Synergistic Activation of the \( \text{N-Methyl-d-aspartate Receptor Subunit 1 Promoter by Myocyte Enhancer Factor 2C and Sp1}^{*} \)

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The \( \text{N-Methyl-d-aspartate (NMDA)} \) subtype of glutamate receptor plays important roles in neuronal development, plasticity, and cell death. NMDA receptor subunit 1 (NR1) is an essential subunit of the NMDA receptor and is developmentally expressed in postnatal neurons of the central nervous system. Here we identify on the NR1 promoter a binding site for myocyte enhancer factor 2C (MEF2C), a developmentally expressed neuron/muscle transcription factor found in cerebrocortical neurons, and study its regulation of the NR1 gene. Co-expression of MEF2C and Sp1 cDNAs in primary neurons or cell lines synergistically activates the NR1 promoter. Disruption of the MEF2 site or the MEF2C DNA binding domain moderately reduces this synergism. Mutation of the Sp1 sites or the activation domains of Sp1 protein strongly reduces the synergism. Results of yeast two-hybrid and co-immunoprecipitation experiments reveal a physical interaction between MEF2C and Sp1 proteins. The MEF2C DNA binding domain is sufficient for this interaction. Dominant-negative MEF2C interferes with expression of NR1 mRNA in neuronally differentiated P19 cells. Growth factors, including epidermal growth factor and basic fibroblast growth factor, can up-regulate NR1 promoter activity in stably transfected PC12 cells, even in the absence of the MEF2 site, but the Sp1 sites are necessary for this growth factor regulation, suggesting that Sp1 sites may mediate these effects.

The NMDA\textsuperscript{1} subtype of glutamate receptor plays an important role in normal brain development as well as in long term potentiation (thought to be important for some forms of learning and memory). However, in a variety of pathologic conditions, including stroke, central nervous system (CNS) trauma, and various neurodegenerative disorders, overactivation of NMDA receptors contributes to neuronal injury or death (1–3).

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EXPERIMENTAL PROCEDURES

Plasmid Construction

All reporter constructs were prepared as described previously (19, 21). Site-directed mutagenesis of the NR1 promoter was performed by applying mutated primers to form a megaprimer in two steps of a polymerase chain reaction (PCR) (20, 33). Linearized pG356 and pG919 were used as templates in all PCRs. Site-directed mutagenesis of the tandem Sp1 sites was accomplished as described previously (21). For mutation of MEF2C, a megaprimer was synthesized with GB81 (\texttt{5' GTCTATGecTAAATAAATAACCAAAATTTC-3'}) and an upstream primer GB78 (\texttt{5'CCTAGAGACTCATACTTATGAGTAG-3'}), covering a native \texttt{KpnI} site at \texttt{919}. The megaprimer was used in the secondary
PCR with GB79 (5'-GAGTACCTTAGGGTCGAGTGGG-3') and XbaI adapter sequences (XbaI/I/I) to determine the presence of pGEM3Z plasmid and salmon sperm DNA were used as a carrier. pGEM3Z plasmid and salmon sperm DNA were used as blank controls. A luciferase cDNA fragment (XbaI/I/EcoRI/EcoRI/V, 1296 base pairs) was radiolabeled by random primer extension in the presence of [α-32P]dCTP and used to probe for the luciferase gene on blots. After stringent washing, blots were analyzed using a Phosphor Imager system (Molecular Dynamics). The amount of luciferase gene in each DNA sample was calculated based on the linear signal of standard luciferase cDNA on each blot. Copy numbers among pooled PC12 cell lines were similar to each other.

Quantification of mRNA

Total RNA was extracted from stably transfected PC19 cells using TRIZOL reagent (Life Technologies, Inc.). NR1 mRNA was measured with a reverse transcriptase-PCR using a PCR kit (CLONTECH). For synthesis of the first strand of cDNA, 1 µg of RNA was annealed with 100 ng of an NR1 promoter/reporter-PCR primer (TCCACACGCTGGTCTGTA, CLONTECH). The cDNA synthesis was carried out using avian myoblastosis virus reverse transcriptase (Invitrogen) at 42 °C for 1 h and stopped at 95 °C for 5 min. Then, 5' primers of NR1 (GTCCTCTGCTCATGGTTT) and glyceraldehyde-3-phosphate dehydrogenase (ACACAGTCTGACCCATAC) were added to Hot Tub polymerase (Amer sham) for 30 cycles on a Perkin-Elmer 9600 thermocycler as follows: 95 °C for 20 s, 60 °C for 10 s, and 72 °C for 1 s.

Yeast Two-hybrid System

An oligo(dT)-primed activation-tagged cDNA expression library was constructed from 22-week-old human frontal cortex, as described previously. The library contains 3.5 × 10^9 clones, >80% of which have a cDNA insert between 1 and 2 kilobase pairs. One-third of its members express a fusion protein containing a COOH-terminal with an acidic activation domain in a vector that allows galactose-inducible expression of the resulting fusion protein. We used a selection strain that contained 2LexAop-LEU2 and 2LexAop-LacZ reporters and a LexA fusion bait with the amino-terminal 86 residues of MEF2C (LexA-MEF2C86), which comprise the DNA-binding region of the protein. Although this bait could activate transcription in haploids, we found that this background transcription could be reduced by using a diploid yeast strain. The vector pJG4-5 expresses library-encoded proteins under the control of a galactose-inducible GAL1 promoter fused to a nuclear localization sequence, a hemagglutinin epitope tag, and a B42 activation domain. An interaction-trap selection was performed in a diploid yeast strain (EGY191xRFY206) which contains two reporters: a 2LexAop-LEU2 construct that replaced the yeast chromosomal LEU2 gene and an episomal 2opLexA-Gal1-LacZ gene. This strain was transformed with the brainto interaction library using the lithium acetate method (45) and with the “bait” LexA-MEF2C1–86. Full-length MEF2C could not be used because it strongly activated the reporters on its own. Isolated cDNAs were sorted into 12 classes based on their restriction mapping pattern. Partial sequence analysis of one class showed that these plasmids contained independent cDNAs derived from the same Sp1 mRNA. Full-length Sp1 was then reintroduced into the EGY-48 yeast strain that contained an 8opLacZ reporter gene and a 6opLexA-LEU2 gene. Alternatively, full-length MEF2C was cloned into a pG4 activation vector and introduced into the EGY-48 strain together with LexA and a panel of unrelated baits. Activation of the LacZ reporter was determined in β-galactosidase assays using the EGY48 yeast strain. Activation of the LEU2 reporter was determined by observing the growth rate of yeast colonies on complete minimal medium lacking leucine. The ability of the LexA-fusion to bind operator DNA was confirmed by a repression assay (45). We also verified that all LexA and B42 fusions were expressed at similar levels by immunoblotting with an antibody to LexA or to the hemagglutinin epitope tag on the B42 fusion vector (data not shown). The K_d of the MEF2C/Sp1 interaction is comparable to measured K_d values for large stretches of DNA (J = 10−8 s for 10^6 bases) (46, 47).

Immunoprecipitation

Metabolic labeling and precipitations were performed as described previously (45), except that cells were lysed in 250 mM NaCl, 50 mM HEPES-KOH (pH 7.5), 5 mM EDTA, 0.1 mM Na3VO4, 50 mM NaF, 0.1% Triton, 1 mM phenylmethylsulfonyl fluoride, and 30 µg/ml pepstatin for 45 min at 4°C. Pellets were run on 10% SDS-gels. For V8 mapping, bands were excised and partially digested in the gel with Staphylococcus aureus V8 protease (45). Products, including those resulting from digestion of in vitro translated MEF2C, were separated on 17% SDS-polyacrylamide gels. In vitro translation of MEF2C was performed according to the manufacturer’s instructions (Promega).

EMSA

The DNA-binding assays were carried out as described previously (22). Crude nuclear extracts of rat cerebrocortical cultures after 7 days

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RESULTS

MEF2C Binds to the MEF2 Site on the NR1 Promoter—Our previous results had demonstrated that Sp1 protein in crude cell extracts binds to the tandem Sp1 sites in the proximal region of the promoter of the NR1 gene (19). Here, we identified a sequence, TTATTTATAGA (positions −805 to −796), in this promoter (19), which matches the consensus of the MEF2 motif (CT/TA(AT)2TA(G/A) (48). Using an EMSA, we tested whether this putative site can be functionally bound by nuclear proteins in embryonic cerebrocortical neurons. As shown in Fig. 1, an A/T-rich sequence (positions −808 to −793) encompassing the MEF2 site in the NR1 promoter specifically formed a slowly migrating complex (lane 1). This complex was abrogated by competition with a 100-fold excess of unlabeled MEF2C consensus oligonucleotide (lane 2), but not by a similar concentration of previously characterized MEF2 mutant oligonucleotide (lane 3) (22). To further identify the complex, we used a MEF2C-specific antibody that does not cross-react with other MEF2 proteins (22). This antibody diminished this complex (lane 5) and confirmed that the putative MEF2 site in the NR1 gene is recognized by MEF2C. However, even at high antibody concentrations, part of the complex still remained (lane 5), suggesting the possible involvement of other members of the MEF2 family.

Synergistic Activation of the NR1 Promoter by MEF2C and Sp1—We next examined the contribution of MEF2C and Sp1 in the transcription of the NR1 gene. We co-transfected MEF2C and/or Sp1 cDNAs with an NR1 promoter luciferase reporter in Drosophila SL2 cells, which lack proteins related to mammalian MEF2C and Sp1 (49). The NR1 promoter, designated pNRL919, contains the MEF2 site and the tandem Sp1 sites. Sp1 alone activated the reporter 40-fold, whereas MEF2C stimulated expression less than 10-fold (Fig. 2). Surprisingly, co-expression of MEF2C plus Sp1 stimulated reporter expression >140-fold, which is at least 2.8 times the additive activities of these two factors, indicating a synergistic effect.

This synergism was mildly decreased either after co-expression of Sp1 with mutated MEF2C R24L, which does not bind to DNA (34), or after co-transfection of MEF2C and Sp1 with a promoter-reporter containing a mutation in the MEF2 site (pNRL919mtMEF2). These findings suggest that, after overexpression, MEF2C may act through protein-protein interactions with Sp1 rather than solely through DNA binding to the MEF2 site in order to produce the synergistic effect. Consistent with this notion, deletion of Sp1 domains A and C, which are responsible for protein interactions, or mutation of the Sp1 sites in this promoter, strongly reduced not only the basal level of promoter activity, but also the MEF2C-Sp1 synergism (Fig. 2). However, in the absence of Sp1 protein, mutation of the MEF2 site (pNRL919mtMEF2) or MEF2C protein (MEF2C-R24L) resulted in a 50% reduction of the reporter activity (Fig. 2). These results suggest that the MEF2 site also contributes to the basal activity of the NR1 promoter.

Interaction of MEF2C with Sp1 in a Yeast Two-hybrid System—In an attempt to directly confirm interactions between MEF2C and Sp1 protein, we used a two-hybrid system (50) as described by Gyuris et al. (45, 51) to identify MEF2C accessory proteins. From 3 million colonies, we recovered 52 plasmids
that expressed potential interactors. On retransformation, 18 of these produced an unambiguous blue color in galactose medium and were analyzed further. Two of these plasmids contained a partial cDNA for Sp1 (Sp1308–743 and Sp1431–708). We verified the specificity of the MEF2C-Sp1 interaction with strains containing reporters that exhibit differential sensitivity to activators (52). In EGY48, which carries an 8LexAop-LacZ reporter, expression of activation-tagged Sp1 together with LexA-MEF2C alone led to a 26-fold increase in β-galactosidase activity compared with yeast expressing LexA-MEF2C alone (Fig. 3A). The approximate strength of this interaction (Kd = 10^{-7}–10^{-8} M) was estimated by correlation of two-hybrid affinity data with in vitro measurements for λ repressor (52). Sp1 did not interact with another MADS protein, MCM1, or with unrelated control proteins (Fig. 3, A and B). In addition, Sp1 interacted with itself (Sp183–778) and with its “fingerless” derivative lacking the three zinc fingers of the DNA binding domain (Sp183–621) (49).

**FIG. 3.** Binding of Sp1 to MEF2C in yeast and HeLa cells. A, activation-tagged Sp1 interacts with MEF2C. Full-length Sp1 was fused to the B42 transcriptional activation domain of JG-45 and transformed into yeast cells containing MEF2C or other proteins fused to the DNA binding domain of LexA. In addition to LexAop-LEU2, a reporter requiring Leu in the medium for growth, this strain harbored an episomal LexAop-LacZ reporter. Basal stimulation of the reporter constructs was determined by the requirement for Leu (the Leu+ phenotype) and by β-galactosidase activity on glucose/Leu- plates. Interactions were monitored by comparison of these phenotypes with those obtained in galactose rather than glucose-containing medium. Values shown reflect the mean number of assays performed with at least five independent colonies. B, activation-tagged MEF2C interacts with Sp1. Full-length MEF2C was fused to the transcriptional activation domain, expressed in yeast cells containing the indicated LexA-fusions and tested as above. C, schematic diagram of the Sp1 deletion derivatives that were fused to LexA and tested in A and B. D, association of MEF2C with Sp1 in HeLa cells. HeLa cells that had not yet reached confluence were transfected with the expression constructs Sp183–778 and MEF2C. After 48 h of culture, immunoprecipitation with Sp1 antibody (Sp1 Ab) or with preimmune serum (Sp1 pre) was performed. The immunoprecipitated complexes were fractionated on SDS-polyacrylamide gel and exposed to x-ray film. M, molecular weight markers. MEF2C IVT, in vitro translated MEF2C protein. Sp1 pre, preimmune serum for Sp1 polyclonal antibody. Sp1 Ab, polyclonal Sp1 antibody. E, partial proteolysis of proteins with indicated amounts (ng) of *S. aureus* V8 protease. MEF2C IVT represents in vitro translated MEF2C. MEF2C represents the band excised from the anti-Sp1 immunoprecipitation gel, as shown in D.

The NH₂-terminal DNA Binding Domain of MEF2C Is Required for Interaction with Sp1—To test whether full-length MEF2C is required to interact with Sp1, we introduced activation-tagged MEF2C into a panel of EGY48-derived strains containing different baits (Fig. 3B). As judged by the Leu2 and LacZ phenotypes, MEF2C specifically interacted with Sp183–778.
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**Fig. 4. Effects of MEF2 and Sp1 sites on NR1 promoter activity in rat cerebrocortical cultures.** Cultured cerebrocortical cells were transfected with the indicated reporter plasmids, as detailed under “Experimental Procedures.” Relative luciferase activity represents values normalized to the activity of cotransfected β-galactosidase. For statistical analysis we used a single planned comparison between pNRL919 and pNRLmMEF2 by a two-tailed t test (*p < 0.005).

and Sp13.6–241 but not with several unrelated proteins. MEF2C derivatives that contained the DNA-binding domain associated with themselves, confirming that this portion of the protein mediates its homo-oligomerization (54). As further judged by interaction phenotype (52, 53), the association of MEF2C and MEF2C1–86 with Sp1 were of comparable affinity, suggesting that regions outside of the DNA-binding domain of MEF2C are not required for the interaction.

**Interaction of MEF2C with Sp1 Protein in Living Cells—**To explore MEF2C–Sp1 interactions in mammalian cells, we used Sp1 antisem to immunoprecipitate protein complexes from 35S-labeled extracts of HeLa cells that were transfected with plasmids expressing MEF2C and Sp13.6–758. Co-precipitation with Sp1 antisem revealed two major bands, with apparent molecular masses of 95 and 55 kDa (Fig. 3D), that were not precipitated with preimmune serum. The 95-kDa band corresponded to Sp13.6–758, whereas the 55-kDa band migrated with a mobility similar to that of in vitro translated MEF2C. To confirm the identity of the 55-kDa band, we recovered it from the gel and subjected it to partial V8 proteolysis for an analysis of the products on an SDS gel (Fig. 3E). The pattern resulting from the digestion was identical to that obtained from in vitro synthesized MEF2C, suggesting that MEF2C forms stable complexes with Sp1 in mammalian cells. Immunoprecipitation was also observed in the presence of 0.1 mg/ml ethidium bromide (data not shown), a concentration sufficient to disrupt interactions of these proteins with DNA (54), suggesting that the proteins interact even in the absence of DNA.

**MEF2C Is Involved in Developmental Expression of the NR1 Gene—**To study the regulation of the NR1 gene under more physiological conditions, we transfected different promoter fragments into primary cultures of embryonic cerebrocortical neurons. Transfection of the cultured neurons with NR1 promoter fragments containing both MEF2 and Sp1 sites (pNRL919) resulted in ~130-fold activation of the reporter over the promoter-less construct alone (Fig. 4). The construct bearing a mutated MEF2 site (pNRL919mtMEF2) displayed significantly reduced promoter activity (56% of wild type), while the construct containing Sp1 mutations (pNRL919mtSp1) manifests only 6.5% of wild-type activity. Mutations or deletion of both MEF2 and Sp1 sites (pNRL919mtMEF2/Sp1 or pNRL239) abrogated virtually all of the NR1 promoter activity in the cultured cerebrocortical cells. These results show that mutation of the MEF2 site abolishes 44% of NR1 promoter activity, but disruption of the Sp1 sites abolishes more than 93% of the promoter activity even in the presence of an intact MEF2 site. Cerebrocortical cells express endogenous MEF2C protein, which may interact with Sp1 protein even in the absence of DNA binding at the MEF2 site. Thus, the retained 56% activity of promoter-bearing MEF2 site mutations may be attributed to both Sp1 and MEF2C–Sp1 complexes. These findings further support the notion that MEF2C protein interacts with Sp1 protein to regulate NR1 gene expression in developing neurons. However, the MEF2 site may also help recruit MEF2C protein to the promoter, which then contributes to synergism by interacting with Sp1 protein. Therefore, these results suggest that maximal activation of the NR1 promoter requires both the MEF2C and Sp1 sites although less synergism was evident than in Drosophila cells.

To gain more direct evidence that endogenous MEF2C is involved in NR1 gene expression in vivo, we monitored NR1 mRNA levels during neuronal differentiation in the presence or absence of a dominant-negative MEF2C protein. We stably transfected the plasmid pG/DN, which contains the cDNA sequence of the NR1 gene under more physiological conditions, with plasmids expressing MEF2C and Sp1 sites although less synergism was evident than in Drosophila cells. NR1 is also observed in the presence of 0.1 mg/ml ethidium bromide.
Marin and co-workers recently showed that Sp1 is essential for NR1 gene expression, MEF2C is also expressed at early embryonic stages in nervous and myogenic tissues (31, 32). Preceding NR1 gene expression, MEF2C levels continue to increase in cultured cerebrocortical neurons obtained from embryonic day 15–17 rat brain, while Sp1 expression is already high. Taken together, these in vivo and in vitro results suggest that the interaction of MEF2C, a tissue specific factor, and Sp1, a ubiquitous factor, plays an important role in the developmental expression of the NR1 gene in neurons.

The expression of some neuronal genes, including NR1, are thought to be suppressed in peripheral tissues by a recently characterized silencer termed neuron-restrictive silencer factor or RE1-silencing transcription factor (60, 61). Our recent results show that the NR1 gene carries a functional binding site for this silencer in its 5’ untranslated region which is included in the promoter fragments used in this study (43). Neuron-restrictive silencer factor/RE1-silencing transcription factor exists in non-neuronal tissues and may prevent gene expression driven by MEF2-Sp1 interactions in muscle, where the MEF2 family is also expressed beginning at an early embryonic stage. The combinatorial actions of neuronal activators via protein-protein interactions and non-neuronal silencers may foster tissue-restricted expression of a given gene, such as NR1. Our recent studies also show that other factors, including single-stranded DNA binding proteins, may also act on the proximal promoter region and participate in directing the neuronal expression of this gene. Such effects may possibly compensate for the lack of MEF2 family members since MEF2C is expressed in the brain in a more restricted manner than NR1 (4, 10–12, 22).

In contrast to the majority of other interactions of Sp1 with accessory proteins (62–64), the MEF2C-Sp1 interaction occurs between the DNA-binding region of MEF2C and the activation domain of Sp1. Interestingly, MEF2C need not be bound to DNA to interact with Sp1. Sp1-MEF2C synergism on the NR1 promoter only requires that Sp1 protein be bound to DNA. Consistent with previous reports, this suggests that Sp1 can recruit free tissue-specific factors to the promoter to foster cell-type specific expression (64). Considering the fact that MEF2 family members share high homology in their DNA binding domain (MADS domain), this MEF2C-Sp1 interaction may also occur with other MEF2 members.

Additionally, Sp1 and MEF2C may interact after each has bound to its cognate site on the promoter. The $K_d$ we estimate for the MEF2C/Sp1 interaction is quite high ($10^{-7}$ to $10^{-8}$ $\mu$M) (52). This suggests that the free energy of this interaction should be sufficient to overcome the entropy loss due to DNA loop formation incurred by an interaction of MEF2C and Sp1 after they bind to their cognate sites located thousands of bases apart. Many neuronal genes, including $\gamma$-aminobutyric acid type A (GABA) receptor $\delta$ subunits, type II sodium channels, neurogranin, synapsin II, neurofilament-light chain, and brain-specific creatine kinase (65–70), contain putative MEF2 and Sp1 sites separated by large distances. Therefore, it is possible that such long distance interactions commonly play a role in the complex transcriptional programs that characterize neuronal gene expression in the developing brain.

Finally, our finding that Sp1 binding sites mediate growth factor effects may represent a generalized phenomenon since Sp1 sites are present in many neuronal genes that respond to growth factor signals but lack serum response element (17). Although a variety of Sp family members bind to Sp1 sites (71),

3 D. Krainc and S. A. Lipton, unpublished observations.
our screening of a human brain cDNA library revealed that Sp1 is the only member of the Sp family that interacts with MEF2C. Therefore, it seems likely that Sp1 is the factor which directly interacts with MEF2C for NR1 gene regulation.

In summary, we describe a novel form of transcriptional control of the neuronal NR1 gene involving a muscle/neuron-specific transcription factor (MEF2C) acting in conjunction with a ubiquitous factor (Sp1). These transcription factors interact in a synergistic manner on the NR1 promoter. The protein-protein interaction is predominately dependent on Sp1 binding rather than MEF2C binding to DNA. In view of the correlated expression pattern of MEF2C and NR1 during CNS development, our results suggest that the MEF2C-Sp1 interaction contributes to the developmental regulation of the NR1 gene.

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