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Autonomously Binding Protein Detected on ets Box of c-*fos* Serum Response Element in Proliferating Cells

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The serum response element (SRE) in the c-*fos* promoter contains an ets box whose integrity is required for full activation of this proto-oncogene by nerve growth factor (NGF) in PC12 rat pheochromocytoma cells. Electrophoretic mobility shift assays (EMSA) detect a protein in nuclear extracts that binds to the wild-type SRE, but not to an SRE containing a mutated ets box. Competition studies using unlabeled probes, and supershift experiments using antibodies and in vitro translated core serum response factor (SRF) indicate that the protein in question is not YY1, SAP-1, nor Elk-1 and that it does not exhibit ternary complex factor (TCF) activity, so that it may correspond to an autonomously binding Ets family protein. The complete disappearance of this "Ets-like autonomous binding factor" upon terminal differentiation of both L6α2 myoblastic and PC12 pheochromocytoma cells points to a possible role in the proliferation/differentiation process.

PC12 c-*fos* Serum response element (SRE) Ets Nerve growth factor (NGF)

THE serum response element (SRE) of the c-*fos* promoter is an important target for transduction of the nerve growth factor (NGF) signal in PC12 cells (1,18,19,28). Among the proteins reported to bind to the SRE and to activate it (31,32), the serum response factor (SRF) and the ternary complex forming proteins (TCFs) are believed to play a central role in signal transduction. The SRE is a target for at least two signal transduction pathways, one of which includes $p21^{RAS}$ and MAP kinases and results in the phosphorylation of SAP-1 and ELK-1 (7), and one which directly affects SRF through an unknown mechanism (8). SRF is a member of the MADS box family of proteins that recognizes a sequence (CArG box) in the center of the SRE; it binds constitutively to the CArG box as a homodimer (21). TCFs such as SAP-1 (4), Elk-1 (9), and Net/Erp/Sap-2 (5,15,23) bind to a sequence, adjacent to the CArG box, called the ets box and form complexes with the SRF protein. Elk-1 and SAP-1 levels vary greatly among cell lines, and even when considerable amounts of protein are present, binding and/or ternary complex formation on the c-*fos* SRE may in some cases be minimal or undetectable (16). TCFs belong to the Ets family, which includes c-Ets1, c-Ets2, Erg-1/erg-2, Elk-1, Elk-2, Spi-1, E74, PEA3, Fli1, GABAα, e1g, Elf-1, SAP-1, and TCF-α; all the members share a highly conserved 85 amino acid domain, the Ets domain, which recognizes a specific DNA sequence present in the transcriptional regulatory regions of a variety of genes and consisting mainly of the purine-rich core sequence C/AGGAA/T (33). Se-

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quences flanking the GGAA/T core influence the DNA binding affinity of the different Ets proteins (29,34).

We demonstrate the existence of a protein that binds to the ets box of the c-*fos* SRE independently of SRF. This protein, which appears to be different from those previously reported to bind to the SRE, is downregulated after differentiation of PC12 pheochromocytoma and L6α2 myoblastic cell lines, suggesting a possible role in regulating the proliferation/differentiation balance.

MATERIALS AND METHODS

Cells and Culture

PC12 rat pheochromocytoma cells were maintained in RPMI-1640 (Gibco Life Technologies, Cergy-Pontoise, France) supplemented with L-glutamine, antibiotics, 10% heat-inactivated horse serum (Biochrom KG, Berlin), and 5% heat-inactivated fetal calf serum (FCS; Biochrom KG). NIH 3T3 (mouse fibroblast) cells were maintained in DMEM (Gibco) containing 4.5 g/l glucose supplemented with 10% heat-inactivated FCS. L6α2 rat and C2C12 mouse myoblastic cell lines were maintained in DMEM supplemented with 20% heat-inactivated FCS.

Plasmids and Oligonucleotides

pT7SAP-1 and pT7Elk-1 were kind gifts from R. Treisman (4). HIVSRF1-4 (full-length SRF) and HIVSRF2-3 (core SRF, comprising amino acids 131–262) were constructed by subcloning the coding sequence of SRF from pG3.5 (a gift from R. Treisman) as described previously (17). pGEX2TK was obtained from Pharmacia (Saclay, France), and the GSTYY1 plasmid was a gift from T. Shenk (26). pGSTSRF contains the SRF coding sequence for amino acids 10–508, and pGSTSAP-1 the coding sequence for full-length SAP-1a, cloned into the pGEX2TK vector. All constructs were verified by direct nucleotide sequencing using a USB sequencing kit (USB, Cleveland, OH).

The blunt-ended double-stranded oligonucleotides used for EMSA are presented in Fig. 1; for simplicity, only one strand is shown. The GpIIb oligonucleotide covers a region of the human glycoprotein IIb promoter that contains a FLI-1 octanucleotide consensus sequence (37). The oligonucleotide containing the –60 YY1 binding sequence from the adeno-asso-

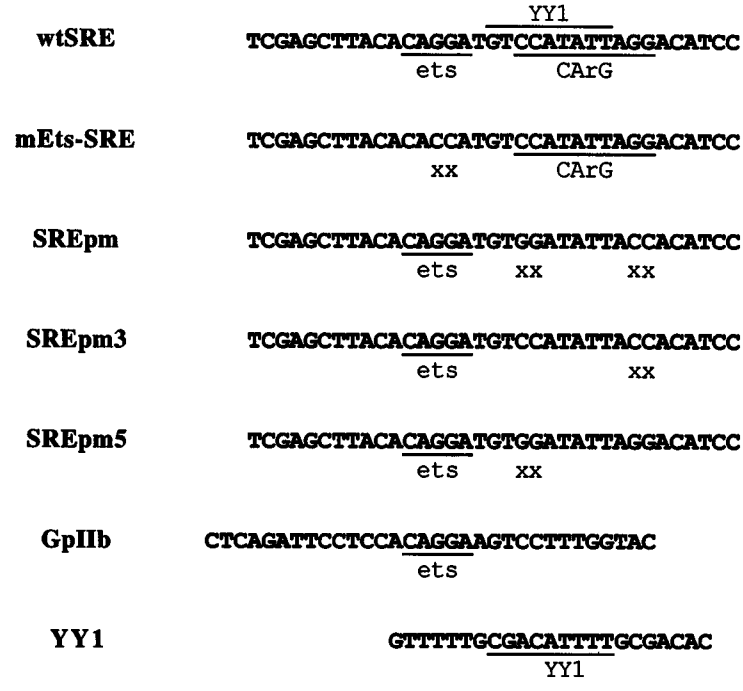

FIG. 1. Double-stranded oligonucleotide probes used in this study. wtSRE: wild-type c-*fos* proximal SRE (–332 to –298). mEtsSRE: the corresponding oligonucleotide with a double mutation that inactivates the ets box. SREpm, SREpm3, SREpm5: SRE oligonucleotides containing mutated CArG boxes. GpIIb: sequence from the human glycoprotein IIb promoter containing a FLI-1 octanucleotide consensus sequence. YY1: oligonucleotide containing the –60 YY1 binding sequence from the adeno-associated-virus P5 promoter. Horizontal lines delineate ets and CArG boxes, as well as the YY1 binding site. Mutated bases are indicated by an "x" placed below the sequence.

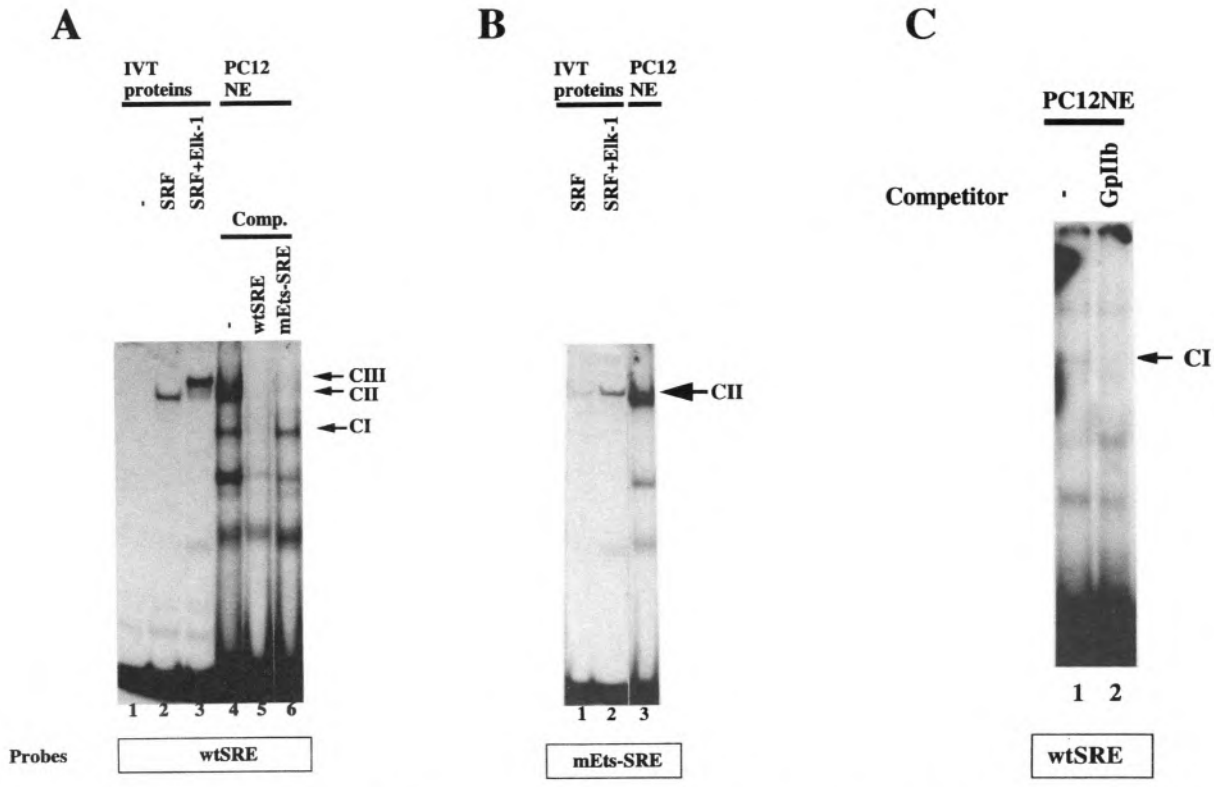

FIG. 2. Gel retardation analysis (EMSA) of nuclear proteins binding to the c-*fos* SRE. In vitro translated (IVT) full-length SRF and Elk-1 proteins, as well as nuclear extracts (NE) from untreated PC12 cells, were tested in EMSA using the indicated probes. For specificity analyses, a 100-fold excess of unlabeled oligonucleotide was incubated with PC12 NE for 15 min at room temperature prior to incubation with the radiolabeled probe. Sequences for all probes and competitors are shown in Fig. 1. (A) PC12 NE proteins form three specific complexes on the c-*fos* wtSRE. Lane 1: control reticulocyte lysate; lane 2: in vitro translated full-length SRF; lane 3: in vitro translated full-length SRF and Elk-1; lane 4: PC12 NE in absence of competitor; lane 5: PC12 NE in presence of wtSRE; lane 6: PC12 NE in presence of mEts-SRE. Arrows indicate the positions of the three complexes (CI, CII, CIII). (B) PC12 NE form only one complex on the mEts-SRE probe, migrating at the position of CII. Lane 1: in vitro translated full-length SRF; lane 2: in vitro translated full-length SRF and Elk-1; lane 3: PC12 NE. (C) Competition with unlabeled GpIIb oligonucleotide prevents detection of CI on the wtSRE probe. Lane 1: PC12NE; lane 2: PC12NE in presence of GpIIb oligonucleotide. This particular example is taken from the same gel that is presented in Fig. 6A, and involves a PC12 NE that had been subjected to heparin agarose affinity purification. Lane 1: PC12 NE in absence of competitor.

ciated-virus P5 promoter was a gift from N. Raich (24).

EMSA

In the majority of experiments, nuclear extracts were prepared from PC12 cells (38). Exponentially growing cells were incubated with 0.5% horse serum overnight and stimulated with NGF or 20% horse serum for 10 min. For differentiation, PC12 cells were incubated with 50 ng/ml NGF in complete medium for 2 weeks. In some experiments we used partially purified protein preparations. Heparin agarose affinity purification was performed using the method of Ryan et al. (25), with slight modifications. Nuclear extracts were diluted three times with buffer A and loaded onto a 1.5-ml heparin agarose (Sigma, St. Quentin Fallavier, France) column. The column was washed with buffer A solution and eluted with buffer C containing 0.4 M KCl in 50-μl sequential fractions. Nuclear extracts were prepared from 1) L6α2 cells that had or had not been induced to differentiate, 2) C2C12 cells, and 3) NIH 3T3 cells in the same manner. To induce differentiation, confluent L6α2 cells were incubated in DMEM with 0.5% FCS for 48 h. Full-length SRF, core SRF, SAP-1a, and Elk-1 were produced using a TNT in vitro translation kit (Promega). Recombinant GST and GST-YY1 proteins were prepared using a method described elsewhere (11). EMSA were carried out as described by Zinck et al. (38), with modifications. Double-stranded oligonucleotides were labeled with [γ-^{32}P]dATP (Amersham, Les Ulis, France) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Nuclear extracts (20 μg) were incubated for 10 min at room temperature with the labeled probe in a total volume of 10 μl containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.05% skim milk, 5% glycerol, 10 mM dithiothreitol, and 1 μg salmon sperm DNA.

For specificity analyses, 100-fold molar excesses of unlabeled oligonucleotide competitors were added. Where indicated, reaction mixtures were incubated with antibodies before adding labeled oligonucleotides. Samples were loaded onto 4% native polyacrylamide gels. Gels were run at 120 V at 4°C for 3 h, then dried and exposed to X-ray films or analyzed using a Bas1000 image analyzer (Fuji Photo Film, Tokyo).

Antibodies

Anti-SRF and anti-SAP-1 antibodies were raised in rabbits by immunization with GST-SRF or GST-SAP-1a, and affinity purified on SRF or SAP-1a CNBr-activated Sepharose (Pharmacia), respectively; normal rabbit serum purified with protein A Sepharose was used as control. Anti-Elk-1, anti-SAP-1a, and anti-YY1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

*Analysis of PC12 Nuclear Extract Proteins Binding to the c-*fos *SRE*

The ets box has been shown to be a key element in SRE induction by NGF in PC12 cells [(1), and our unpublished observations]. To better understand the pathway involved in transmission of the NGF signal to the SRE in PC12 cells, nuclear extracts were analyzed by EMSA using a double-stranded oligonucleotide probe encompassing the c-*fos* wild-type SRE (Fig. 2A). Several specific complexes were detected in EMSA (CI, CII, CIII) that could be competed away by unlabeled wtSRE probe (lane 5 vs. lane 4), and two of which (CII, CIII) were also removed by excess unlabeled DNA containing an intact CArG box but a mutated ets box (lane 6 vs. lane 4). Besides being competed away by unlabeled CArG-containing probes, CII forms on CArG-containing SRE probes irrespective of ets box integrity, and in each case mi-

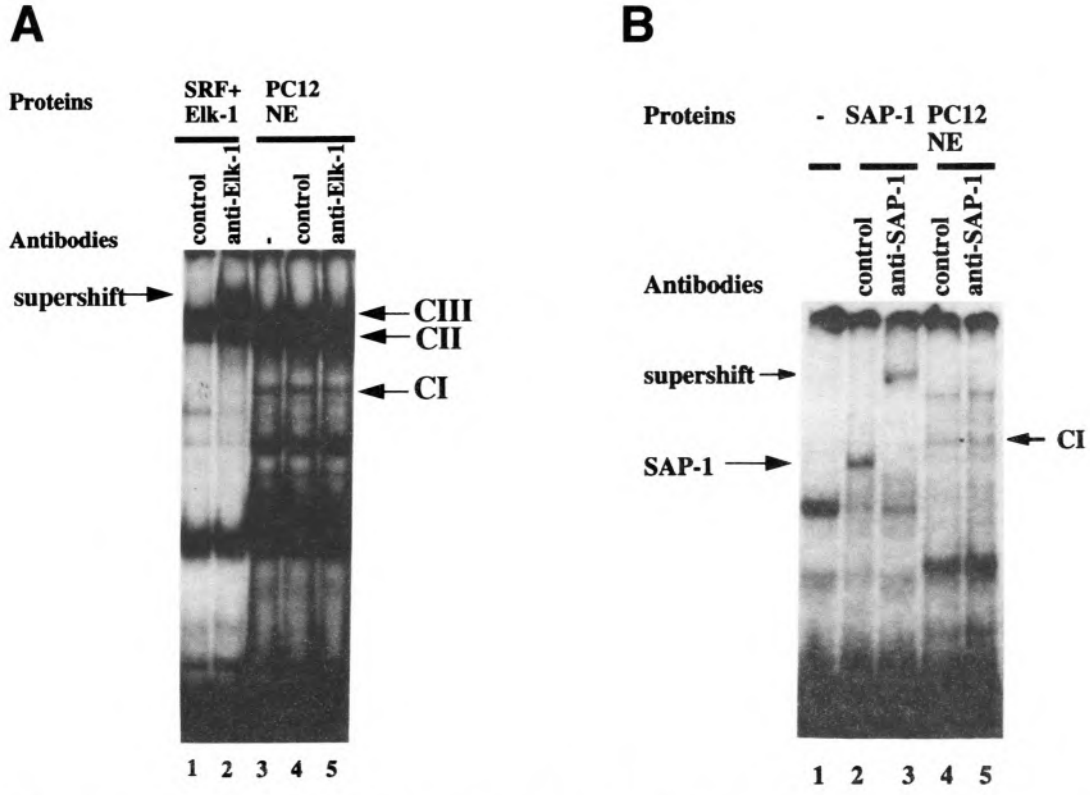

FIG. 3. CI does not contain any known TCFs. In vitro translated (IVT) proteins or PC12 nuclear extracts (NE) were incubated with control, anti-Elk-1, or anti-SAP-1 antibodies for 15 min at 4°C, before testing in EMSA on radiolabeled wtSRE probe. (A) Anti-Elk-1 antibodies do not interfere with CI detection, nor shift its position. Lane 1: in vitro translated full-length SRF and Elk-1 proteins with control antibody; lane 2: in vitro translated full-length SRF and Elk-1 proteins with anti-Elk-1 antibody; lane 3: PC12 NE alone; lane 4: PC12 NE with control antibody; lane 5: PC12 NE with anti-Elk-1 antibody. (B) Anti-SAP-1a antibodies do not interfere with CI detection, nor shift its position. The arrows on the left indicate autonomous binding of SAP-1a (lower arrow) and a supershift of this complex (upper arrow) by anti-SAP-1a antibodies. Lane 1: control reticulocyte lysate; lane 2: in vitro translated Sap-1 protein with control antibody; lane 3: in vitro translated Sap-1 protein with anti-Sap-1 antibody; lane 4: PC12 NE with control antibody; lane 5: PC12 NE with anti-Sap-1 antibody.

grates at the same position as does in vitro translated SRF (Fig. 2A, lanes 4 and 2; Fig. 2B, lanes 3 and 1); thus, CII appears to be SRF. CIII was not observed when the probe was mutated in either the ets box (Fig. 2B, lane 3) or the CArG box (not shown), and besides being removed by competitors containing an intact CArG box as noted above, CIII migrates at the same position as the Elk-1/SRF complex formed with in vitro translated proteins (Fig. 2A, lanes 4 and 3); hence, CIII corresponds to a ternary complex. Like CIII, CI was detected on a wild-type SRE probe (Fig. 2A, lane 4) but not on a probe containing a mutated ets box (Fig. 2B, lane 3); unlike CIII, when tested on an intact SRE, CI was not affected by competition with a probe containing an intact CArG but a mutated ets box (Fig. 2A, lane 6 vs. lane 4). CI was, however, competed away by GpIIb probe, which contains a high-affinity Ets binding site (Fig. 2C). These results collectively suggest that CI corresponds to the binding of an Ets protein.

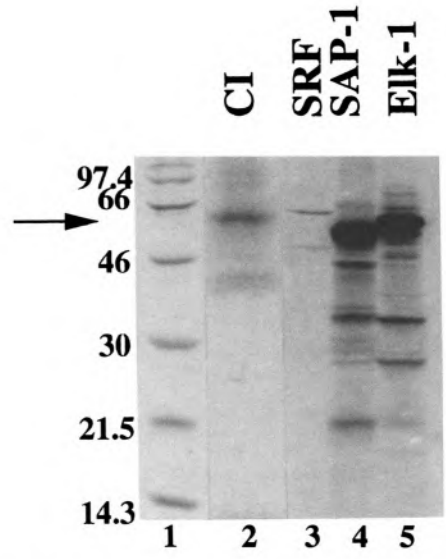

FIG. 4. UV cross-linking analysis. Nuclear extracts were incubated with the radiolabeled wtSRE probe followed by UV irradiation. The bands in EMSA corresponding to the complex were excised and eluted. The CI eluate and ^{35}S-labeled in vitro translated SRF, SAP-1a, and Elk-1 were analyzed by SDS-PAGE. Lane 1: CI; lane 2: SRF; lane 3: SAP-1a; lane 4: Elk-1. The positions of the ^{14}C-radiolabeled rainbow molecular weight marker are indicated. The arrow shows the position of CI protein.

CI Protein Seems to Be Distinct From the TCFs SAP-1 and Elk-1

We next investigated the possibility that CI protein might correspond to one of the known TCFs. As seen in Fig. 3, when tested in EMSA on the wtSRE probe, CI was not influenced by polyclonal anti-Elk-1 antibodies (Fig. 3A, lane 5 vs. lane 4); in contrast, the band corresponding to the ternary complex (CIII) formed by in vitro translated proteins (lane 2 vs. lane 1) was supershifted by this antibody. Similarly, addition of polyclonal anti-SAP-1 antibodies did not affect CI (Fig. 3B, lane 5 vs. lane 4), whereas the band corresponding to the autonomous binding of in vitro translated SAP-1a (18) was greatly shifted by the antibody (lane 3 vs. lane 2). UV cross-linking analysis suggested a molecular weight of approximately 52 kDa for the protein associated with CI after taking into account the oligonucleotide "tail" (Fig. 4). In EMSA experiments the migration of CI complex is quite distinct from SAP-1 or ELK-1 complexes, CI migrating more slowly (see, e.g., Fig. 3B, lane 2 vs. lane 4 or 5).

*CI Protein Does Not Require the Assistance of SRF in Order to Bind to the c-*fos *SRE*

To verify that formation of CI was not dependent on the presence of SRF, we further analyzed the PC12 nuclear extract by heparin agarose fractionation. CI was eluted by low concentrations of KCl, whereas SRF was eluted with higher concentrations of KCl (not shown), suggesting that CI is in fact independent of SRF. Although CI protein binds to the SRE in an autonomous manner, we next investigated whether the protein might be capable of ternary complex formation. PC12 nuclear extracts, either from untreated cells or from cells treated with 50 ng/ml NGF for 10 min, were incubated with core SRF, a deleted version of SRF that includes the dimerization domain and the DNA binding domain as well as the domain of interaction with TCFs (27). Addition of core SRF did not change the CI migration pattern (Fig. 5A, lanes 8 and 9 vs. lanes 6 and 7), whereas a ternary complex was detected with in vitro translated Elk-1 (Fig. 5A, lane 3 vs. lane 2; lane 5 vs. lane 4). Furthermore, whereas addition of anti-SRF antibody produced the expected supershift of ternary complex detected in nuclear extracts analyzed on a wild-type c-*fos* SRE probe, the treatment did not influence CI migration (Fig. 5B, lane 2 vs. lane 1). Thus, CI protein does not appear to have TCF activity. Finally, short-term NGF treatment does not influence CI complex (Fig. 5A, compare lanes 8 and 9, for example).

CI Does Not Involve YY1

The ubiquitous protein YY1 (12) is known to bind to the c-*fos* SRE without displacing SRF (20). Its

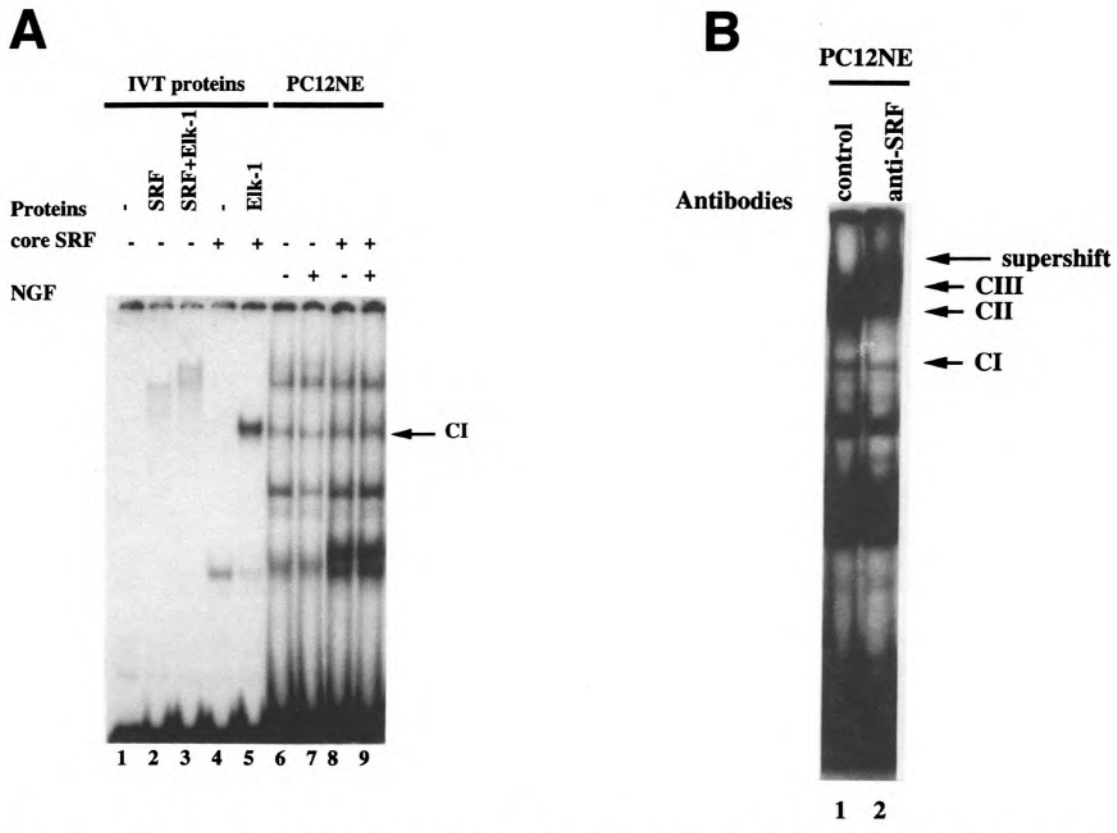

FIG. 5. CI protein binds to c-*fos* wtSRE in the absence of SRF. (A) In vitro translated (IVT) full-length SRF and/or Elk-1, or PC12 nuclear extracts (NE) were tested in EMSA for complex formation on the wtSRE, with addition of IVT core SRF protein where indicated. PC12 NE were prepared from untreated or NGF-treated (50 ng/ml, 10 min) cells as indicated. Lane 1: control reticulocyte lysate; lane 2: in vitro translated full-length SRF; lane 3: in vitro translated full-length SRF and Elk-1 proteins; lane 4: in vitro translated core SRF protein; lane 5: in vitro translated Elk-1 and core SRF proteins; lane 6: PC12 NE (untreated cells); lane 7: PC12 NE (cells treated with NGF); lane 8: PC12 NE (untreated cells) and core SRF; lane 9: PC12 NE (NGF-treated cells) and core SRF. The position of CI is indicated by an arrow. (B) PC12 NE were incubated with control (lane 1) or affinity-purified anti-SRF antibodies (lane 2) for 15 min before adding radiolabeled wtSRE probe. Lane 1: PC12 NE with control antibody; lane 2: PC12 NE with anti-SRF antibody. Positions of CI, CII, CIII, and supershifted complex are indicated by arrows.

consensus sequence is (C/g/a)(G/t)(C/t/a)CATN(T/a)(T/g/c) (10), and its reported elution pattern on heparin agarose (25) is similar to that we found for CI protein (see above). Because the YY1 binding site is nearly contiguous with the ets box, ets box modifications might theoretically be able to influence YY1 binding; hence, we sought to determine whether CI contains YY1. As shown in Fig. 6A, CI detection on the wtSRE probe was blocked by an excess of SREpm (lane 3) or SREpm5 (lane 4), neither of which is able to bind YY1.

Furthermore, addition of either anti-YY1 antibodies (lane 7) or an excess of unlabeled YY1-consensus probe (lane 2) did not change the electrophoretic mobility of CI, despite the efficiency of these two agents in binding GST-YY1 fusion protein as determined by EMSA on the YY1 probe (Fig. 6B). These results clearly indicate that CI does not contain YY1; they also provide additional indirect evidence for the Ets-like nature of CI protein, because all competitors containing an intact ets box (SREpm, SREpm5, SREpm3; lanes 3–5) effectively prevented CI detection.

Disappearance of CI on Terminal Differentiation

As shown in Fig. 7A, CI protein can be detected in nuclear extracts from continuously growing mouse fibroblast cells (NIH 3T3, lane 7) and mouse myoblastic cells (C2C12, lane 6), as well as rat pheochromocytoma cells (PC12, lane 5). Stimulation of PC12 cells by serum or by NGF for short periods of time (up to 20 min) did not appear to alter patterns of CI expression in experiments in which NGF effectiveness was demonstrated by a rapid phosphorylation of MAP kinase p42, as revealed by Western blot-

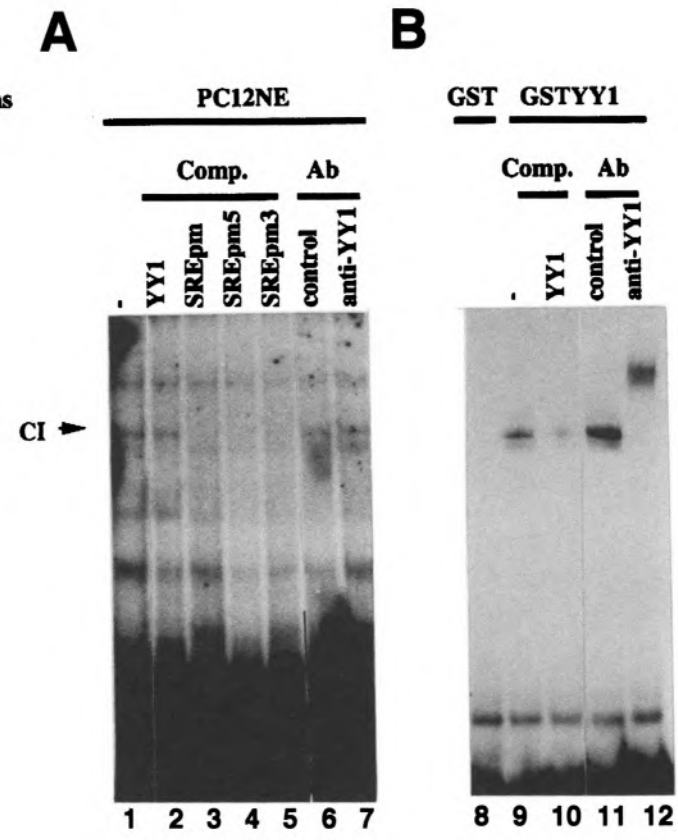

FIG. 6. CI protein is not YY1. Comp.: competitor oligonucleotide (sequences are given in Fig. 1); Ab: antibody. (A) PC12 nuclear extract (NE) was subjected to heparin agarose affinity column fractionation; aliquots were then incubated with the indicated unlabeled competitors or antibodies for 15 min before addition of wtSRE probe and analysis by EMSA. Lane 1: PC12 NE assayed in absence of competitor/antibody; lane 2: PC12 NE in presence of recombinant GST-YY1 protein; lane 3: PC12 NE in presence of SREpm oligonucleotide; lane 4: PC12 NE in presence of SREpm5 oligonucleotide; lane 5: PC12 NE in presence of SREpm3 oligonucleotide; lane 6: PC12 NE with control antibody; lane 7: PC12 NE with anti-YY1 antibody. (B) Bacterially produced GST and GST-YY1 fusion protein were assayed for binding to wtSRE probe, either directly or after 15-min preincubation with the indicated unlabeled competitor oligonucleotide or antibody. Lane 8: GST (control); lane 9: GST-YY1 in the absence of competitor; lane 10: GST-YY1 in presence of YY1 oligonucleotide (see Fig. 1); lane 11: GST-YY1 with control antibody; lane 12: GST-YY1 with anti-YY1 antibody.

ting with anti-ERK2 monoclonal antibodies (not shown).

We next determined the effect of differentiation on the expression of CI protein. PC12 cells were induced to differentiate by 2 weeks' exposure to NGF (Fig. 7B), and L6α2 rat myoblastic cells by 48-h partial serum starvation of confluent cells (Fig. 7C); in both cases, CI was detected in continuously proliferating but not in differentiated cells, consistent with the hypothesis that CI protein may play a role in the proliferation/differentiation process.

DISCUSSION

While studying NGF-stimulated activation through the c-*fos* SRE in PC12 cells, we used EMSA to analyze the SRE binding proteins in nuclear extracts, and detected a complex (CI) whose formation requires ets box, but not CArG box, integrity. Addition of either anti-SRF antibodies or core SRF did not influence migration of the complex, suggesting that it does not contain SRF and, indeed, that the protein responsible for the complex (CI protein) does not exhibit TCF activity. Despite this lack of TCF activity, we first thought that CI protein might correspond to one of the Ets family members known to form ternary complexes on the c-*fos* SRE: although full-length Elk-1, SAP-1, and Net/Erp/Sap-2 are generally considered to exhibit negligible autonomous binding to the c-*fos* SRE (23,31,32), under our experimental conditions full-length SAP-1a, and to a much lesser extent Elk-1, are in fact able to bind to and transactivate the c-*fos* SRE in the absence of SRF (18). However, CI protein can be distinguished from Elk-1 and SAP-1a by the fact that antibodies to the latter two

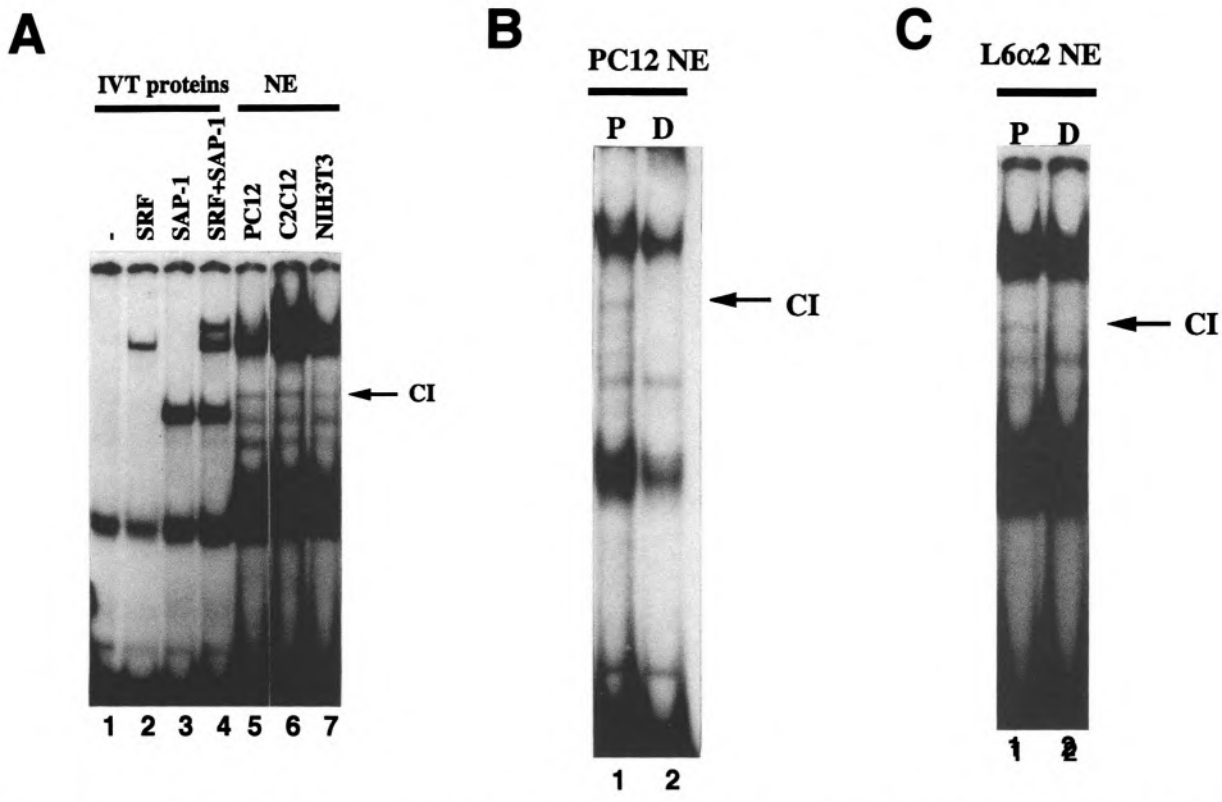

FIG. 7. Patterns of expression of CI protein. In vitro translated (IVT) full-length SRF and/or SAP-1a protein(s) as well as nuclear extracts (NE) from the indicated cell types were analyzed by EMSA on radiolabeled wtSRE probe. (A) Proliferating PC12, C2C12, and NIH 3T3 cells all contain CI protein. Lane 1: control reticulocyte lysate; lane 2: in vitro translated full-length SRF; lane 3: in vitro translated Sap-1a; lane 4: in vitro translated full-length SRF and Sap-1a proteins; lane 5: PC12 NE; lane 6: C2C12 NE; lane 7: NIH3T3 NE. (B) Disappearance of the CI protein after 2 weeks' exposure of PC12 cells to NGF. P: proliferating cells; D: differentiated cells. Lane 1: proliferating PC12 cells; lane 2: differenciated PC12 cells. (C) Downregulation of the CI protein after differentiation of L6α2 cells. P: proliferating cells; D: differentiated cells. Lane 1: proliferating L6α2 cells; lane 2: differentiated L6α2 cells.

proteins did not interfere with complex formation nor affect its mobility; and it can be distinguished from Net/Erp/SAP-2 both by its size—approximately 52 kDa, compared to 46 kDa reported (6) for Net from nuclear extracts—and by the inability of the latter to bind the c-*fos* SRE autonomously (23). Removal of the C-terminal domain of SAP-1, Elk-1, or SAP-2 has been shown to favor not only autonomous binding but also ternary complex formation (23), so that CI protein's lack of TCF activity tends to argue against the hypothesis that CI might involve a truncation product of one of the known TCFs, although this possibility has not been completely ruled out.

Among the other Ets proteins, P3F (14) and PEA3 (35,36) may fit some of the characteristics of CI protein. But in contrast with CI protein, P3F binds SRF in vitro (14); and anti-PEA3 antibodies had no effect on CI (not shown). Among the non-Ets factors reported to bind to the c-*fos* SRE, YY1 (also called CF1, δ, NF-E1, UCRBP, LBP, or p62DBF/MAPF1) was an interesting candidate protein: it binds to a sequence juxtaposing the ets box, its elution (25) and expression (13) patterns resemble those of CI protein, and it is downregulated after muscle differentiation (13). However, previous reports showed that YY1 does not bind oligonucleotides that have mutations in the 5′ portion in the CArG box [SREpm and SREpm5 (25)]—mutations that in our hands do not affect CI formation. Further, neither antibodies against YY1 nor competition with a YY1 consensus probe had any effect on CI, indicating that CI protein is not YY1.

The involvement of CI protein in regulation through the SRE is not immediately obvious, because its binding was not modified after short-term NGF or serum stimulation. However, a 20-min stimulation by serum or NGF likewise did not significantly alter TCF patterns, despite its efficacy in modulating MAP kinase (ERK 2) phosphorylation (not shown). Zinck et al. (38) showed that ternary complex formation mediated by full-length SRF was only slightly affected by cytokine stimulation, and proposed a model for c-*fos* regulation involving modification of consti-

tutively bound TCF; one could imagine an analogous scenario for CI protein. Alternatively, one might postulate that CI protein acts as a negative regulator, and is relatively unaffected by early events following NGF stimulation. This is plausible, because other proteins recognizing ets sequences have been shown to play an inhibitory role (3,6).

In contrast to its apparent unresponsiveness to short-term NGF exposure, binding of CI protein was clearly downregulated after 2 weeks' treatment of PC12 cells with NGF, or 48-h serum starvation of L6α2 cells, suggesting a possible role in the differentiation/proliferation process. This would be consistent with the "Ets-like" nature of CI protein, because several Ets family members are known to be involved in regulating differentiation events. For example, increased T-cell apoptosis and terminal B-cell differentiation was induced by inactivation of Ets1 (2); Ets2 is implicated in the process of skeletal formation (30); and drosophila Ets proteins Pointed and Yan seem to intervene in eye development (3,22).

In conclusion, we report here a protein that binds to the ets sequence of the c-*fos* SRE in an autonomous manner and that is downregulated during cell differentiation. Besides their role in SRE activation, ets binding proteins may play a role in the regulation of differentiation via regulation of the c-*fos* promoter.

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