Transcriptional Regulation of Neuronal Nicotinic Acetylcholine Receptor Genes

A POSSIBLE ROLE FOR THE DNA-BINDING PROTEIN Purα*

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Qun Du, Alan E. Tomkinson, and Paul D. Gardner‡

From the Department of Molecular Medicine, Institute of Biotechnology, University of Texas Health Science Center, San Antonio, Texas 78245-3207

Nicotinic acetylcholine receptors constitute a multigene family (α2–α9, β2–β4) expressed in discrete temporal and spatial patterns within the nervous system. The receptors are critical for proper signal transmission between neurons and their targets. The molecular mechanisms underlying receptor gene expression have not been completely elucidated but clearly involve regulation at the level of transcription. We previously identified a novel 19-base pair (bp) transcriptional regulatory element in the promoter region of the rat β4 subunit gene. This 19-bp element interacts specifically with DNA-binding proteins enriched in nuclear extracts prepared from adult rat brain. Using a combination of cellulose-phosphate, DNA-cellulose, and DNA sequence-specific affinity chromatographies, we purified the 19-bp element binding activity approximately 19,000-fold. Analysis by denaturing gel electrophoresis revealed the presence of four polypeptides in the most purified fraction, ranging in molecular masses between 31 and 114 kDa. Peptide sequence analysis revealed that one of the polypeptides is the bovine homologue of the transcriptional regulatory factor, Purα. Electrophoretic mobility shift assays indicated that Purα interacts directly and specifically with the 19-bp element. In addition, mobility shift assays using an anti-Purα monoclonal antibody revealed the presence of Purα, or an immunologically related protein, in nuclear extracts prepared from brain tissue. We hypothesize that the interaction between Purα and the 19-bp element is critical for proper expression of the β4 subunit gene.

One of the key events that takes place during development of the nervous system is the formation of synapses. Numerous lines of evidence indicate that expression of neurotransmitter sensitivity is central to synaptogenesis (reviewed in Ref. 1). It is also clear that changes in sensitivity to neurotransmitters are due to changes in the expression of neurotransmitter receptors. Recent advances toward a molecular understanding of the formation of nicotinic cholinergic synapses in the nervous system were made with the isolation of a family of genes (α2–α9 and β2–β4) encoding subunits of the nicotinic receptors for acetylcholine (nACh); reviewed in Ref. 2). Functionally diverse nACh receptors can be generated by distinct combinations of the subunits in vitro (3–5). In addition, each of the subunits exhibits discrete temporally and spatially restricted expression patterns in vivo (2). Despite these important advances, however, the cellular and molecular mechanisms controlling the expression of the nACh receptor genes are relatively obscure, although several recent studies suggest that both positive and negative transcriptional regulatory mechanisms are involved in receptor gene expression (6–16).

Three of the nACh receptor genes, those encoding the β4, α3, and α5 subunits, are tightly linked within the rat genome, spanning only approximately 60 kilobase pairs (17). This genomic organization, coupled with the high nucleotide sequence similarities between the three genes, suggests that the genes arose through tandem duplication of a common ancestral gene as has been suggested previously (17). On the other hand, the genomic organization may also reflect a regulatory mechanism that ensures co-expression of the genes in the appropriate cell types at the appropriate developmental time. Such a mechanism would be consistent with recent data indicating the presence, in vivo, of receptors consisting of β4, α3, and α5 subunits (18). However, the expression of these genes is not completely overlapping (2) and, thus, some other mechanism must account for this disparity. One possibility is that while each of the subunit genes is independently regulated transcriptionally, they share some common regulatory features, which are active in the appropriate developmental and cellular environments. Several laboratories, including ours, have characterized distinct promoter regions for the β4 (10, 11, 14) and α3 (9, 15, 16) subunit genes. Within these regions, several transcriptional regulatory elements have been identified. Thus far, the only regulatory element common to both the β4 and α3 genes is a consensus Sp1-binding site, which appears critical for transcription of the two genes (9, 14). Our analysis of the rat β4 subunit promoter region led to the identification of three elements, E1–E3, which may play roles in β4 gene expression (14). E2 is the aforementioned Sp1-binding site, E3 shares some sequence homology with the consensus AP1-binding site, and E1 is a novel 19-bp regulatory element (14). E1, characterized by three CCCT repeats, forms specific complexes with proteins present in nuclear extracts prepared from neuronal cells (11, 14). We have now used E1 as an affinity ligand in a purification scheme to isolate, from bovine brain nuclear extracts, the nuclear proteins which bind specifically to this element. In the most highly purified fraction, four polypeptides with apparent

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‡ To whom correspondence should be addressed: Dept. of Molecular Medicine, Institute of Biotechnology, University of Texas Health Science Center, 15355 Lambda Dr., San Antonio, TX 78245-3207. Tel.: 210-567-7251; Fax: 210-567-7247; E-mail: gardner@uthscsa.edu.

1 The abbreviations used are: nACh, nicotinic acetylcholine; NARP, neuronal nACh receptor promoter-binding protein; bp, base pair(s); EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
molecular masses of 31, 43, 65, and 114 kDa were detected. We termed these E1-binding proteins, neuronal nACh receptor-promoter-binding proteins (NARP41, -43, -65, and -114). Peptide sequence analysis indicated that NARP43 is the bovine homologue of Purα, a DNA-binding protein previously shown to stimulate transcription of the myelin basic protein gene in oligodendrocyte cells (19). Electrophoretic mobility shift assays (EMSA) demonstrated that Purα is present in nuclear extracts prepared from brain and binds directly and specifically to E1, raising the possibility that this interaction plays a role in the expression of the β4 subunit gene.

EXPERIMENTAL PROCEDURES

Buffers—Buffer C consisted of 25 mM HEPES (pH 7.8), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 20% glycerol. Buffer D consisted of 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Buffer E consisted of 20 mM HEPES (pH 7.5), 50 mM NaCl, 5 mM EDTA, 1 mM DTT, 10% glycerol. Binding buffer consisted of 20 mM HEPES (pH 7.4), 5 mM DTT, 1 mM MgCl₂, 50–100 mM KCl, and 10% glycerol. Buffer Z consisted of 25 mM HEPES (pH 7.6), 10 mM MgCl₂, 1 mM DTT, 20% glycerol, and 0.1% Nonidet P-40.

Preparation for Electrophoretic Mobility Shift Assays—The sequence of E1 used as a probe and competitor in EMSA is shown in Fig. 1, along with the sequence of a mutated form of the element used as a competitor in EMSA. The E1 complementary oligonucleotides (from Crucchem, Inc.) were annealed and then radioactively labeled with γ-[32P]ATP (DuPont NEN) using T4 polynucleotide kinase (Promega). The specific activities of the probes were typically between 14,000 and 17,000 cpm/fmol. Competitor oligonucleotides were prepared by annealing equal amounts of non-radioactive complementary oligonucleotides.

Preparation of Nuclear Extracts—Nuclear extracts were prepared as described by Christy et al. (20). Bovine brains were obtained within 1 h of death from a local slaughterhouse (Kiolbassa Provision Company), while fresh rat brains were from adult female Harlan Sprague Dawley animals (Charles River Laboratories). The final rat brain nuclear extract pellet was resuspended in buffer C, while the final bovine brain nuclear extract pellet was resuspended in buffer D in preparation for phosphocellulose chromatography (see below). The nuclear extracts were dialyzed overnight in the buffer in which they were resuspended. Insoluble material was removed by centrifugation.

Protein Assays—During phosphocellulose and DNA-cellulose chromatography, protein was monitored by measuring absorbances at 280 nm. Protein concentrations were determined by using bicinchoninic acid (Pierce).

Electrophoretic Mobility Shift Assays—E1 binding activity was detected using EMSA as described previously (11). To demonstrate sequence specificity of the DNA-protein complexes, competition experiments were done with a 15-min preincubation of unlabeled double-stranded oligonucleotides prior to the addition of labeled oligonucleotide. The amount of DNA present in specific DNA-protein complexes was quantified using a Molecular Dynamics PhosphorImager and Imagequant software. Antibody supershift experiments were performed by incubating 1 μg of a monoclonal antibody directed against Purα (9C12, Ref. 24) or a nonrelated preimmune serum with 0.5 μg of bovine brain nuclear extract for 10 min at 37 °C before the addition of specific probe. Following a 1-h incubation at 4 °C, the binding reactions were analyzed by electrophoresis through 6% native acrylamide gels and visualized by autoradiography.

Purification of E1-binding Proteins—Crude nuclear extract from bovine brain (365 mg) was applied directly onto a 1.6 × 50-cm 511 phosphocellulose column (Whatman) equilibrated with 50 mM Tris-HCl (pH 7.5) and 50 mM NaCl. After washing with three column volumes of the same buffer, bound proteins were eluted with a 1-liter linear gradient of NaCl from 50 mM to 800 mM in 50 mM Tris (pH 7.5). Approximately 9-ml fractions were collected and assayed for E1 binding activity using EMSA as described above. Fractions with binding activity (11 mg) were pooled, concentrated by ultrafiltration, dialyzed against buffer E, and then loaded onto a native calf thymus DNA-cellulose column (10 ml), which was equilibrated with buffer E. After washing with three column volumes of the same buffer, bound proteins were eluted with a 100-mI linear gradient of NaCl from 50 mM to 1.0 mM in buffer E. Fractions containing E1 binding activity (2.5 mg) were pooled, and dialyzed against buffer E in preparation for DNA sequence-specific affinity chromatography. Preparation and regeneration of the E1 affinity column was done essentially as described by Kadonaga and Tjian.

RESULTS AND DISCUSSION

We previously identified several transcriptional regulatory elements, E1–E3 (Fig. 1), in the rat β4 subunit gene (14). One of these elements, E2, is a binding site for the regulatory factors, Sp1 and Sp3 (14),2 while the identities of the proteins interacting with E1 and E3 are unknown. The study described below focuses upon E1 and the proteins with which it interacts. A DNA Binding Activity That Interacts with E1 Is Conserved between Rat and Bovine.—Our earlier analyses of the β4 subunit gene focused upon the rat gene. As there are advantages for using bovine brain versus rodent brain as a source of nuclear extract, we determined whether nuclear extracts from

2 C. B. Bigger, I. N. Melnikova, and P. D. Gardner, unpublished results.
are sequence-specific and bind to the column (Fig. 3).

The origin of this nonspecific DNA-protein complex is unknown, although it is clearly not complexes B or C as shown. The migratory species was not DNA sequence-specific (data not shown). Competition experiments indicated that this faster migrating species was not detected in the nuclear extract did not bind to the column (Fig. 2).

In addition, the faster migrating species was not present in
all nuclear extract preparations (data not shown). An approximately 6-fold purification of the specific E1 binding activity was achieved on the phosphocellulose column.

The E1 binding activity was fractionated further by native DNA-cellulose chromatography. The binding activity eluted from the column as a sharp peak between 600 and 750 mM NaCl (Fig. 3B), indicating that the protein responsible for E1 binding also binds to DNA in a sequence-independent manner. DNA-cellulose chromatography afforded an approximately 3-fold greater purification of the E1 binding activity.

The final chromatographic step was recognition site affinity chromatography on a column with covalently linked, catenated E1 oligonucleotides. The E1 binding activity bound to the affinity column and eluted at 350–500 mM KCl (Fig. 3C). Two passages of the E1 binding activity over the affinity column resulted in approximately 960-fold purification with a 40% yield in this step. A summary of the purification data is presented in Table I and indicates that, relative to the nuclear extract, the final purification was approximately 19,000-fold with a 5% recovery of activity.

Analysis of the protein content of the E1 binding activity by silver staining after SDS-PAGE revealed four major polypeptides with molecular masses of 31, 43, 65, and 114 kDa (Fig. 3D). We refer to these proteins as neuronal nACh receptor promoter-binding proteins, or NARP (i.e. NARP31, NARP43, etc.; Ref. 14).

NARP43 Is the Bovine Homologue of Purα—In several preparations of the E1 binding activity, NARP43 was the most abundant species; therefore, it was the first NARP to be subjected to amino acid sequence analysis. Following electrophoresis, NARP43 was electrophoblated onto a polyvinylidene difluoride membrane and digested with trypsin. The cleavage products were separated by high performance liquid chromatography, and four peptides were sequenced. The four amino acid sequences of these peptides were identical to sequences in the previously described Purα sequence (Fig. 4; Ref. 25). Purα is a sequence-specific DNA-binding protein that has recently been implicated in cell type-specific transcriptional regulation of myelin basic protein in oligodendrocytes (19). Its recognition element is purine-rich (25), as is the sequence of E1 (Fig. 1).

Pura Interacts Specifically with E1—To determine whether Purα does in fact interact with E1, EMSA were carried out with a GST-Purα fusion protein (24). As shown in Fig. 5, GST-Purα forms two major complexes with the radiolabeled E1 oligonucleotide, the larger of which is specific in that it can be competed away by an excess of unlabeled wild type E1 oligonucleotide but not by an excess of a mutant E1 oligonucleotide (see Fig. 1 for the sequences). GST alone does not form any specific complexes with E1 (Fig. 5).

To confirm that Purα interacts specifically with E1, the GST moiety was cleaved from the GST-Purα fusion protein, the GST moiety was removed by thrombin cleavage (see "Experimental Procedures"), and the recovered Purα protein was used in EMSA. As shown in Fig. 5, purified Purα interacts specifically with E1 forming one major complex that can be competed by an excess of wild type E1 oligonucleotide but not by mutant E1 oligonucleotide.

Pura or an Antigenically Related Protein Is Present in Brain Tissue—The results presented thus far strongly implicate Purα as playing a role in regulating the expression of the β4 subunit gene via interactions with E1. To determine whether Purα (or an immunologically related protein) is present in a more physiological context, we carried out supershift EMSA using a monoclonal antibody against Purα and crude nuclear extracts prepared from bovine brains. The extracts were incubated with one of three radiolabeled E1 probes (the coding strand, the noncoding strand, or double-stranded E1) and an anti-Purα.
Fig. 3. Purification of E1-binding proteins. A, phosphocellulose chromatography. The upper portion of the panel shows the protein profile as determined by monitoring the absorbances at 280 nm of every other fraction. E1 binding activity was detected by EMSA of every other fraction. The lower panel is an autoradiograph showing the E1 binding activity in the active fractions. Fractions 51–85 were pooled for further purification on DNA-cellulose. FT, flow-through. B, DNA-cellulose chromatography. The upper portion of the panel shows the protein profile as determined by monitoring the absorbances at 280 nm of every other fraction. E1 binding activity was detected by EMSA of every other fraction. The lower panel is an autoradiograph showing the E1 binding activity in the active fractions. Fractions 21–27 were pooled for further purification on the E1-affinity resin. FT, flow-through. C, E1 affinity chromatography. An autoradiograph showing the E1 binding activity in the active fractions after two rounds of affinity chromatography. Fractions 5–10 were pooled for SDS-PAGE. There is no protein profile as the quantity of protein was too low to detect by A_280 readings. FT, flow-through; W, wash fraction. D, SDS-PAGE. Nuclear extract (10 μg), the phosphocellulose pool (20 μg), the DNA-cellulose pool (10 μg), and the affinity-purified E1 binding activity (~0.2 μg) were analyzed by 10% SDS-PAGE and visualized by silver staining. The positions of molecular size markers run in an adjacent lane are indicated.
monoclonal antibody (24) prior to electrophoresis (see “Experimental Procedures”). Both single- and double-stranded probes were used to gain more insight into the interactions between Pur α and E1; Pur α has been shown to bind to both single- and double-stranded DNA targets (19). As shown in Fig. 6, incubation of the coding strand probe (ss) with nuclear extract led to the appearance of a single DNA-protein complex (lower arrow). The mobility of this complex was unaltered by the addition of anti-Pur α antibody or a preimmune serum. Similarly, incubation of the noncoding strand probe (ss′) or the double-stranded probe (ds) with nuclear extract led to the appearance of one major DNA-protein complex (Fig. 6, upper arrow). These complexes migrated more slowly than that seen with the coding strand probe raising the possibility that different proteins can interact with the two strands. In support of this hypothesis is the observation that the addition of anti-Pur α antibody led to a slight decrease in the mobilities of the complexes formed with the noncoding and double-stranded probes (Fig. 6, arrowhead). Although the decrease seen following addition of anti-Pur α antibody was not as large as might be expected given the size of the antibody, it may be a consequence of the overall electrostatic charge of the DNA–Pur α anti-Pur α antibody complex, such that the binding of antibody may not greatly change the overall charge of the DNA–protein complex. Given that separation through native acrylamide gels is based on charge rather than size, the small change in overall charge upon binding of antibody may not result in a large decrease in mobility. Nonetheless, these data suggest the presence of Pur α or an antigenically related protein, in bovine brain and further suggest that different proteins are capable of interacting with the cod-
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