein was found to be highly sensitive to proteolytic degradation (Conti-Tronconi et al., 1985) whereas the polypeptide described in this paper was observed to be relatively resistant to proteolysis, remaining intact even after relatively long purification procedures. In this respect, the 48 000 molecular weight polypeptide from the mAb 35 binding component resembles the α subunits from electric organ and muscle receptors, which, as discussed above, are also relatively resistant to the action of endogenous proteases.

Cholinergic transmission in chick ciliary ganglia is not blocked by αBgt. As described above, these cells have a synaptically located mAb 35 binding component (Jacob & Berg, 1983). Biochemical studies have shown that it has several of the properties described here for the mAb 35 binding component from chick brain. It is an integral membrane glycoprotein which sediments on a sucrose gradient slightly larger than *Torpedo* monomers and whose amount is modulated by cholinergic ligands (Smith et al., 1985a,b). It also does not bind αBgt. Most significantly, we have recently found that antisera raised against the mAb 35 binding component purified from chick brain specifically block the acetylcholine-induced depolarization of chick ciliary ganglion neurons in cell culture when applied at a 1:100 dilution (J. Stollberg, D. Berg, P. Whiting, and J. Lindstrom, unpublished results), clearly suggesting cross-reactivity between these two components and also that both are physiologically relevant neuronal acetylcholine receptors.

We have presented here clear evidence that the mAb 35 binding component from chick brain is a neuronal acetylcholine receptor. More studies are necessary to further define the structure of the receptor from chick brain and its developmental and evolutionary relationship to receptor from muscle and αBgt binding sites on neurons. mAbs raised against this molecule will be useful tools for biochemical and molecular genetic studies, and for investigating the function of this molecule. Additionally, they may provide probes for the mammalian neuronal nicotinic acetylcholine receptor, about which currently very little is known.

Added in Proof

Receptors immunoisolated from extracts of chicken and rat brains have been shown to be high-affinity nicotine binding sites, and 80% of the high-affinity nicotine binding sites in brain are capable of binding to antibodies raised against the purified receptors described in this paper (Whiting and Lindstrom, submitted for publication). Monoclonal antibodies to the purified receptors described here have been shown to label rat brain sections with the same pattern as does [^{3}H]-nicotine (Larry Swanson, Paul Whiting, Donna Simmons, and Jon Lindstrom, unpublished results).

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Registry No. BAC, 17139-54-7; carbachol, 51-83-2.

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