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A Calcium-Responsive Transcription Factor, CaRF, that Regulates Neuronal Activity-Dependent Expression of BDNF

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Summary

Transcription of the brain-derived neurotrophic factor (BDNF) gene is regulated in a calcium- and neuron-selective manner; however, the mechanisms that underlie this selectivity are not known. We have characterized a new calcium-response element, CaRE1, that is required for activity-dependent transcription of *BDNF* exon III and have cloned a transcription factor, CaRF, that activates transcription from *BDNF* promoter III in a CaRE1-dependent manner. The transcriptional activity of CaRF is regulated in a calcium- and neuron-selective manner, suggesting that CaRF may confer selectivity upon the activity-dependent induction of *BDNF* exon III expression.

Introduction

Neuronal activity is a driving force for the development and function of the brain, leaving a long-lasting imprint on both neuronal structure and function through the induction of new gene expression. Activity-regulated gene transcription is required for the ability of membrane depolarization to promote neuronal survival (Ghosh et al., 1994; Mao et al., 1999) and for maintenance of the late phases of both long-term potentiation (LTP) and long-term depression (Ahn et al., 1999; Nguyen et al., 1994). The expression of a large number of genes has been shown to be rapidly induced by neuronal activity (Lanahan and Worley, 1998; Nedivi et al., 1993). These genes include both transcription factors, such as *c-fos* (Greenberg et al., 1986), that function in a wide variety of cell types to mediate adaptive responses to extracellular stimuli, as well as a second class of genes, such as *Narp* (O'Brien et al., 1999), *Homer* (Tu et al., 1998), and *Cpg15* (Nedivi et al., 1998), whose expression is enriched in the nervous system and which act to regulate synaptic function. Neuron-selective mechanisms that may regulate transcription of these synaptic gene products are not well understood.

One of the most extensively studied of the neuronally enriched class of activity-induced genes encodes the neurotrophin brain-derived neurotrophic factor (BDNF). BDNF was initially identified as a brain-derived survival factor for central neurons (Leibrock et al., 1989; Schwartz et al., 1997), but has subsequently been shown to have a wide range of effects on both central and peripheral neurons (Kafitz et al., 1999; Lohof et al., 1993; McAllister et al., 1995; Poo, 2001; Ringstedt et al., 1998; Wang et al., 1995). In concordance with its role as an effector of neuronal activity in the brain, the transcription of *BDNF* is exquisitely sensitive to regulation by neuronal activity. The expression of *BDNF* mRNA is highly induced by seizure (Zafra et al., 1990) and by stimuli used to induce LTP in the hippocampus (Patterson et al., 1992). *BDNF* expression is also modulated in vivo by endogenous synaptic activity during behaviors (Castren et al., 1992; Liang et al., 1998; Tokuyama et al., 2000).

Since the observation was first made that the transcription of genes such as *BDNF* is regulated by neuronal activity, a number of laboratories have contributed to a working model of the mechanisms that drive activity-induced neuronal gene expression (Bito et al., 1997; Cruzalegui and Bading, 2000; West et al., 2001). Briefly, neurotransmitter reception and membrane depolarization lead to the influx of calcium across the plasma membrane, initiating cascades of intracellular signaling pathways that carry the signal to the nucleus to activate transcription factors. These transcription factors bind to key regulatory elements within the promoter regions of the responsive genes and recruit the basal transcription machinery.

We have been studying the activity-dependent transcription of *BDNF* as a means to identify transcription factors that mediate activity-regulated gene expression in neurons. The *BDNF* gene has a complex organization, with four initial noncoding exons, each of which can be alternatively spliced in a tissue- and stimulus-specific manner to a single 3′ exon containing the coding sequence (Timmusk et al., 1993, 1995). Exon III-containing transcripts are the most highly induced in response to elevated KCl-mediated membrane depolarization of embryonic cortical neuron cultures (Tao et al., 1998). A region of 170 bp upstream from the exon III initiation site is sufficient to recapitulate activity-dependent transcription of a reporter gene. Within this fragment, a sequence resembling the calcium/cAMP response element (CRE) bound by members of the CREB family of transcription factors is required for activity-dependent induction of the reporter gene (Shieh et al., 1998; Tao et al., 1998). However, mutation of a distinct region upstream of the CRE-like sequence also blocks activity-dependent induction of the reporter gene (Shieh et al., 1998; Tao et al., 1998). This result indicates that CREB alone is not sufficient to drive expression of *BDNF* exon III transcription, and suggests that there must be other transcription factors cooperating with CREB to mediate activity-dependent expression of *BDNF* exon III. A critical unanswered question is what specific functions the transcription factors that bind to the region 5′ to the CRE-like sequence play in mediating activity-dependent transcription of *BDNF* in neurons.

In this report, we demonstrate that the induction of

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BDNF exon III expression in neurons is selectively activated by calcium influx, but not by other stimuli such as those that lead to the elevation of cAMP, even though both types of stimuli lead to the activation of CREB. In addition, we find that the induction of *BDNF* exon III transcription in response to calcium influx is selectively found in neurons, but not in the neuroendocrine cell line PC12, although CREB is activated by calcium influx in both cell types. These findings led us to investigate whether transcription factors binding to the region 5′ to the CRE-like sequence in *BDNF* promoter III might confer calcium and neural selectivity on the induction of *BDNF* exon III expression. We have conducted detailed mutagenesis of the region of *BDNF* promoter III 5′ to the CRE-like sequence and find that the 5′ element comprises a binding site for two distinct transcription factors. In this study, we characterize the most distal calcium-response element which we term CaRE1. We find that CaRE1 is required for membrane depolarization-mediated induction of a *BDNF* promoter III reporter gene in neurons in a calcium- and neural-selective manner. Using CaRE1 in a yeast one-hybrid screen, we have cloned a CaRE1-dependent transcription factor, CaRF, whose activity is regulated in a calcium- and neuron-selective manner, suggesting that CaRF may confer calcium and neural selectivity upon *BDNF* promoter III. The identification of CaRF as a neuronal, calcium-regulated, transcriptional activator of *BDNF* exon III expression suggests that this transcription factor may play a key role in neuronal development and plasticity.

Results

The Induction of *BDNF* Exon III Expression Is Calcium and Neuron Selective

As a first step toward identifying the molecular mechanisms that regulate transcription of *BDNF* exon III, we investigated the range of stimuli that lead to induction of *BDNF* exon III mRNA expression. Using a quantitative RT-PCR analysis, we find that *BDNF* exon III expression is regulated in a calcium- and neuron-selective manner. In cultured rat cortical neurons, *BDNF* exon III mRNA expression is induced over 100-fold by membrane depolarization and subsequent calcium influx; however, elevation of intracellular cAMP levels leads to little if any induction of *BDNF* exon III (Figure 1A). By contrast, both stimuli drive similar increases in the expression of c-*fos* (Figure 1B). Regulation of *BDNF* exon III expression was found to be cell type selective, with induction occurring in neurons, but not in several other cell types. For example, in the neuroendocrine-like pheochromocytoma cell line PC12, membrane depolarization failed to stimulate transcription of *BDNF* exon III (Figure 1C), despite the fact that PC12 cells express functional L-type voltage-sensitive calcium channels (L-VSCCs; Greenberg et al., 1986) and show rapid induction of c-*fos* (Figure 1C).

These data demonstrate that the induction of *BDNF* exon III transcription is restricted to a limited range of cell types and stimuli. Notably, although *BDNF* expression is regulated in part by the transcription factor CREB, the range of stimuli that lead to induction of *BDNF* exon III expression is more limited than the range of stimuli that lead to the activation of CREB, indicating that CREB is not sufficient to drive *BDNF* expression. This finding

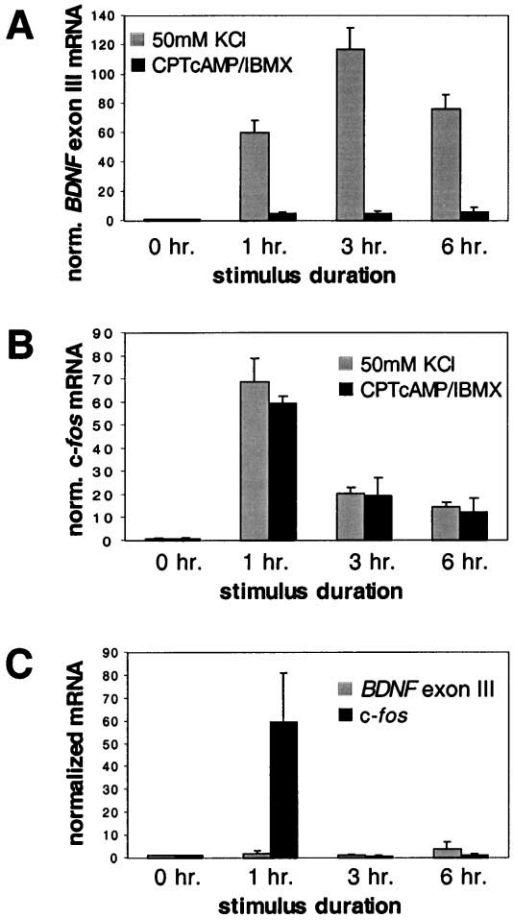

Figure 1. The Induction of *BDNF* Exon III Expression Is Calcium and Neuron Selective

(A) Real-time quantitative PCR was used to measure *BDNF* exon III expression levels in unstimulated 6DIV neurons or after 1, 3, or 6 hr of membrane depolarization (gray) or elevation of cAMP (black). *BDNF* exon III levels were normalized for expression of *GAPDH*.

(B) Real-time quantitative PCR was used to measure c-*fos* mRNA expression levels in unstimulated 6 DIV neurons or after 1, 3, or 6 hr of membrane depolarization (gray) or elevation of cAMP (black). c-*fos* levels were normalized for expression of *GAPDH*.

(C) Real-time quantitative PCR was used to measure *BDNF* exon III (gray) and c-*fos* (black) expression levels in undifferentiated PC12 cells left unstimulated or after 1, 3, or 6 hr of membrane depolarization. *BDNF* and c-*fos* levels were normalized for expression of *GAPDH*.

is consistent with the previous observation that deletion of a region 5′ to the CRE-like sequence in *BDNF* promoter III blocks induction of expression of a *BDNF* promoter III reporter gene (Shieh et al., 1998; Tao et al., 1998), suggesting that transcription from *BDNF* promoter III may require the coordinate activation of several transcription factors. We therefore considered the possibility that the element(s) 5′ to the CRE-like sequence and the transcription factors that bind to these elements might confer calcium and neural selectivity upon the induction of *BDNF* exon III transcription.

Characterization of CaRE1, a Calcium-Response Element in *BDNF* Promoter III

Previously we found that 170 bp of the 5′ flanking sequence of *BDNF* exon III is sufficient to drive transcrip-

tion of a transfected reporter gene in response to membrane depolarization of cultured embryonic cortical neurons (Tao et al., 1998). Induction of this promoter is driven by the depolarization-induced influx of calcium through L-VSCCs and the activation of downstream calcium-responsive signaling pathways. To identify the transcription factors that drive *BDNF* exon III expression, we have mapped their presumptive binding sites by using deletion analysis and mutagenesis to identify calcium-responsive enhancer elements contained within the 170 bp of *BDNF* promoter III. Deletion of the sequence between nucleotides −84 and −43 bp relative to the initiation site of *BDNF* exon III or 6 bp substitution of the sequence between nucleotides −77 and −60 eliminated induction of the *BDNF* promoter III reporter in transfected neurons, suggesting that this region of the promoter contains a critical calcium-response element (Tao et al., 1998).

To define calcium-response elements within this region, we made two base pair substitutions of the DNA sequence that lies between nucleotides −77 and −60 in *BDNF* promoter III. Mutations were introduced by PCR into the pIII(170)Luc *BDNF* promoter reporter plasmid, which contains 170 bp of *BDNF* exon III 5′ flanking sequence and exon III fused to the coding region of the firefly luciferase gene. When transiently transfected into cortical neurons, membrane depolarization strongly enhanced the expression of luciferase from plasmids containing the wild-type sequence (Figure 2A). Most mutations between nucleotides −73 and −64 significantly reduced the magnitude of luciferase induction relative to wild-type levels, while mutations between nucleotides −77 and −74 or −63 and −60 had no effect. These data identify a 10 bp calcium-response element spanning the sequence between nucleotides −73 and −64 in *BDNF* promoter III that we have named *ca*lcium *r*esponse *e*lement *1* (CaRE1). The CaRE1 sequence is conserved in the rat, human, and mouse *BDNF* genes. A search of the TRANSFAC database (http://www.cbil.upenn.edu/tess/index.html) revealed that the sequence of CaRE1 (5′-CTATTTCGAG-3′) does not match the binding site sequence for any known transcription factor, suggesting that this element may bind to a novel transcription factor(s).

Mutagenesis of the region between CaRE1 and the CRE-like sequence in *BDNF* promoter III has revealed the presence of a third CaRE, which we have termed CaRE2 (W.G. Chen et al., submitted). Figure 2B shows a diagram of the position of these three CaREs with respect to each other and the *BDNF* exon III transcriptional initiation site.

Having found that CaRE1 is necessary for membrane depolarization-mediated induction of *BDNF* promoter III, we next sought to determine if this element is sufficient to mediate a transcriptional response to membrane depolarization. To measure the activity of CaRE1, we isolated it from other elements in *BDNF* promoter III. Three copies of the CaRE1 sequence were cloned into the pGL3 vector immediately 5′ to a minimal promoter driving expression of the firefly luciferase gene. Membrane depolarization of neurons transfected with this construct led to a significant induction of luciferase expression (Figure 2C; $p < 0.0001$). In addition to demonstrating that the CaRE1 element is sufficient to mediate a transcriptional response to membrane depolarization and subsequent calcium influx, the ability of this element to function independently of other CaREs from the *BDNF* promoter suggests that the calcium responsiveness of the element may be conferred by direct calcium signaling pathway-dependent regulation of the transcription factor(s) that binds to CaRE1.

We have hypothesized that the activation of transcription factors binding to the elements upstream of the CRE/CaRE3 sequence may confer calcium and neuron selectivity upon the induction of *BDNF* promoter III. Consistent with this hypothesis, elevation of cAMP does not lead to an induction of CaRE1-dependent transcription when the reporter is expressed in cortical neurons (Figure 2D). The induction of CaRE1-dependent transcription is also selective for neurons, as the reporter is not activated by membrane depolarization of PC12 cells (Figure 2E). These data indicate that CaRE1-dependent transcription is activated in a calcium- and neuron-selective manner.

CaRF Is a CaRE1 Binding Protein

To determine if there are neuronal nuclear proteins that bind CaRE1 and thereby mediate the calcium-responsive induction of *BDNF* transcription through this element, we performed electrophoretic mobility shift assays (EMSAs) with a radiolabeled 25 bp fragment of DNA corresponding to the *BDNF* promoter region containing the CaRE1 element. When nuclear extract from E18 rat brain cortex was incubated with the DNA probe, one major complex was identified (Figure 3A). An equivalent shift was seen from nuclear extracts prepared from unstimulated neurons, or neurons depolarized for 1, 2, or 3 hr, suggesting that depolarization does not directly regulate protein binding to CaRE1 or the nuclear localization of the CaRE1 binding protein (Figure 3B). The binding of this protein(s) to CaRE1 was specific, as formation of the complex was competed by the addition of an excess of an unlabeled DNA fragment with the wild-type CaRE1 sequence, but not by the addition of unlabeled fragments in which the CaRE1 element was mutated to sequences that do not support a calcium response in the context of the *BDNF* pIII(170)Luc reporter (from Figure 2A). This correlation between the binding of the nuclear factor to CaRE1 sequences and the ability of these sequences to support calcium induction of the *BDNF* pIII(170)Luc reporter suggests that the nuclear protein(s) that binds to CaRE1 is the endogenous protein(s) that regulates calcium-dependent transcription through CaRE1 in membrane-depolarized neurons.

Because the sequence of CaRE1 does not resemble a known transcription factor binding site, we used a yeast one-hybrid screen to clone CaRE1 binding proteins. Three independent clones were isolated from the screen; however, extracts from only one of these clones contained a protein that specifically bound CaRE1 by EMSA (Figure 3C). Sequencing of this clone revealed that it is a novel protein that we have named CaRF for *ca*lcium *r*esponse *f*actor (Figure 3D). Human, murine, bovine, and porcine ESTs with homology to this sequence have been deposited in GenBank; however, we identified no homologs in *Drosophila* or *C. elegans*. The human CaRF cDNA is 3.1 kb and codes for a protein of 725 amino acids. The mouse sequence was obtained

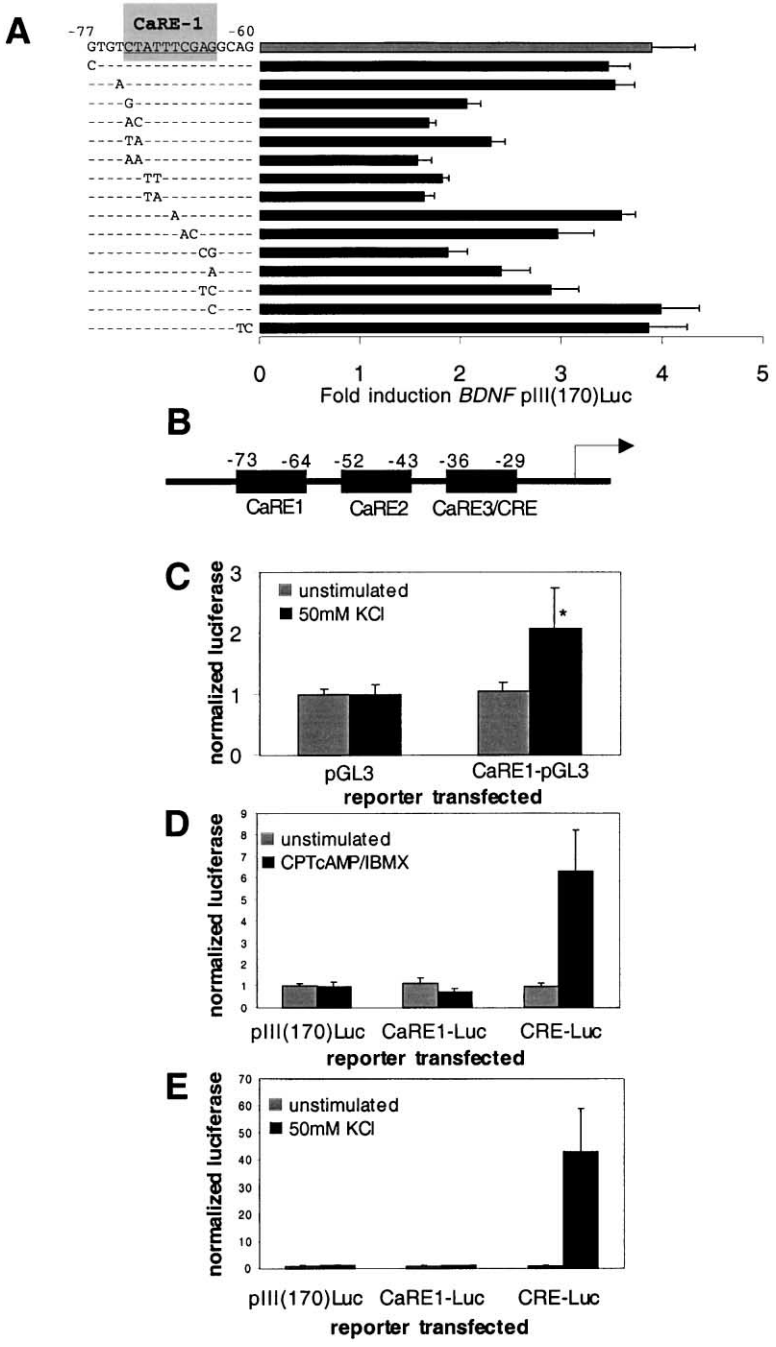

Figure 2. CaRE1 Is a Calcium-Response Element in *BDNF* Promoter III

(A) A *BDNF* promoter III luciferase reporter plasmid with wild-type or mutant sequences was transfected into E18 + 3DIV rat cortical neurons. Two days after transfection, neurons were left unstimulated or stimulated for 9 hr with 50 mM KCl, then homogenized and assayed for luciferase and β-galactosidase expression from cotransfected pEF-*lacZ*. Fold induction is defined as the ratio of β-gal normalized luciferase from stimulated cells relative to the normalized luciferase from unstimulated cells. The gray box indicates the region of *BDNF* promoter III that corresponds to CaRE1.

(B) Diagram of the three CaREs in *BDNF* promoter III is shown. The arrow indicates the transcriptional start site for *BDNF* exon III, and all numbering is relative to this site.

(C) Neurons transfected with either the empty pGL3 vector or the CaRE1-luciferase plasmid were either left unstimulated (gray) or depolarized with 50 mM KCl for 9 hr (black), then harvested for luciferase assay. Values are normalized for cotransfected renilla luciferase. The data presented is the average of at least three measurements from each of three independent experiments. Asterisk (*) indicates a significant increase in luciferase expression ($p < 0.0001$).

(D) The *BDNF* pIII(170)Luc, CaRE1-luciferase, and CRE-luciferase plasmids were transfected into cortical neurons. Two days later, these cells were either left unstimulated (gray) or stimulated with 300 μM each CPT-cAMP and IBMX for 9 hr (black), then lysates were processed for luciferase assays. Values are normalized for cotransfected renilla luciferase.

(E) The *BDNF* pIII(170)Luc, CaRE1-luciferase, and CRE-luciferase plasmids were transfected into PC12 cells. Two days later, these cells were either left unstimulated (gray) or stimulated with 50 mM KCl for 7 hr (black), then lysates were processed for luciferase assays. Values are normalized for cotransfected renilla luciferase.

by hybridization of a human CaRF probe to a P0 mouse brain lambda phage library, and is 71% identical overall (80% conserved). A fragment of CaRF encoding amino acids 144–725 was recovered from the yeast one-hybrid screen. RACE was used to identify the 5′ and 3′ ends of both the mouse and human clones. The 5′ end of each clone contains multiple stop codons in all three frames upstream of the triplet codon that encodes the first methionine, suggesting that we have identified the 5′ end of the CaRF coding sequence. In addition, the start methionine of the CaRF cDNA lies within a Kozak consensus sequence. Alignment with the Celera and NIH human genome databases revealed that the CaRF coding sequence is composed of 14 exons spanning about 50 kb of chromosome 2q33. Multiple splice variants of CaRF were identified by RACE, RT-PCR, and database searches, including two C-terminally truncated forms of CaRF that use alternative polyadenylation sites within introns (Figure 3D).

Extensive analysis of the CaRF sequence has shown that it has no significant homology to any known proteins in the GenBank database and no domains that would identify it as a member of a family of transcription factors. However, consistent with its role as a putative transcriptional regulator of *BDNF* exon III expression, the CaRF sequence contains a predicted nuclear localization signal (NLS; Figure 3D), two glutamine-rich helices which are found in many transcriptional activators (Trie-

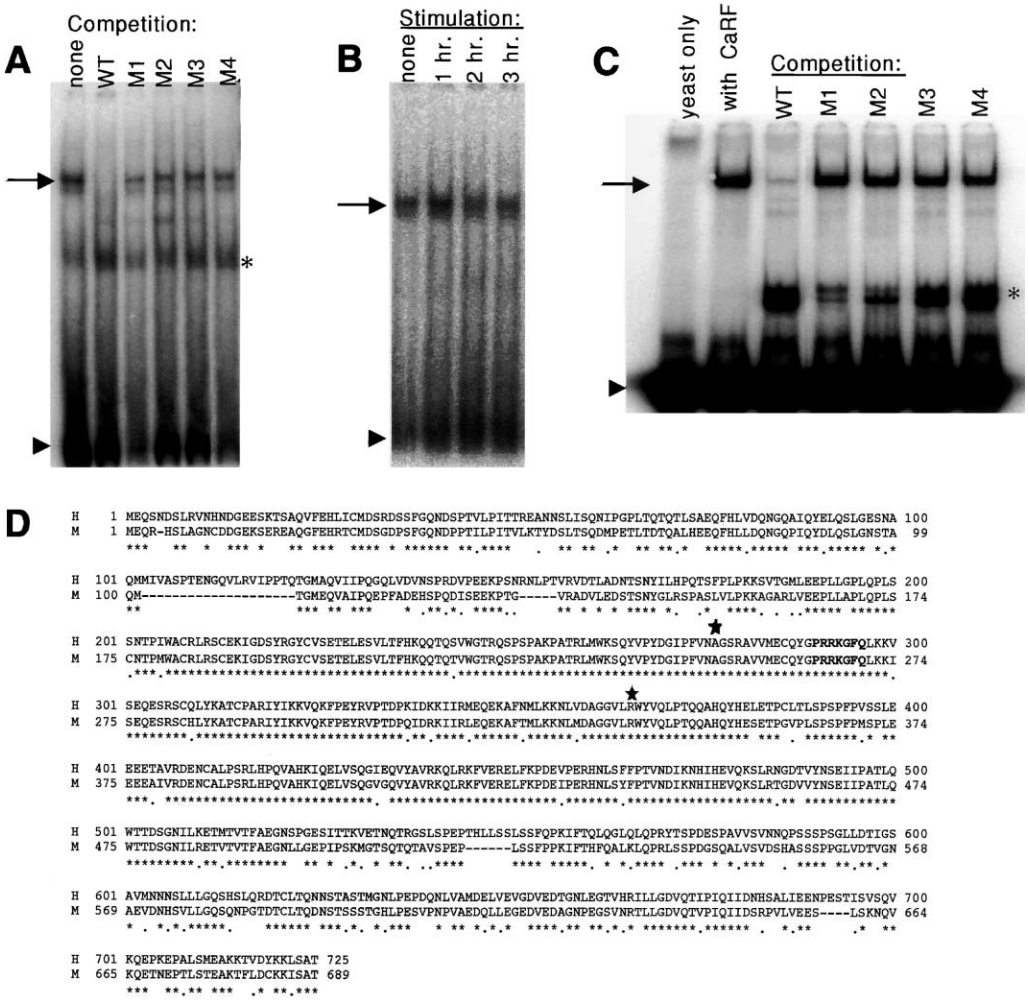

Figure 3. CaRF Is a CaRE1 Binding Protein

(A) Nuclear extracts from cortical neurons were mixed with a radiolabeled CaRE1 probe, then subjected to EMSA. Association of a nuclear protein with CaRE1 (black arrow) causes a retardation in the migration of the labeled probe (arrowhead). This binding can be competed by addition of an excess of unlabeled wild-type probe, but not by addition of an excess of unlabeled CaRE1 probes containing mutations that block the function of CaRE1 in the reporter gene assay shown in Figure 2A (M1-4). The mutant sequences used were: M1-CTtccgCcAG, M2-CTA TTTCGcttagc, M3-CTATTTCGcG, M4-CTATTTtaAG (the CaRE1 sequence is underlined). A nonspecific band is marked by the asterisk (*).

(B) Cultured cortical neurons were depolarized with 50 mM KCl for 0, 1, 2, or 3 hr, before nuclear extracts were harvested and subjected to EMSA with the CaRE1 probe. The arrow shows the retarded protein-DNA complex and the arrowhead marks the unbound probe.

(C) Nuclear extracts from yeast or yeast overexpressing CaRF were assayed by EMSA for binding to radiolabeled CaRE1. In labeled lanes, an excess of unlabeled wild-type or mutant (M1-4) CaRE1 probe was added to test for competition of binding to CaRE1. The mutant sequences used are the same as those for Figure 3A. The arrow marks the CaRE1-CaRF complex. The asterisk (*) marks a nonspecific retarded band not competed by addition of wild-type unlabeled probe.

(D) shows sequence of mouse (M) and human (H) CaRF. Identical amino acids are indicated by asterisks, conserved amino acids by dots. Gaps are indicated by dashes. The predicted NLS is highlighted in bold. Stars above the sequence indicate the location of two alternative polyadenylation signals that lead to early truncations. GenBank accession numbers are AF454946 (human) and AF454947 (mouse).

zenberg, 1995), and consensus phosphorylation sites for a number of kinases including calcium-calmodulin kinase II, mitogen-activated protein kinase, and protein kinase C.

Given the novelty of the CaRF sequence, we established a set of criteria to determine if CaRF is a bone fide transcription factor capable of supporting calcium-responsive induction of *BDNF* exon III transcription. To this end, we asked the following questions: (1) Will CaRF bind selectively to CaRE sequences that support calcium-dependent induction in the context of the *BDNF* pIII(170)Luc reporter gene? (2) Will an antibody raised against recombinant CaRF recognize the endogenous CaRE1 binding protein in nuclear extracts? (3) Is CaRF expressed in the nuclei of neurons that show activity-dependent expression of *BDNF* exon III? (4) Does CaRF act as a CaRE1-dependent transcriptional activator?

CaRF Is a Neuronal CaRE1-Dependent Transcriptional Activator

If CaRF is a regulator of activity-dependent *BDNF* expression through CaRE1, CaRF might be expected to bind to wild-type CaRE1 sequences, but not to sequences that fail to support calcium-dependent induc-

tion in the context of the *BDNF* pIII(170)Luc reporter gene. CaRF binds a radiolabeled CaRE1 fragment by EMSA and this binding is competed by addition of excess wild-type unlabeled CaRE1 probe (Figure 3C). However, formation of the CaRE1-CaRF complex is not competed by addition of a panel of mutant CaRE1 sequences that do not support the depolarization-mediated induction of the *BDNF* pIII(170)Luc reporter plasmid (Figure 3C). This correlation between the binding of CaRF to CaRE1 sequences and the ability of these sequences to support calcium-dependent transcription from *BDNF* pIII(170)Luc suggests that CaRF is identical or closely related to the protein from neuronal nuclear extracts that binds CaRE1 and contributes to the regulation of *BDNF* exon III transcription.

To further test the possibility that CaRF is the CaRE1 binding protein in neuronal nuclear extracts, we raised antibodies against recombinant CaRF and asked if the antibodies could alter the CaRE1-neuronal nuclear protein complex seen by EMSA. Preincubation of neuronal nuclear extracts with the anti-CaRF serum blocked formation of the CaRE1 complex (Figure 4A). By contrast, incubation with preimmune serum from the same rabbit had no effect, and addition of the antibodies to the CaRE1 fragment in the absence of added nuclear extract had no effect on the CaRE1 fragment. These data indicate that the endogenous CaRE1 binding protein from neuronal nuclear extracts is either CaRF or a protein immunologically related to CaRF.

Using the antibodies raised against recombinant human CaRF, we further assessed the subcellular localization of endogenous CaRF in rat neurons. These antibodies recognize recombinant human CaRF by Western blot analysis, though rather than the predicted molecular weight of 80 kDa, the protein migrates at 96 kDa when synthesized by in vitro transcription and translation or when overexpressed in HEK-293T cells (Figures 4B, 4C, and 6C). As predicted from the rodent amino acid sequence, rat CaRF is slightly smaller (94 kDa) than the human form. This 94 kDa band is not recognized by preimmune serum from the same rabbit used to raise the anti-CaRF antibody, and recognition of the band is fully competed by preincubation of the antibody with recombinant GST-CaRF, but not by preincubation of the antibody with recombinant GST (Figure 4C). Consistent with the presence of a putative NLS in the CaRF sequence, we found that rat CaRF is highly enriched in nuclear extracts from cultured rat cortical neurons as compared to the cytoplasmic fraction (Figure 4B). The band detected at 55 kDa in the cytoplasmic fraction may reflect expression of one of the C-terminally truncated forms of CaRF, as the shortest predicted form lacks the putative NLS (Figure 4B). The function of this truncated form of CaRF is unknown.

For CaRF to be a regulator of *BDNF* expression, it should be expressed in neurons that show activity-dependent induction of *BDNF* exon III transcription. We first characterized the tissue distribution of CaRF by Northern analysis of multiple adult mouse tissues (Figure 5A). A 5 kb band reacting with the CaRF probe was detected at low levels in several tissues but at a high level in brain, a pattern consistent with a role for CaRF in the regulation of *BDNF* exon III transcription (Timmusk et al., 1993). A smaller 3 kb transcript was also highly expressed in testis. The expression of CaRF in tissues outside of the nervous system suggests that CaRF may have important biological functions in addition to its role in the activity-dependent regulation of *BDNF* expression. To determine which cells in the brain express

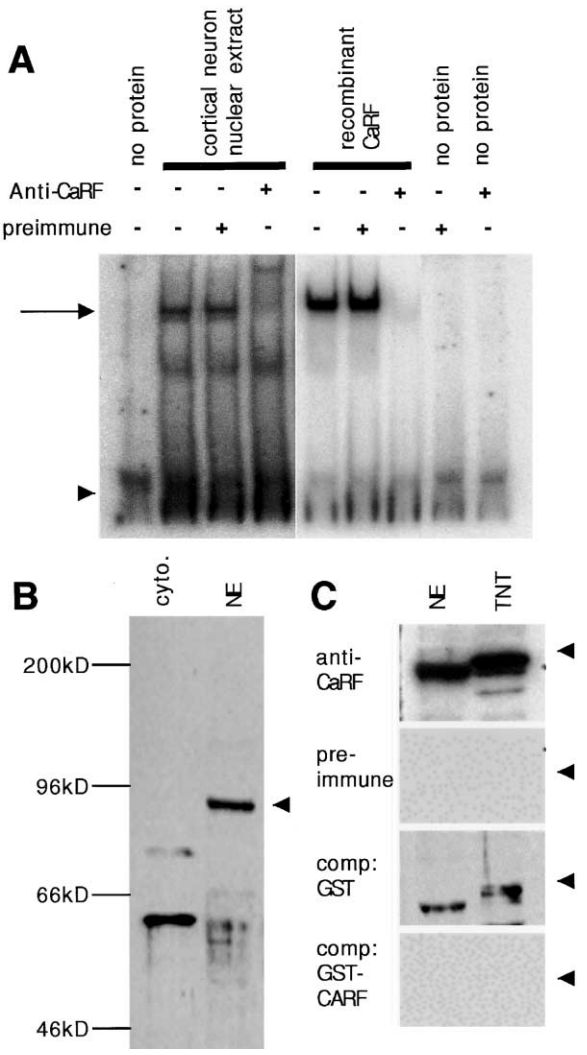

Figure 4. CaRF Is a Neuronal Nuclear Protein

(A) Nuclear extracts from cortical neurons were incubated with radiolabeled CaRE1 probe in the presence or absence of preimmune or anti-CaRF serum. The arrowhead indicates the unbound labeled probe. The arrow marks a band retarded by addition of cortical neuron nuclear extract or recombinant CaRF which is eliminated by preincubation of the extract or recombinant protein with anti-CaRF serum but not preimmune serum. No shift of the probe is seen when sera are added to the CaRE1 probe alone.

(B) Nuclear extracts were prepared from E18 + 5DIV cortical neurons in culture. Equal protein from either the cytoplasmic (cyto.) or the nuclear fraction (NE) was loaded on an 8% SDS-PAGE gel, transferred, and reacted with an antibody raised against recombinant CaRF. The arrowhead indicates a band of approximately 94 kDa that is highly enriched in neuronal nuclei.

(C) Nuclear extracts (NE) or in vitro transcribed and translated CaRF (TNT) were studied by Western analysis. The arrowheads mark the 96 kDa molecular weight marker. Blots were probed with anti-CaRF serum, preimmune serum, or anti-CaRF serum that had been preincubated for 1 hr with recombinant GST or recombinant GST-CaRF.

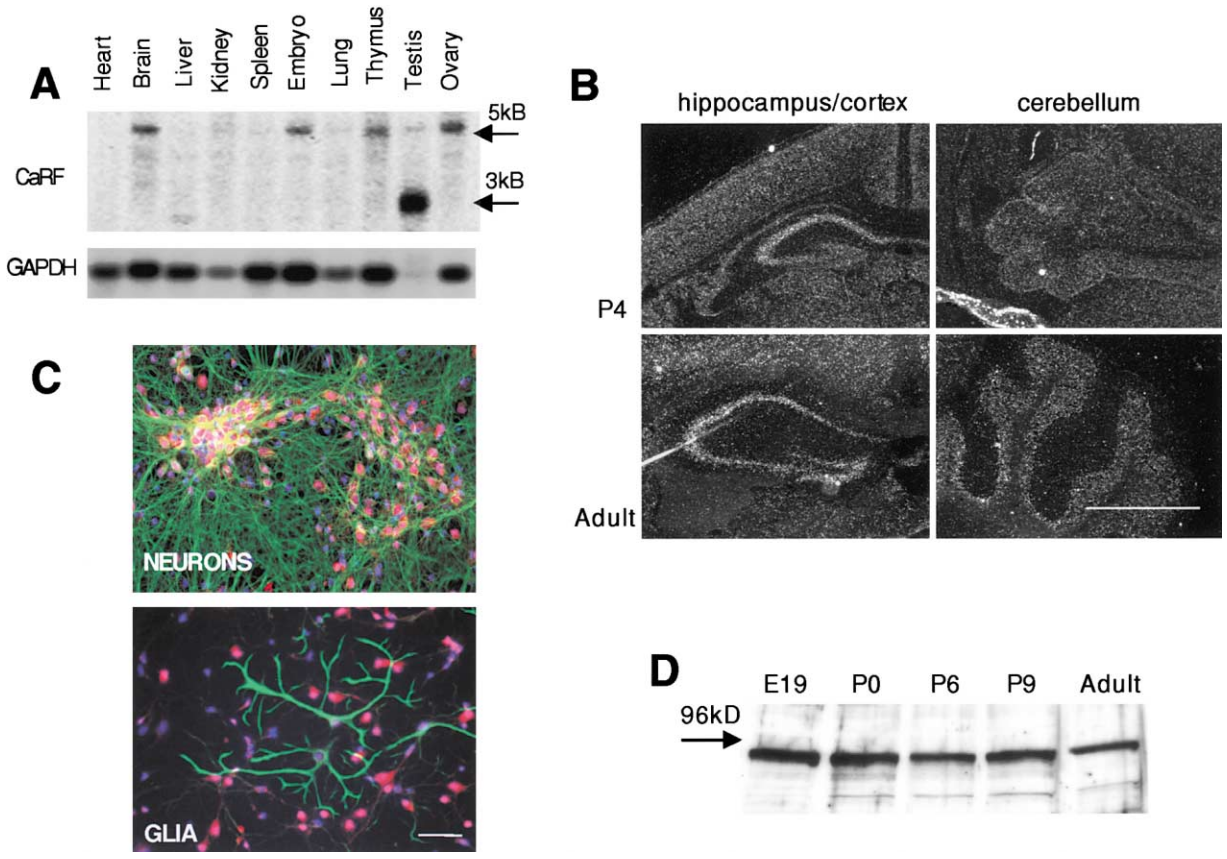

Figure 5. Pattern of CaRF Expression

(A) A mouse tissue Northern blot containing 1 μg polyA RNA per lane was probed with a radiolabeled RNA probe containing the CaRF open reading frame. All tissues except embryo were taken from adult mouse. After the CaRF probe decayed, the blot was reprobed for GAPDH to compare total RNA loading per lane.

(B) Brains of mice at P4 or adult were fixed and sunk in sucrose, then coronal cryostat sections were prepared and hybridized with an antisense ^{33}P labeled RNA probe representing the full CaRF cDNA. The bar indicates 1 mM.

(C) E19 hippocampal cultures were grown for 5DIV, then fixed and stained with the anti-CaRF antibody (red) and an antibody against the neuronal marker β-tubulin III or the glial marker GFAP (both in green). Nuclei are labeled with Hoechst dye in blue. Overlap of CaRF with nuclei is indicated by the pink color. The bar indicates 30 μm.

(D) Nuclear extracts were made from rat brains at E19, P0, P6, P9, and adult. Equal amounts of protein were loaded into each lane and separated by SDS-PAGE electrophoresis. The gel was transferred and probed with the anti-CaRF antibody. The arrow marks the 96 kDa molecular weight marker.

CaRF, we performed in situ hybridization of mouse brain sections. The highest levels of CaRF expression were found in the hippocampus of developing and adult mouse, with lower levels of diffuse expression throughout the cortex, areas where activity-dependent transcription of *BDNF* exon III containing transcripts occurs (Figure 5B). Lower levels of CaRF mRNA were detected in the granule cells of the adult cerebellum. To determine if the expression of CaRF in the brain is restricted to neurons, we used the anti-CaRF antibody to assay for endogenous CaRF in the nuclei of cultured hippocampal neurons and astroglial cells. CaRF was detected in the nuclei of cells expressing the neuronal marker protein β-tubulin III, but not in the nuclei of cells reactive for the astroglial lineage marker GFAP (Figure 5C). No nuclear staining was seen when cells were incubated with preimmune serum from the same rabbit (data not shown). Finally, Western analysis of nuclear extracts made from rat brain revealed that CaRF is expressed in nuclei throughout development and into adulthood (Figure 5D), consistent with the temporal pattern of *BDNF* exon III expression in brain (Timmusk et al., 1994).

A feature of transcription factors is that they often have modular DNA binding and transcriptional activation domains. To determine if there is a specific region of CaRF that mediates binding to CaRE1, we assessed the ability of in vitro transcribed and translated N- and C-terminally truncated versions of CaRF to bind to CaRE1 by EMSA. Deletion of the first 144 amino acids from the N terminus had no effect on binding to CaRE1; however, a deletion removing the N-terminal 226 amino acids or an internal deletion of amino acids 187–277 completely abolished the ability of CaRF to bind CaRE1 (Figure 6A). By contrast, deletion of the C-terminal 126 amino acids had no effect on DNA binding. All truncated constructs and the wild-type CaRF protein were expressed at similar levels as judged by Western analysis (data not shown). These data suggest that the DNA bind-

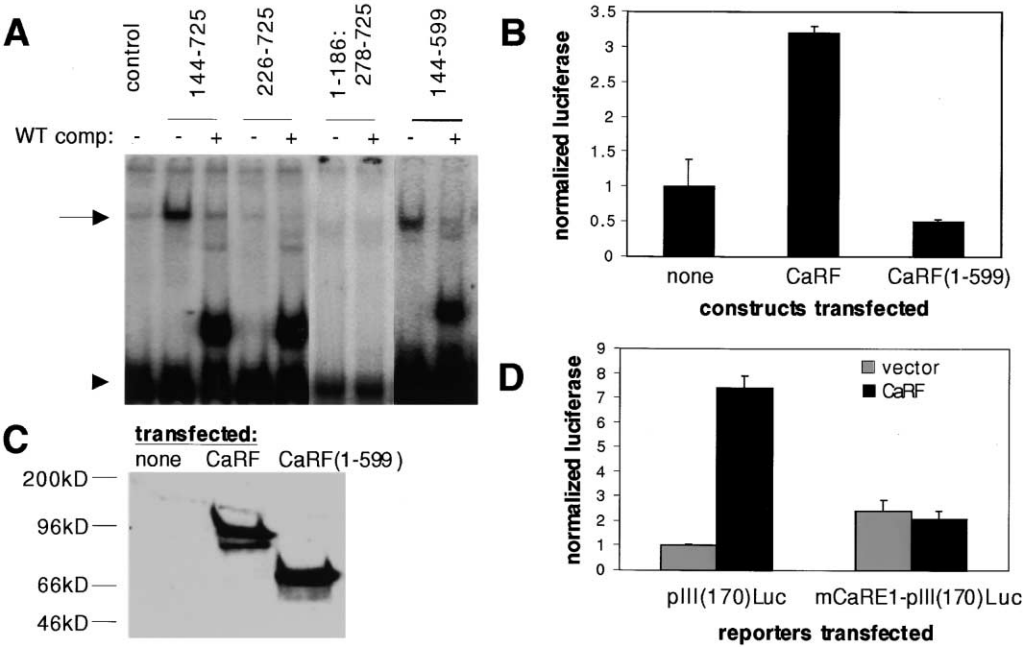

Figure 6. DNA Binding and Transcriptional Activation Domains of CaRF

(A) N- and C-terminally truncated or internally deleted CaRF constructs were transcribed and translated in vitro, then assayed for binding to radiolabeled CaRE1 by EMSA. The specificity of binding was assessed by competition with wild-type unlabeled CaRE1. The arrow marks the CaRE1-CaRF complex, the arrowhead points to the unbound probe.

(B) The CaRE1 reporter was transfected into 293T cells along with either full-length CaRF, a truncated CaRF lacking the C-terminal 126 amino acids (CaRF 1-599), or the pEF vector. A renilla luciferase plasmid was cotransfected as a control for transfection efficiency and sample handling. Two days later, the cells were homogenized and processed for renilla and firefly luciferase.

(C) Nuclear extracts were made from 293T cells transfected at the same time as cells used for the luciferase analysis in (Figure 6B), then analyzed by Western blot with an anti-CaRF antibody.

(D) 293T cells were transfected with the *BDNF* promoter reporter plasmid (pIII(170) Luc) or with a BDNF reporter that has the CaRE1 site mutated to a sequence (CTtccgCcAG) that does not bind CaRF (mCaRE1-pIII(170) Luc). Full-length CaRF (black) or the pEFmyc-his vector (gray), as well as TK-renilla luciferase, were cotransfected, and two days later the cells were homogenized and analyzed for renilla and firefly luciferase.

ing domain is located in the N-terminal half of CaRF, and requires the region between amino acids 187 and 277. As this region shares no significant homology with the DNA binding domains of other known transcription factors, we speculate that the DNA binding domain of CaRF may function in a novel manner.

We next asked if CaRF when bound to CaRE1 can activate or repress transcription. To assay the basal transcriptional activity of CaRF, we assessed the ability of transfected CaRF to drive expression of reporter genes cotransfected into HEK-293T cells, as these cells contain no detectable endogenous CaRF by Western or Northern analysis (Figure 6C and data not shown). Coexpression of CaRF with the CaRE1-luciferase reporter or with the *BDNF* pIII(170)Luc reporter led to a significant enhancement of luciferase expression over that seen in cells transfected with the reporter genes alone (Figures 6B and 6D). Although we did detect basal transcription activity of overexpressed CaRF in these cells, notably we saw no induction of CaRF activation in response to ionomycin-mediated elevation of intracellular calcium levels (data not shown), consistent with the idea that the induction of CaRF activity requires a cell type-specific regulatory event. Mutation of the CaRE1 element to a sequence that does not support calcium induction of *BDNF* in neurons, or the binding of CaRF by EMSA, blocks the ability of overexpressed CaRF to drive transcription from the reporter plasmid. Deletion of the C-terminal 126 amino acids of CaRF completely abolishes the ability of CaRF to drive transcription, even though the full-length and C-terminally deleted CaRF constructs are expressed at similar levels in HEK-293T cell nuclear extracts (Figures 6B and 6C). Although the C terminus of CaRF does not resemble any characterized transcriptional activation domain, these experiments indicate that this discrete region of CaRF is required for transcriptional activation and raise the possibility that further studies may show that CaRF functions in a novel manner to promote transcription.

In summary, these data demonstrate that CaRF is a neuronal nuclear protein with discrete DNA binding and transcriptional activation domains that can activate transcription from the CaRE1 element in *BDNF* promoter III. CaRF therefore fulfills all of the criteria required to be a bone fide transcriptional regulator of *BDNF* exon III expression, suggesting that CaRF itself is an important mediator of activity-dependent *BDNF* exon III transcription.

The Transcriptional Activity of CaRF Is Regulated in a Calcium- and Neuron-Selective Manner

Given that the induction of both *BDNF* exon III expression and CaRE1-dependent transcription are calcium- and neuron-selective, and since CaRF binds to CaRE1,

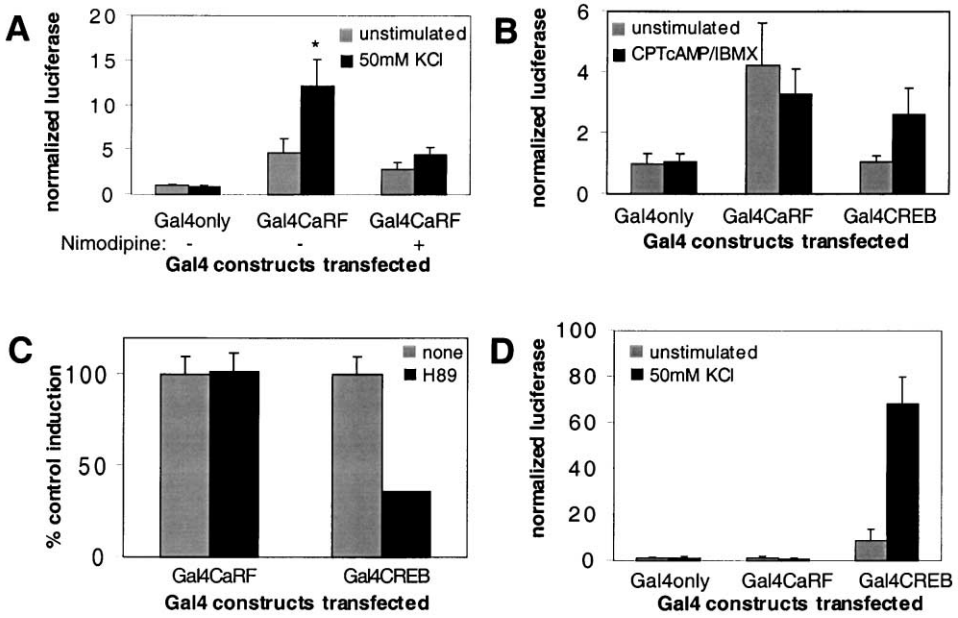

Figure 7. CaRF Is a Calcium-Regulated Transcriptional Activator in Neurons

(A) A Gal4-CaRF fusion protein was cotransfected into E18 + 4DIV rat cortical neurons with a Gal4-UAS reporter gene. TK-renilla luciferase was transfected as a control for transfection efficiency and sample handling. After two days, the neurons were either left unstimulated (gray) or stimulated for 6 hr with 50 mM KCl (black), either in the presence of the L-VSCC blocker Nimodipine, or with an equal dilution of the nimodipine vehicle EtOH. The cells were then lysed and processed by luciferase assay. Asterisk (*) indicates a significant increase in luciferase expression ($p < 0.0001$).

(B) Neurons were transfected with either Gal4only, Gal4CaRF, or Gal4CREB along with the Gal4UAS reporter gene and renilla luciferase. Two days later, the cells were left unstimulated (gray) or exposed to 300 μM each CPT-cAMP and IBMX for 9 hr (black). The cells were then harvested and processed by luciferase assay.

(C) Neurons were transfected with either Gal4CaRF or Gal4CREB along with the Gal4UAS reporter gene and renilla luciferase. Two days later, the control cells were depolarized as for Figure 7A (gray) while another set was depolarized in the presence of 10 μM H89 (black). The fold induction of the control cells is graphed as 100%.

(D) PC12 cells were transfected with either Gal4only, Gal4CaRF, or Gal4CREB along with the Gal4UAS reporter gene and renilla luciferase. Two days later, the cells were left unstimulated (gray) or depolarized for 7 hr (black). The cells were then harvested and processed for luciferase assay.

we investigated the possibility that the calcium and neural selectivity of *BDNF* promoter III might be mediated by CaRF. To first determine in neurons if CaRF activity is regulated by calcium influx into neurons, we isolated the C-terminal half of CaRF, containing the transcriptional activation domain, by tethering it to the DNA binding domain of Gal4. This fusion protein was cotransfected into neurons along with a reporter gene containing firefly luciferase gene under control of a minimal promoter enhanced by tandem copies of the Gal4 UAS element. When transfected neurons were membrane depolarized, Gal4 CaRF drove a significant increase in luciferase expression (Figure 7A, $p < 0.0001$). This increase was blocked when the neurons were depolarized in the presence of the L-VSCC blocker Nimodipine, indicating that depolarization-induced calcium entry through L-VSCCs is required for CaRF activation. No increase in luciferase expression was seen in response to membrane depolarization when only the Gal4 DNA binding domain was expressed with the UAS-luciferase reporter, indicating that the transcriptional activation domain of CaRF was required for the induction of luciferase expression. These data suggest that the transcriptional activity of CaRF is induced in response to calcium influx in neurons, consistent with the hypothesis that CaRF drives the calcium-dependent regulation of *BDNF*.

We next asked if the transcriptional activity of CaRF is regulated in a calcium- and neuron-selective manner, consistent with the neuron-selective calcium activation of *BDNF* exon III transcription. We found that elevation of cAMP is not sufficient to drive Gal4 CaRF activity in neurons, although this stimulus activates the transcriptional activity of a Gal4CREB fusion protein (Figure 7B). This result demonstrates that elevation of cAMP is not sufficient to drive CaRF activation, but since calcium influx can lead to activation of protein kinase A (PKA) through the action of calcium-sensitive adenylate cyclases, we also asked if PKA is required for the regulation of CaRF in response to membrane depolarization. Inhibition of PKA during membrane depolarization significantly depressed the induction of Gal4CREB activity, but had no effect on the induction of Gal4CaRF-dependent transcription (Figure 7C). These data indicate that, unlike the situation for CREB, the calcium-dependent activation of CaRF is independent of the activation of PKA. Finally, to ask if the activity of CaRF is regulated in a neuron-selective manner, we studied the transcriptional activity of Gal4CaRF in PC12 cells. In support of selectivity, we found that Gal4CaRF is not activated by membrane depolarization of PC12 cells although this stimulus robustly activates Gal4CREB-dependent transcription (Figure 7D).

From the data in total, we conclude that CaRF is a novel transcriptional activator that functionally mediates the calcium- and neuron-selective induction of *BDNF* exon III transcription.

Discussion

We have cloned and characterized an activity-regulated transcription factor, CaRF, that functionally regulates neuronal calcium-dependent expression of *BDNF* exon III. Several lines of evidence support the hypothesis that CaRF is an endogenous regulator of *BDNF* expression. CaRF binds to a 10 bp calcium-response element, CaRE1, that is required for the depolarization-mediated induction of a *BDNF* promoter III reporter gene in cultured embryonic cortical neurons. CaRF binds to wild-type CaRE1 sequences, but not to mutant sequences that do not support activity-dependent expression of BDNF. An antibody raised against recombinant CaRF recognizes the protein from neuronal nuclear extracts that binds CaRE1, blocking formation of the CaRE1 complex. CaRF expression is enriched in neuronal nuclei within the cerebral cortex and hippocampus, brain regions that show robust activity-induced *BDNF* exon III expression. CaRF is a CaRE1-dependent activator of transcription, and its role as a transcriptional activator is promoted by calcium influx in neurons. Finally, we have shown that like *BDNF* exon III transcription, the regulation of CaRF as a transcriptional activator is both calcium and neuron selective, suggesting that CaRF may confer calcium and neuron selectivity upon the induction of *BDNF* exon III expression.

Calcium Signaling Pathways Regulate the Activity of CaRF

Although we have demonstrated that the transcriptional activity of CaRF is regulated by calcium influx in neurons, the mechanism of this activation remains to be determined. Because CaRE1 when placed upstream of a heterologous promoter is sufficient to mediate a transcriptional response to membrane depolarization, one potential mechanism of activation is that calcium signaling pathways induce a posttranslational modification of either CaRF or a critical CaRF-interacting protein in a manner that promotes transcription. We did not detect depolarization-mediated changes in the nuclear targeting of CaRF or the binding of CaRF to CaRE1 (Figure 3B and data not shown), but a remaining possibility is that a posttranslational modification of CaRF or a critical CaRF-interacting protein directly promotes transcription. This scenario is similar to the activation of CREB, in which phosphorylation at Ser133 of CREB promotes the recruitment of the transcriptional coactivator CBP (Shaywitz and Greenberg, 1999). Several kinase cascades are known to be activated by calcium influx in neurons, including the calcium-calmodulin-activated kinases (CaMKs), PKA, and the mitogen-activated protein kinase (MAP kinase), and all three pathways are known to lead to the phosphorylation of CREB at Ser133 (Impey et al., 1998; Shaywitz and Greenberg, 1999). Our data indicate that PKA is neither required nor sufficient for the activation of CaRF; however, which signaling pathways are mediating the effects of membrane depolarization on CaRF activity remains to be determined.

If kinases regulate the transcriptional activity of CaRF, they may do so by phosphorylating CaRF directly. The CaRF sequence contains consensus phosphorylation sites for CaMKII and MAP kinase, and preliminary two-dimensional isoelectric focusing of neuronal nuclear extracts indicates that CaRF focuses in several discrete spots, consistent with the idea that CaRF is posttranslationally modified by phosphorylation (data not shown). Phosphorylation of CaRF could act to enhance its binding to a transcriptional coactivator, or phosphorylation of a coactivator could alter its ability to bind CaRF. Alternatively, phosphorylation could release binding of a transcriptional repressor, a mechanism shown to contribute to the calcium-dependent regulation of the transcription factor MEF2 (Youn et al., 1999). The identity of CaRF binding proteins remains to be determined, but preliminary experiments indicate that CaRF does not interact with CBP. Significant clues to the mechanism of CaRF activation may be found in a comparison of neurons and PC12 cells. Though PC12 cells express CaRF as detected by both Northern and Western analysis (data not shown), CaRF-dependent transcription in these cells is not activated by calcium influx. Whether PC12 cells lack the signaling pathways required for activation of CaRF or the expression of a transcriptional coactivator, these cells will provide a convenient system for reconstituting the activation of CaRF-dependent transcription.

Transcriptional Integration Contributes to Calcium Specificity of Gene Induction

Previously, we and others demonstrated that the activity-regulated transcription factor CREB is necessary but not sufficient for the expression of *BDNF* exon III, and suggested that CREB cooperates with factor(s) binding CaREs upstream of the CaRE3/CRE to drive expression (Shieh et al., 1998; Tao et al., 1998). Consistent with this hypothesis, CaRE1, though sufficient to mediate a calcium response when isolated from the *BDNF* promoter, in its native context cooperates with other elements including the CaRE3/CRE. In addition, in a separate study (W.G. Chen et al., submitted), we have identified a third calcium-response element in *BDNF* promoter III, CaRE2, that lies between CaRE1 and the CaRE3/CRE. As is seen for CaRE1 and the CaRE3/CRE, mutation of CaRE2 also blocks induction of the *BDNF* promoter III reporter gene. Thus, coordination of the activity of transcription factors bound at all three elements is minimally required for expression of *BDNF* exon III in cultured embryonic cortical neurons.

Why should three calcium-responsive elements be required to drive induction of *BDNF* promoter III? The data we have presented suggest that calcium signaling specificity may arise from the coordinated action of complexes of transcription factors that form on the promoters of calcium-inducible genes such as *BDNF*. Several studies have shown that not all intracellular calcium rises lead to equivalent activation of downstream gene expression (Bading et al., 1993; Ghosh et al., 1994). Cooperativity between CaRF and other calcium-regulated transcription factors, such as CREB, could be a

general mechanism for determining the specificity of gene induction in response to calcium influx. Different genes may be more or less broadly inducible depending on the number of enhancer elements they require for the response of the promoter, in combination with the stimulus and cell type selectivity of the elements. One outstanding question regarding the three elements in *BDNF* promoter III is the mechanism of cooperativity between the three factors. Cooperativity could potentially arise from cooperatively assisted DNA binding, regulated changes in genomic DNA accessibility, protein-protein interactions between the transcription factors, or association with a common transcriptional coactivator.

Regulation of Calcium- and Neural-Selective Transcription by CaRF

The identification of CaRF as a neuronal calcium-dependent transcriptional activator has implications for furthering the understanding of adaptive gene transcription in neurons. Unlike many of the other calcium-regulated transcription factors known to be important for inducible gene expression in neurons, such as CREB, MEF2, and NF-AT, which were first identified in non-neuronal cells, CaRF was cloned from a functional screen for calcium-dependent transcription factors expressed in neurons. Given that the regulation of CaRF activity appears to be selectively induced by calcium influx into neurons, this factor and others regulated in a similar fashion would not have been found in studies of non-neuronal cells, raising the possibility that other important regulators of activity-dependent transcription in neurons may remain to be discovered.

Since CaRF is selectively activated by calcium influx into neurons, it is a good candidate for conferring neuron-selective induction upon the expression of neuronally enriched genes required for brain-specific functions, such as synaptic transmission. The mechanisms that regulate the transcription of synaptically enriched gene products remain to be characterized. Once the consensus binding sequence for CaRF is fully characterized, it will be important to search the available genome databases to determine if the promoters of genes such as *Narp*, *Homer*, and *Cpg15* contain the CaRF binding site and also to determine if these or other activity-regulated genes in the brain are selectively induced by calcium and regulated by CaRF. Given that activity-dependent gene expression is required for neuronal development, survival, and long-term changes in synaptic efficacy, it may be anticipated that CaRF will also be found to play an important role in these biological processes.

Experimental Procedures

Plasmids

BDNF pIII(170)Luc, EF-β-gal, pSG424 (Gal4only), Gal4CREB, and Gal4-UAS-Luc were described previously (Abdollah et al., 1997; Sheng et al., 1991; Tao et al., 1998). We generated 2 bp substitutions of *BDNF* pIII(170)Luc by PCR with a 5′ oligo containing two random bases at 2 bp intervals from nucleotides −77 to −60 in promoter III. CaRE1-Luc has three copies of the *BDNF* promoter III sequence from −79 to −58 upstream of the SV40 minimal promoter in the vector pGL3 (Promega). CRE-Luc is from Stratagene and TK-pRL is from Promega. CaRF and its deletion mutants were cloned into pEFmyc-his (Invitrogen) for expression. Gal4CaRF was cloned by PCR with primers against bases 951 to 2352, generating a fragment of CaRF expressing amino acids 325–725. This CaRF fragment was fused in frame to the C terminus of amino acids 1–147 of Gal4 under control of the EF promoter in the vector pEF-Bos.

Cell Culture, Transfection, and Stimulation

Cortical neurons from E18 Long Evans rats (Charles River) were cultured as described (Tao et al., 1998). Neurons were transfected at 3-4DIV by calcium phosphate precipitation (Xia et al., 1996). Neurons were depolarized 2 days after transfection with 50 mM KCl (Tao et al., 1998). CPT-cAMP (Sigma) and IBMX (Sigma) were added at concentrations of 300 μM each. Cells were stimulated for 1, 3, or 6 hr for quantitative RT-PCR of endogenous gene expression, and for 6–9 hr for luciferase assays. L-VSCCs were blocked by the addition of 5 μM Nimodipine (Roche Molecular Biochemicals) and PKA was blocked by addition of 10 μM H89 (Calbiochem) for 5 min prior to depolarization. PC12 cells were transfected with Lipofectamine 2000 and stimulated 2 days later as for the neurons.

Luciferase Assays

Luciferase assays were carried out 2 days after transfection using the Dual Assay Luciferase kit (Promega). Cotransfected EF-β-gal or TK-renilla luciferase was used to normalize samples for transfection efficiency and sample handling. The calcium-mediated induction of these viral promoters also served as a control for the potential induction of Gal4-CREB and Gal4-CaRF protein expression during membrane depolarization. The data presented are the average of at least three measurements from each of three independent experiments. Statistical significance was determined by ANOVA using the Statview program.

Quantitative RT-PCR

RNA was prepared with the Absolutely RNA kit (Stratagene). Eight hundred nanograms RNA was used for reverse transcription with the First Strand Superscript II kit (Invitrogen). PCR was carried out in an iCycler (BioRad) using SYBR-green (P-E Applied Biosystems). Each independent sample was assayed in triplicate. Threshold cycle for each sample was taken from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. *BDNF* and c-*fos* levels were normalized for each well to *GAPDH*. Single PCR products were verified both by assessing that the melting temperature of the product had a single value, and by viewing the PCR product on an agarose gel.

Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts and EMSAs were done as described (Tao et al., 1998). The CaRE1 probe was synthesized as complementary oligonucleotide probes 5′-GAGTGTCTATTTCGAGGCAGAGGAGG-3′ and 5′-CCTCCTCTGCCTCGA-3′. Wild-type and mutant unlabeled probes were synthesized by PCR using primers 5′-GGTAATTCGTGCAC TAG-3′ and 5′-CGAGAGGGCTCCACGCTGC-3′. In competition assays, unlabeled probes were added to nuclear extracts in 100-fold molar excess to the radiolabeled probe for 30' at 4°C prior to addition of the labeled probe. In antibody supershift assays, 1 ul anti-CaRF antibody or preimmune serum was added to the nuclear extract and incubated at 40°C for 1 hr prior to addition of radiolabeled probe.

Yeast One-Hybrid Screen

The yeast one-hybrid screen for CaRE1 interaction proteins was carried out using the Matchmaker Yeast one-hybrid system (Clontech). Eight repeats of the CaRE1 sequence (from nucleotides −79 to −58) were cloned upstream of the *HIS3* gene and four copies were cloned upstream of *LacZ*. These plasmids were integrated to generate a yeast reporter strain, and the strain was transfected with a human fetal brain library containing 3.5×10^6 independent clones fused to the transcriptional activation domain of the yeast Gal4 protein (Clontech). Colonies that grew on minimal medium lacking histidine were lifted on filters and screened for β-gal expression.

Library Hybridization and RACE

Filter lifts of colonies infected with a mouse P0 brain library in lambda phage (Stratagene) were hybridized with a probe from bases 685 to 825 of human CaRF. One positive clone was recovered and sequenced. 3′ RACE (Invitrogen) was performed with a polyA anchored 3′ primer, and 5′ RACE was performed with the RLM-RACE kit (Ambion).

Antibodies

His-tagged CaRF (amino acids 145–725) in the pET vector (Novagen) was transfected into the BL21 strain of *E. coli* and expression of the protein was induced with IPTG. The protein was purified from lysed bacteria over nickel resin (Invitrogen) and isolated by SDS-PAGE. Purified protein was injected into rabbits (Covance) and the serum was collected. Anti-CaRF preimmune and immune serum was used at 1:1000 for Western blotting and 1:500 for cell staining. The mouse TuJ1 anti-β-tubulin III antibody (Babco) was used at 1:300, and mouse anti-GFAP (Roche Molecular Biochemicals) was used at 1:250.

Northern Blotting and In Situ Hybridization

Mouse multiple tissue northern was purchased from Ambion. In situ hybridization was performed as described (Corfas et al., 1995). Frozen sections were cut from paraformaldehyde fixed P4 mouse heads or perfused adult brains. An antisense ^{33}P-labeled CaRF probe was made against the complete coding sequence.

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Accession Numbers

The CaRF sequences reported in this paper were deposited in GenBank under accession codes AF454946 (human) and AF454947 (mouse).