The calcium-responsive transactivator (CREST) is targeted to nuclear bodies and is required for the normal development of neuronal dendritic trees. Here we report the identification of a multifunctional domain (MFD) of CREST that is involved in transcription transactivation, nuclear body targeting, and dimerization. MFD is located near the C terminus of CREST from amino acid 251 to 322 and is required and sufficient for CREST homodimerization. When fused with a GAL4 DNA-binding domain, MFD was effective in transcription transactivation of a luciferase reporter system. A C-terminal 339–401 amino acid sequence of CREST was shown to contain a nuclear localization signal (NLS), which was able to direct a yellow fluorescence protein (YFP) to nucleus. A CREST deletion mutant containing both the MFD and NLS, which spanned the C-terminal amino acid sequence 251–401, was able to target YFP to the nucleus and nuclear bodies. However, MFD alone failed to target YFP and was largely cytosolic. The addition of a SV40 NLS to MFD domain restored nuclear body targeting. When YFP-MFD was expressed in cultured rat embryonic cortical neurons, it was effective in inhibiting depolarization-induced dendritic growth, suggesting that CREST dimerization may be necessary for its function in neuronal dendritic development.

The calcium-responsive transactivator (CREST) has been shown to be required for activity-dependent dendritic development in neurons (1). Mutant mice lacking CREST had a marked decrease in dendritic growth and branching, whereas axonal growth was largely unaffected (1). Sequence analysis showed that CREST is highly homologous to the SYT proto-oncogene that is involved in synovial sarcoma with 54% amino acid identity (1, 2). CREST is composed of three main functional domains: a N-terminal region that has been suggested to play an auto-regulatory role, an internal methionine-rich domain with unknown functions, and a large C-terminal glutamine-rich domain responsible for transactivation (1). Immunohistochemical localization showed that CREST is a nuclear protein (1). Moreover, CREST was found in nuclear body structures in the rat pheochromocytoma PC12 cells and when it was expressed in various cell types (3). A possible functional significance of CREST nuclear bodies is the recruitment of additional transcription activators/co-activators. Deletion analysis suggested that the nuclear body-targeting signal was localized to its C-terminal region of CREST (3).

CREST has been shown to interact with cAMP-response element-binding protein and p300 (1), and such interactions may be crucial for the Ca^{2+}-dependent transactivation of CREST and its function in activity-dependent neuronal dendritic development. Activity-dependent gene transcription in the central nervous system is mediated by second messengers such as Ca^{2+} and cAMP (4–6). For instance, the transcription factor CREB binds to the Ca^{2+} - and cAMP-responsive element CRE and is shown to play an essential role in synapse formation and memory storage (7–9). In the current study, we have identified a domain that is involved in CREST transactivation, nuclear body targeting, and homodimerization. Furthermore, we have demonstrated that this domain was able to inhibit neuronal dendritic growth in cultured neurons, suggesting that CREST dimerization is necessary for its function.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Mutagenesis—**The FLAG-(251–322) CREST was generated by PCR. A point mutation at 751 bp was engineered, and full-length FLAG CREST was used as the template. Primers used were as follows: forward, 5′-CTCTTCCCAGCGTACCT-GGGCCA-3′, and reverse, 5′-TGGCCCATGACCTGCTGGA-3′. A possible functional significance of CREST nuclear bodies is the recruitment of additional transcription activators/co-activators. Deletion analysis suggested that the nuclear body-targeting signal was localized to its C-terminal region of CREST (3).

**CREST** has been shown to interact with cAMP-response element-binding protein and p300 (1), and such interactions may be crucial for the Ca^{2+}-dependent transactivation of CREST and its function in activity-dependent neuronal dendritic development. Activity-dependent gene transcription in the central nervous system is mediated by second messengers such as Ca^{2+} and cAMP (4–6). For instance, the transcription factor CREB binds to the Ca^{2+} - and cAMP-responsive element CRE and is shown to play an essential role in synapse formation and memory storage (7–9). In the current study, we have identified a domain that is involved in CREST transactivation, nuclear body targeting, and homodimerization. Furthermore, we have demonstrated that this domain was able to inhibit neuronal dendritic growth in cultured neurons, suggesting that CREST dimerization is necessary for its function.
with a digital camera. All of the dendrites in each region of interest 17 embryonic cortical cultures were transfected with a GFP expression (1:500) was used. To isolate the dendrites, the neurons were stained the cells at 4 °C overnight. Rhodamine-conjugated donkey anti-goat IgG then blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. PA). For the endogenous CREST staining in neurons, the neurons were monoclonal anti-FLAG (10

3% bovine serum albumin in PBS for 30 min at room temperature. The cells were cultured for 24 h before transfection using Lipofectamine 2000 (Invitrogen). The cells were incubated with 8 μg of pG5 luciferase vector (Promega). The pG5Luc vector contains five GAL4-binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. After 24 h, the media was changed and the cells were treated with 70 mM KCl. 48 h after transfection, the cells were collected in 1× cell culture lysis buffer. The samples were vortexed briefly and then centrifuged at room temperature for 30 s. 100 μl of luciferase reagent (Promega) was added to the cell lysate and mixed, and the sample tube was placed in the luminometer (TD-20/20, Turner Designs) to initiate reading.

Cell Cultures and Transfection—PC12 and HEK293 cells were cultured and transfected by polyethyleneimine and calcium phosphate method, respectively, as described previously (3). Rat embryonic cortical neurons were cultured as described previously (3). The cortices from day 17 embryos of Sprague-Dawley rat were dissected and dissociated by trituration. Cells were plated onto 35-mm culture dishes precoated with 100 μg/ml poly-D-lysine (Sigma) and cultured in neurobasal media (Invitrogen) with 0.5% fetal bovine serum and N2 supplement (Invitrogen). The cells were cultured for 24 h before transfection using Lipofectamine 2000 (Invitrogen). The cells were incubated with 8 μg of pG5 luciferase vector (Promega). The cells were then blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. Goat anti-CREST (1:2,000) antibody was incubated with the cells at 4 °C overnight. Rhodamine-conjugated donkey anti-mouse IgG (1:500) was from Jackson Immunoresearch Laboratories (West Grove, PA). For the endogenous CREST staining in neurons, the neurons were fixed and permeabilized with 90% cold ethanol for 5 min. The cells were then blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. Goat anti-CREST (1:2,000) antibody was incubated with the cells at 4 °C overnight. Rhodamine-conjugated donkey anti-goat IgG (1:500) was used. To isolate the dendrites, the neurons were stained with monoclonal anti-MAP-2 antibody (1:1000, Sigma) at 4 °C overnight. Rhodamine-conjugated donkey anti-mouse IgG (1:500) was used as secondary antibody.

Analysis of Dendritic Length—For analysis of dendritic length, day 17 embryonic cortical cultures were transfected with a GFP expression vector and analyzed. Images of the transfected neurons were captured with a digital camera. All of the dendrites in each region of interest were manually traced, and the length was measured using IPLab 3.5 (Scanalytics, Fairfax, VA). For each experiment, at least 100 cells were measured.

RESULTS
CREST Dimerization

CREST Targeting Is Mediated by Its C-terminal Domain—We have previously shown that CREST is specifically targeted to nuclear bodies in a variety of cell types including HEK293, COS-7, PC12, and MCF-7 cells. A significant amount of CREST was detected in cultured rat embryonic neurons and neuronal-like PC12 cells (3). Using a polyclonal goat-anti-CREST antibody, we were able to show that the CREST protein was primarily localized to the nucleus of cultured rat cortical neurons, as previously reported (1). Under a ×100 objective, CREST nuclear bodies were clearly identifiable (Fig. 1), which is similar to the endogenous CREST localization in a PC12 cell line (3).

We have previously located the nuclear body-targeting domain of CREST to the C-terminal 238–401 amino acid sequence (3). To further define the nuclear body-targeting domain, we made several additional CREST mutants that were fused with the YFP. An internal deletion mutant, CREST-(251–322), in which the amino acid sequence was removed, failed to target to nuclear bodies (Fig. 2). Interestingly, CREST-(Δ251–322) was still targeted to the nucleus (Fig. 2A), suggesting that a nuclear localization signal for CREST exists outside the nuclear body-targeting domain. A search based on known NLS criteria did not yield any classical NLS in CREST. However, a deletion mutant C (251–401) was found to target YFP to nucleus and nuclear bodies (Fig. 2A). Removing amino acid residues 251–322 abolished nuclear body localization, whereas nuclear localization remained unaffected. These results suggest that the C-terminal 339–401 contained the NLS. Moreover, it indicates that nuclear import and nuclear body localization are closely coupled events.

To test whether a nonspecific NLS could facilitate the nuclear body targeting, we fused a SV40 NLS to the N-terminal of C-(251–322). As shown in Fig. 2B, nearly all of YFP-NLS-C (251–322) were targeted to the nucleus. Moreover, ~45% showed nuclear body localization by the fusion protein. This suggested that the sequence from amino acids 251–322 contain the element that is needed for nuclear body localization, although the efficiency was reduced compared with the wild-type CREST.

Dimerization of CREST—A majority of the endogenous CREST in rat brain existed as multimeric complexes when analyzed by ultracentrifugation sedimentation experiments. As shown in Fig. 3, there were two distinct CREST species that were separated on a density gradient. A small fraction of CREST had an apparent sedimentation coefficient (S) of 5, and a majority of CREST had a S value of ~17. Because the endogenous CREST may have multiple binding partners, we transiently expressed CREST in HEK293 cells and performed chemical cross-linking of the exogenous proteins. As shown in Fig. 3B, a product with an apparent molecular mass of 120 kDa was detected, suggesting that CREST could exist as homodimers.

To further investigate whether there is a minimal domain of the protein necessary for the dimerization, we determined the
dimerization domain by co-immunoprecipitation experiments. CREST and CREST deletion mutants were fused to YFP and co-expressed with FLAG-tagged CREST or its mutants. As shown in Fig. 4, YFP-CREST was able to interact with FLAG-CREST and was co-immunoprecipitated. The deletion of the N-terminal 1–145 or C-terminal 322–401 amino acid sequences did not affect dimerization, whereas the removal of amino acid 189–401 abolished dimerization, suggesting that the dimerization domain was in the C terminus.

An internal deletion mutant, CREST-(251–322), in which the nuclear body-targeting domain was removed, failed to interact with the full-length CREST (Fig. 4B). However, amino acid sequence 251–401 containing the nuclear body targeting and NLS domains were fully functional in dimerization (Fig. 4B). To further dissect the dimerization domain, we co-expressed FLAG-CREST with YFP-C-(251–322) or YFP-C-(322–401) and performed co-immunoprecipitation using a monoclonal anti-FLAG antibody. As shown in Fig. 4C, the FLAG-CREST was co-immunoprecipitated with YFP-C-(251–322). However, YFP-C-(339–401) did not interact with the FLAG-CREST (data not shown). These results demonstrated that the nuclear body-targeting domain was also responsible for the homodimerization of CREST.

**Transactivation activity of C-(251–322)—CREST has been shown to be a Ca\(^{2+}\)-dependent transactivator by Ghosh and colleagues (1). When CREST was fused with a GAL4 DNA-binding domain, the fusion protein was able to activate transcription of a reporter gene under the control of GAL4 promoters in a Ca\(^{2+}\)-dependent manner. Using similar strategies, it was shown previously that the CREST homolog SYT also activated transcription, although no attempts were made to determine its calcium dependence (11). We have used the rat pheochromocytoma PC12 cells to assay CREST transactivation activities. Undifferentiated PC12 cells express l-type Ca\(^{2+}\) channels, and the cells have been extensively used for investigating neuronal functions such as differentiation and Ca\(^{2+}\)-dependent exocytosis (12). A high concentration of KCl leads to membrane depolarization and Ca\(^{2+}\) influx in PC12 cells, which allows assaying for Ca\(^{2+}\)-dependent transcription similar to that in cultured neurons.

As shown in Fig. 5, GAL4-CREST fusion protein activated the transcription of luciferase from the GAL4 promoters that was enhanced by KCl treatment, which was in agreement with the previous results from cultured neurons (1). Interestingly, Ca\(^{2+}\)-independent transcriptional activation was also prominent, similar to those previously reported for GAL4-SYT fusion protein (11). Thus, it seems likely that, in addition to the Ca\(^{2+}\)-dependent transactivation, CREST plays an important role in basal transcriptional activation in the absence of Ca\(^{2+}\) influx.

The GAL4-C-(251–322) was also able to activate transcription of the luciferase reporter in a Ca\(^{2+}\)-independent manner,
and the activity was markedly higher compared with the wild-type CREST (Fig. 5). This was probably because of the removal of the putative N-terminal inhibitory domain. However, no enhancement was observed under KCl depolarization, and KCl treatment seemed to further depress transcription (Fig. 5). These results suggested that amino acid sequence 251–322 of CREST contained a basic transactivation domain, whereas the Ca\textsuperscript{2+}-responsive domain lay in other parts of the molecule. Based on the results that the same 251–322 amino acid sequence was involved in CREST targeting, dimerization, and transactivation, we have established the domain multi-functional (MFD).

The MFD Domain Inhibits Depolarization-induced Dendritic Growth in Culture Neurons—Cultured cortical neurons from rat embryos undergo differentiation that includes rapid axonal growth and a somewhat delayed and slower dendritic growth. After 4–5 days in vitro, most neurons have a single long axon and several short dendritic processes that can be distinguished with specific immunological markers such as MAP-2 (Fig. 6A) (15). Under depolarizing conditions, i.e. 56 mM KCl, which activates voltage-dependent calcium channels, the dendritic growth is accelerated, which results in a marked increase in total dendrite length and complexity (13).

To determine the role of CREST dimerization in such activity-dependent dendritic growth/development, we transfected cultured cortical neurons from embryonic day 17 rats with YFP-MFD. The neurons were depolarized with KCl at 4–5 days in vitro, and their axonal and dendritic growth was examined by fluorescence microscopy. As shown in Fig. 6, the expression of YFP-MFD effectively blocked KCl-induced dendritic growth compared with the control neurons expressing YFP. However, axonal growth did not seem to be affected.

Because YFP-MFD resided largely in the cytosolic compartments (Fig. 2), we asked whether it could still form a heterodimer with wild-type CREST and move to the nuclear compartment, especially the nuclear bodies. As shown in Fig. 6D, YFP-MFD became highly co-localized with FLAG-CREST in the nuclear bodies when the two proteins were co-expressed in HEK293 cells. These results suggested that YFP-MFD was able to dimerize with the wild-type CREST, probably in the cytosolic compartment, and then translocate to the nucleus. Furthermore, it suggested that the formation of CREST-YFP-MFD heterodimer interfered with depolarization-induced dendritic growth, probably as a result of reduction in the amount of CREST homodimers.

DISCUSSION

Although the importance of CREST in activity-dependent dendritic tree growth during brain development is well established, the mechanisms underlying its function and how its activity is regulated remain unknown. Structural analysis suggests that the protein may have three major functional domains that include a N-terminal regulatory domain, an internal methionine-rich domain, and a C-terminal transactivation domain (1). The C-terminal domain originally characterized by Azaiwa et al. (2004) spans a large region from amino acid 238 to 401, which also contains a CREB-binding protein-binding site located within the last 9 amino acids (1). We have previously shown that amino acid sequence 1–322 of CREST contains the nuclear body-targeting signal (3). Now we have narrowed down the domain to 251–322 as essential...
for CREST nuclear body targeting. Moreover, we have shown that this is a multi-functional domain that is involved in transactivation, dimerization, and nuclear body targeting. However, it is unknown whether the domain can be further dissected structurally and functionally.

Many transcriptional activators/co-activators contain glutamine-rich activation domains. Thus, it was not surprising that the transactivation domain of CREST resided within its C-terminal region that is rich in glutamine. Interestingly, the activation activity of the MFD was significantly higher than that of the wild-type CREST (Fig. 5). This is probably attributed to the absence of the N-terminal domain, which has been shown to inhibit CREST activity (1). However, this domain failed to respond to KCl-induced membrane depolarization that markedly enhanced the transcriptional activation by wild-type CREST (Fig. 5). Thus, it appears that MFD probably contains the basic and main transactivation domain of CREST, whereas the Ca²⁺-responsive domain lay outside the region. This C-terminal region is also highly homologous to the so-called QPGY domain of SYT, which is enriched with Glu, Pro, Gly, and Tyr residues and is shown to be responsible for transcriptional activation of SYT (2, 11). However, it has not been determined whether the corresponding sequence of 251–322 in SYT is the transactivation domain. Moreover, there is a deletion of 30 amino acid residues from 282–312 in SYT and the functional implication of this region is unknown at present.

Because of the high degree of homology between SYT and CREST, a comparative study between the two proteins should yield insightful information regarding their functions in neuronal and cancer development.

Using several approaches, we have demonstrated that CREST could form a homodimer or oligomer when expressed in HEK293 cells (Fig. 3). Dimerization of transcription factors and activators is a common phenomenon associated with the gene transcription process. For instance, transcription factors of the AP-1 family form homodimers or heterodimers that are crucial for transcriptional activation. By exploiting such properties, researchers have generated dominant c-Jun mutations that block its transcriptional activities in various cell types (14). Using similar approaches, we have shown that YFP-MFD was able to act as a dominant negative mutant that inhibited depolarization-induced dendritic growth in cultured neurons (Fig. 6). Such a mutant should offer a useful tool for investigation into CREST function and its regulation during brain development.

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