Expression of Angiogenic Factor Cyr61 during Neuronal Cell Death via the Activation of c-Jun N-terminal Kinase and Serum Response Factor*

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The immediate early gene, Cyr61, is transcriptionally activated within minutes by serum and serum growth factors. The encoded Cyr61 protein is secreted into the extracellular matrix and promotes cell adhesion and migration. In this study, we sought to examine the expression profile of Cyr61 gene during neuronal cell death induced by various toxic stimuli and the mechanisms involved. Our data show that toxic stimuli, such as etoposide, significantly increased Cyr61 mRNA levels in immortalized hippocampal progenitor (H19-7) cells. Cyr61 transcriptional activation was corroborated at the protein level as well. To identify the upstream signaling cascades involved in Cyr61 gene induction, the blocking effect of either JNK or p38 kinase-signaling pathway on Cyr61 induction in response to etoposide was tested. Transfection of the cells with a kinase-deficient mutant of MEKK, an upstream activator of JNK, significantly decreased the Cyr61 expression induced by etoposide. In contrast, Cyr61 mRNA levels did not change after pretreatment with SB203580, the p38 kinase inhibitor. When the induction of Cyr61 was tested by using several of its deleted promoters driving the expression of reporter gene, the promoter activation occurred primarily within the region containing an SRE-like CArG box. In addition, the SRF, which binds to the CArG site, was directly phosphorylated by active JNK. Furthermore, the blockade of Cyr61 gene expression using an antisense encoding cyr61 sequence significantly inhibited the cell death induced by etoposide. Overall, these results suggest that the induction of the immediate early gene, Cyr61, is important for neuronal cell death in the central nervous system hippocampal progenitor cells, and JNK activation, but not of p38, as well as the subsequent SRF phosphorylation are involved in Cyr61 gene induction.

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The immediate early gene (IEG), Cyr61, encodes a secretory, growth regulatory, and heparin-binding protein that is associ- ated with the cell surface and extracellular matrix (1, 2). It is a member of the CCN family that includes CTGF, Nov, Eln, WISP-1, WISP-2, and WISP-3 (3–5). A remarkable feature of the CCN protein family is their organization into four conserved modular domains, which share sequence similarities with the insulin-like growth factor-binding protein, the von Willebrand factor type C repeat, and the thrombospondin type 1 repeat (6). Cyr61 was originally identified in both mouse 3T3 fibroblasts and human umbilical vein endothelial cells, and its mRNA is rapidly and transiently expressed by the serum and the serum growth factors (1). At the molecular level, its recombinant protein is able to support cell adhesion and migration, enhances the proliferative effects of the basic fibroblast growth factor. Cyr61 is expressed during embryogenesis of the circulatory system and the cartilaginous skeleton, and enhances chondrogenesis in vitro (7). In addition, Cyr61 gene plays a role during neuronal differentiation (8) and promotes angiogenesis and the growth of certain tumors, probably through its angiogenic potential (9, 10).

An upstream 2-kb 5′-flanking DNA fragment of Cyr61 gene functions as a serum-inducible promoter (11). This DNA fragment contains a sequence that resembles the serum response element (SRE) originally identified in the c-fos promoter. A deletion of the Cyr61 SRE-like sequence abrogates its serum inducibility. Furthermore, this SRE-like sequence is sufficient to confer induction by the serum and growth factor and binds to a serum response factor (SRF). The SRE mediates c-fos induction in response to the growth factor, cytokines, and other extracellular stimuli that activate the MAPK pathways (12). The SRE in the c-fos promoter is comprised of an inner core known as the CArG box, which is recognized by a dimer of the SRF, and the ternary complex factor (TCF), a family of Ets-domain transcription factors (13). Elk-1 is a member of the TCF

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† The abbreviations used are: IEG, immediate early gene; CAT, chloramphenicol N-acetyltransferase; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MK, MAPK-activated protein kinase; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor; ERK, extracellular signal-regulated kinase; CaM, calmodulin; CMV, cytomegalovirus; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; NMDA, N-methyl-D-aspartic acid, RT, reverse transcriptase; MEKK, MAPK/ERK kinase kinase; SARP, secreted apoptosis-related protein; nt, nucleotide(s); MAPKAP, MAPK-activated protein.
family, which is phosphorylated by JNK and p38 as well as by ERK (14, 15). Several N-terminal phosphorylation sites have been identified within the SRF (16). Specifically, the Ser-133 residue in the SRF is phosphorylated by CaM kinase II and IV (17) and MAPKAP kinase 1 and 2 (also referred to as MK-1 and MK-2) (18). MK-2 is located downstream of the p38 kinase pathway. Although there have been several reports of situations where JNK and/or p38 activation can occur without influencing cell death (19–21), high levels of JNK and p38 activities have been correlated with the induction of apoptosis in many cases (22–24).

Based on these findings, the possibility that cyr61 is expressed during the neuronal cell death induced by variousotoxic stimuli was investigated. The cyr61 mRNA levels were found to increase, and its encoded proteins were expressed and subsequently secreted into the extracellular space during etoposide-induced neuronal cell death in the embryonic hippocampal progenitor cells. Furthermore, the induction of cyr61 was mediated by the JNK-dependent phosphorylation of SRF, and the blocking of cyr61 expression, through use of an antisense-expressing construct, suppressed the neuronal cell death induced by etoposide. These results suggest that the induction of the angiogenic factor cyr61 plays a key role during neuronal cell death.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), the fetal bovine serum, and the LipofectAMINE reagents were purchased from Invitrogen. The U0126 was purchased from New England BioLabs. The SB203580 and 1,9-pyrazoloanthrone were purchased from Calbiochem. Both the Protein A-Sepharose and glutathione-Sepharose 4B were purchased from Amersham Biosciences. The cyr61 promoter-chloramphenicol acetyltransferase (CAT) reporter fusion constructs (2602cyr61/CAT, -1700cyr61/CAT, -1300cyr61/CAT, -926cyr61/CAT, -529cyr61/CAT, BglIIcyr61/CAT, and Xhoicyr61/CAT) were prepared, as described previously (11). Plasmid encoding antisense cyr61, pZeoSV-AS-cyr61, was constructed by inserting a 300-nucleotide antisense strand of cyr61 into the Xhol and EcoRI site of pZeoSV vector (Invitrogen, Carlsbad, CA), causing an overexpression of the anti-cyr61 expression. The clone was stably transfected into H19-7 cells. The cDNA/mRNA hybrids were amplified with the sense and antisense primers by PCR. After an initial denaturation at 94°C for 3 min, temperature cycling was initiated for each cycle as follows: 94°C for 30 s, 68°C for 1 min, and 72°C for 2 min for 25 cycles for cyr61, followed by a final elongation step at 72°C for 10 min.

Metabolic Labeling and Immunoprecipitation of Cyr61—The H19-7 cells were incubated with 1 μCi [32P]methionine (ICN, Costa Mesa, CA) and [3H]leucine and [35S]methionine (1 μCi each) for the indicated time. The cell lysates were prepared, and the recovered culture medium was concentrated using a Centricon YM-30 (with 30-kDa molecular size cut-off). The cell lysates and media were immunoprecipitated with the polyclonal anti-Cyr61 serum and analyzed as described elsewhere (45).

Reporter CAT and Luciferase Assay—The CAT assay was carried out with an enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. The luciferase activity was measured using a luciferase assay kit (Promega) and a luminometer.

Detection of Apoptotic Cells—The detection of apoptotic cells was performed by the TUNEL labeling (TUNEL) using the reaction mixture for 60 min at 37°C. After two washes with phosphate-buffered saline, cells were labeled with peroxidase-conjugated anti-goat antibody and 3,3-diaminobenzidine (DAB) to make antiphase-antibody (Fab fragment) for 30 min at 37°C and stained with a Vectastain ABC kit (Vector Laboratories). To detect interneuronal cell death, fragmentation assay was performed as described elsewhere (45).

RNA Preparations and Northern Blot Analysis—The total cellular RNA from the H19-7 cells was isolated by the TRizol reagent (Invitrogen) according to the manufacturer’s protocol. The total RNA (10 μg) was subjected to electrophoresis in a 1% agarose gel containing 37% formaldehyde for 2 h and transferred onto nylon membranes by capillary transfer. The cyr61 cDNA was labeled with 32P using a Rediprime II kit (Amersham Biosciences) according to the procedure provided by the manufacturer and used as a hybridization probe. Prehybridization and hybridization were carried out in a solution containing 50% formamide, 5× Denhardt’s reagent, 6× SSPE, 0.5% SDS at 42°C for 18 h. After hybridization, the nylon membrane was rinsed twice in 1× SSPE containing 0.1% SDS at 42°C for 20 min, which was then subject to autoradiography at −70°C for 3 days.

Western Blot Analysis with Anti-ERK, Anti-JNK, or Anti-p38 Antibodies—Cell lysates were prepared from 85 μg etoposide induced H19-7 cells. The cell lysates were analyzed by immunoblotting using a 1:1000 dilution of anti-phospho-JNK (New England BioLabs), 1:500 dilution of anti-phospho-p38 antibody (Cell Signaling Technology), 1:1000 dilution of anti-ERK (New England BioLabs), and 1:1000 dilution of anti-Cyr61 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were used as secondary antibodies.

In Vitro Immunoassay Kinase Assay—The cell extracts were prepared after the H19-7 cells were treated with the etoposide, washed twice with phosphate-buffered saline, and lysed with 300 μl of the lysis buffer (20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 10% glycerol, 1 mM β-glycerophosphate (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin). The cell lysates were incubated with 1 μg of the cyr61 cDNA/mRNA hybrids in the presence of 50 μM ATP and [γ-32P]ATP and allowed to incubate at 30°C for 1 h. After 2-h incubation, the Sepharose beads were treated with 2% SDS sample buffer, and the proteins were separated by 8% SDS-PAGE. The resolved proteins were analyzed using autoradiography (autoradiogram analysis system, BioImage). In Vitro In-gel Kinase Assay—A 12.5% SDS-PAGE gel was prepared in the presence of 50 μg/ml of the bacterial recombinant wild type GST-SRF508 or the C-terminal deleted GST-SRF140 proteins as a substrate. The total cell extracts were prepared after stimulating the
control, cells were treated with basic fibroblast growth factor effects cell viability in H19-7 cells (Fig. 2A). When 10 μM etoposide, NMDA, and glutamate confirmed that IEG cyr61 is rapidly induced by these toxic stimuli (Fig. 1A). These results indicated that etoposide-treated H19-7 cells die by means of apoptosis. However, once produced inside the cells, Cyr61 appears to be translocated gradually into the extracellular media.

FIG. 1. Induction of immediate early gene cyr61 by toxic stimuli. The H19-7 cells were treated with either etoposide (85 μM), NMDA (200 μM), or glutamate (200 μM), as indicated, and the total RNAs were prepared. RT-PCR and Northern blot analyses were performed to detect cyr61 expression. For Northern blot analysis, 10 μg of the total RNA was extracted from the cells and hybridized to a 361-bp 32P-labeled cyr61 cDNA fragment. The 32P-labeled cyr61 cDNA probe was made by using a Rediprime™ II kit. As a control for RNA loading, the total RNA was visualized under UV light by ethidium bromide staining.

RESULTS

Various Toxic Stimuli Induce the Expression of Immediate Early Gene cyr61—To examine whether or not IEG cyr61 is induced by various toxic stimuli in H19-7 cells, cyr61 mRNA expression was measured in response to several neurotoxins, such as etoposide, NMDA, or glutamate. As shown in Fig. 1A, RT-PCR analysis clearly showed that the expression of a 361-bp fragment of cyr61 was abundantly amplified after being stimulated with 85 μM etoposide for 1 h. Furthermore, cyr61 gene expression increased significantly after stimulation with either 200 μM glutamate or 200 μM NMDA. As a positive control, cells were treated with basic fibroblast growth factor (10 ng/ml), which resulted in cyr61 induction, as previously reported (8). In a similar way, Northern blot analysis using the total RNAs isolated after being stimulated with the same concentration of etoposide, NMDA, and glutamate confirmed that IEG cyr61 is rapidly induced by these toxic stimuli (Fig. 1B).

Etoposide Induces Apoptosis in H19-7 Cells—To clarify the upstream signal transduction pathways leading to cyr61 induction, the effect of the DNA topoisomerase II inhibitor, etoposide (29), on cyr61 expression was examined. Etoposide stabilizes the DNA-topoisomerase II complexes by blocking DNA relegation. Initially, the alteration of cell viability was determined in response to various etoposide doses to test how etoposide affects cell viability in H19-7 cells (Fig. 2A). When 10–85 μM etoposide was added to the serum-containing medium, the cell viability decreased in a dose-dependent manner, as measured by the trypan blue staining method. Stimulation of the cells with 85 μM etoposide caused an approximate 51% decrease in cell viability, compared with the control cells. To determine whether the neuronal cell death was due to apoptosis or necrosis, the occurrence of rapid cyr61 synthesis by etoposide. However, once produced inside the cells, Cyr61 appears to be translocated gradually into the extracellular media.

The Etoposide-induced Expression of cyr61 Occurs by JNK-dependent Pathway—Next we sought to elucidate the upstream signaling pathways leading to the induction of cyr61. Because the mitogen-activated protein kinases (MAPKs), consisting of JNK, p38 kinase, and ERK, are potential candidates for cyr61 gene activation, we first examined whether they are activated in response to etoposide in H19-7 cells. As shown in Fig. 4A, both JNK1 and JNK2 were transiently activated within 5–15 min post-etoposide stimulation, whereas no significant levels of active p38 kinase or ERK were detected. To investigate whether the mRNA induction of cyr61 directly occurs by the JNK-dependent pathway, cells were transfected with kinase-inactive MEKK mutant, an upstream JNK kinase activator in the JNK-signaling cascade, which selectively inhibits JNK activation. As shown in Fig. 4B, the RT-PCR experiment revealed that blocking the JNK pathway results in a significant decrease in etoposide-induced cyr61 expression, compared with
Fig. 3. Induction of Cyr61 protein in H19-7 cells by etoposide. While metabolically labeled with [35S]methionine, the H19-7 cells were stimulated with 85 μM etoposide for the indicated times. Where specified, Cyr61 in the cell lysates and culture media were immunoprecipitated using monoclonal anti-Cyr61 IgG, resolved by SDS-PAGE (8.0% gel), and visualized by autoradiography. Cyr61 synthesis in cell lysates (A) or media (B) is shown in the individual autoradiograph.

Fig. 4. Etoposide-induced expression of cyr61 is mediated by JNK-dependent signaling pathway. A, etoposide-induced activation of JNK, but not p38 or ERK. The H19-7 cells were treated with 85 μM etoposide for the indicated times. The cell extracts were then resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated with antibodies specific to phosphorylated JNK, p38 kinase, or ERK. The membrane was incubated with peroxidase-conjugated secondary antibodies, and the bands were visualized by enhanced chemiluminescence. B, effect of JNK and p38 activation on etoposide-induced cyr61 expression. The cells were either pretreated for 30 min with the p38 kinase inhibitor, 30 μM SB203580 (SB), or transiently transfected with 6 μg of a kinase-deficient mutant MEKK (mM). Where indicated, cells were untreated (Cont) or pre-treated with 30 μM SB203580 (SB) or 10 μM U0126 (U) for 30 min, and then stimulated with 85 μM etoposide (Etop or E) for 3 h. The CAT activity was measured using an enzyme-linked immunosorbent CAT assay kit according to the manufacturer’s protocol.

Fig. 5. Inhibition of transcriptional activation of cyr61 by blocking JNK. A, time course of transcriptional activation of cyr61 by etoposide in H19-7 cells. The -2062cyr61/CAT fusion plasmid (5 μg) was transiently transfected into the H19-7 cells. The cells were then stimulated with 85 μM etoposide. The activity of the expressed CAT enzyme in 50–70 μg of the cell lysate was measured as described under “Experimental Procedures.” Extracts prepared from the mock-transfected cells were used as a negative control. The results are plotted as a mean plus the range of samples from two independent experiments. B, blocking effect of MAPK pathways on the transcriptional activity of cyr61. 2.5 μg of -2062cyr61/CAT plasmid DNA was transiently transfected into the H19-7 cells either alone or with 2.5 μg of a mutant MEKK (mM). Where indicated, cells were untreated (Cont) or pre-treated with 30 μM SB203580 (SB) or 10 μM U0126 (U) for 30 min, and then stimulated with 85 μM etoposide (Etop or E) for 3 h. The CAT activity was measured using an enzyme-linked immunosorbent CAT assay kit according to the manufacturer’s protocol.

the mock-transfected cells. However, blocking p38 and ERK activity with specific inhibitors did not significantly affect on cyr61 expression, compared with the control cells (Fig. 4A). Furthermore, Northern blot analysis confirmed that the cyr61 mRNA levels induced by the etoposide were considerably decreased after blocking the JNK pathway in the cells. However, blocking either p38 or ERK had little effect (Fig. 4B). This suggests that the etoposide-induced expression of cyr61 occurs by the JNK-dependent pathway but not by either the ERK or p38 kinase pathway.

Transcriptional Activation of the cyr61 Occurs by JNK-dependent Pathway—A 2-kb 5′-flanking DNA fragment of cyr61 gene functions as a serum-inducible promoter (11). This DNA fragment contains a sequence resembling the serum response element. The transcriptional activation of the cyr61 gene was examined using a CAT reporter plasmid linked to a 2062-bp cyr61 promoter fragment (∼2062cyr61/CAT) transiently expressed in H19-7 cells. Treatment of the H19-7 cells with 85 μM etoposide rapidly stimulated cyr61 transcription, which reached a plateau after 4 h, as monitored by CAT activity (Fig. 5A). To analyze the role of the MAPK pathways in the promoter activity of cyr61, the blocking effect of the ERK, JNK, or p38 kinase pathway was tested using either the chemical inhibitors of ERK and p38 kinase or a plasmid encoding kinase-inactive mutant MEKK (mMEKK) (Fig. 5B). Following pretreatment of the H19-7 cells for 30 min with either 30 μM SB203580 or 10 μM U0126, the activation of the cyr61 promoter was unaffected. However, when a plasmid encoding kinase-deficient MEKK mutant and −2062cyr61/CAT-construct were co-transfected...
significant inhibition of cyr61/H11002, the CAT construct (Fig. 6A).

Enzyme-linked immunosorbent CAT assay kit according to the manufacturer's protocol.

into the cells, stimulation with 85 μM etoposide for 3 h, and the CAT activity was measured using an enzyme-linked immunosorbent CAT assay kit according to the manufacturer's protocol.

Fig. 6. CAT assay of intact and deleted cyr61/CAT promoter constructs. A. diagram depicting deletions of the cyr61 promoter. Construct −2062, nt −2062 to +65; construct −1763, nt −1763 to +65; construct ΔBgIII, removed a BgIII fragment (−1285 to −353); construct −529, nt −529 to +65; construct CarG, CarG box is attached to −529 construct. The cyr61 SRE-like sequence, which contains the CarG box, is shown as a filled square. B. CAT assay. The H19-7 cells were mock-transfected (Vehicle) or transiently transfected with 5 μg of DNA of the deletion cyr61 promoter/CAT constructs. The cells were stimulated with 85 μM etoposide for 3 h, and the CAT activity was measured using an enzyme-linked immunosorbent CAT assay kit according to the manufacturer's protocol.

Cis-regulatory Serum Response Element in cyr61 Promoter Is Required for Its Induction by Etoposide—The results described above indicate that the 2062-bp cyr61 promoter contains a domain responsive to a JNK-dependent signaling pathway, which is activated by etoposide. To identify the domains, the cyr61 promoter was subjected to deletion analysis (Fig. 6A). The cyr61 promoter has one SRF-binding domain (SRE or CarG box) between −1950 and −1900. Deleting this SRE (−1763cyr61/CAT) resulted in a complete loss of the etoposide-activated transcription of cyr61, compared with the −2062cyr61/CAT-transfected cells (Fig. 6B). A further deletion of the promoter up to −529 (−529cyr61/CAT) had no effect, whereas an intact SRE-containing construct with an internal deletion (ΔBgIIIcyr61/CAT) showed a significant increase in cyr61 promoter activity, compared with the −2062cyr61/CAT construct (Fig. 6B). The SRE enhancer region of the cyr61 promoter was further analyzed by linking it to the 529-bp fragment (CarG/−529cyr61/CAT). The results suggest that a domain containing a CarG box is sufficient for the full cyr61 transcriptional activation by etoposide.

The SRE was then tested to determine whether it is sufficient for cyr61 induction or other serum-inducible cis-regulatory elements, such as the TCF-binding motif where its presence appears to be crucial for inducing IEGs during neuronal cell death (27, 30). In addition, the TCF-binding motif was examined to determine if it acts together with the SRE to mediate a cyr61 induction in a synergistic way. To test this possibility, two heterologous SRE-c-fos promoter/luciferase constructs involving either the SRE alone (ppm18GL3) or a combined TCF-SRE (pWTGL3) were further analyzed (Fig. 7A). The plasmids, pWTGL3 and ppm18GL3, containing the −335 to −285 region of the murine c-fos promoter were constructed by their fusion into the −53 to +45 region of the human c-fos promoter subcloned into the upstream of the firefly luciferase gene in the pGL3 luciferase vector (Promega) (31). As shown in Fig. 7B, irrespective of the mutation in the TCF site to abolish the binding of p62TCF, two heterologous SRE-c-fos promoter constructs showed similar and significant transcriptional activation, as monitored by their luciferase activity (Fig. 7B). Overall, these results suggest that the toxic signal of etoposide converges to the SRE region in the cyr61 promoter.

Active JNK Directly Phosphorylates SRF in H19-7 Cells Stimulated with Etoposide—Although the SRE-binding factor, SRF, was reported to be phosphorylated by CaM kinase-II and -IV (17) and MAPKAP kinase-2 (18), the likelihood of SRF activation by the JNK-dependent pathways has not been reported. Therefore, we determined whether SRF could be phosphorylated either directly by active JNK or by the JNK-dependent signaling pathways. The cell lysates were prepared from

Fig. 7. SRE region is required for etoposide-induced transcriptional activation of heterologous c-fos enhancer. A. diagram of the heterologous SRE-c-fos/luciferase reporter constructs. A section of the mouse c-fos promoter with SRE and TCF, −355 to −285, was fused to the human c-fos minimal promoter at −53. The mutation of the TCF site, consequently abolishing the binding of p62TCF, is indicated by the “x” (pm18). B, luciferase assay for c-fos promoter. H19-7 cells were transfected with the heterologous wild type (WT) or the mutant SRE-c-fos/luciferase reporter genes (pm18). The transfected cells were untreated (Cont) or stimulated with 85 μM etoposide (Etop), and the cell lysates were assayed for their firefly luciferase activity, as indicated.
the H19-7 cells stimulated with 85 μM etoposide and, consequently, immunoprecipitated with the anti-JNK antibodies. Subsequently, the immunocomplex kinase assay was performed using bacterially recombinant glutathione S-transferase (GST) proteins fused to the whole SRF proteins (residues 1–508: GST-SRF508), the N-terminal SRF peptide (residues 1–140: GST-SRF140), or the C-terminal SRF peptide (residues 198–508: GST-SRF198/508) as a substrate (32). As shown in Fig. 8A, the in vitro kinase assay showed that the SRF is phosphorylated by JNK immunocomplexes. Interestingly, SRF phosphorylation significantly increased when GST-SRF508 and GST-SRF198/508 were used as substrates but not with the N-terminal SRF peptide (GST-SRF140) (Fig. 8A). To test the possibility that one or more other kinases beside JNK in the JNK immunocomplexes phosphorylates the SRF in response to etoposide, an in vitro gel kinase assay was performed using a polyacrylamide gel prepared in the presence of either the GST-SRF508 or GST-SRF140 proteins as a phosphorylation substrate. Equal protein-containing anti-JNK-IgG immunoprecipitates from the H19-7 cells, which had been stimulated with 85 μM etoposide for 60 min, were resolved by SDS-PAGE, renatured, and assayed for the SRF phosphorylation in the gel. The results showed the 46- and 54-kDa bands corresponding to the molecular size of JNK-1 and -2 phosphorylated GST-SRF508 in the gel (Fig. 8B). No significant kinase activity was detected when the N-terminal SRF peptide with its C-terminal 141–508 residues deleted was used as a substrate (Fig. 8B). Furthermore, to determine whether JNK could phosphorylate SRF in a selective way upon the stimulation with etoposide, the cells were either transiently co-transfected with expression vectors encoding kinase-deficient JNK1 and JNK2 mutants or pretreated with 1,9-pyrazoloanthrone, a cell-permeable, and selective JNK inhibitor (44). As shown in Fig. 8C, the overexpression of dominant-negative JNKS as well as the addition of a potent chemical inhibitor of JNKS significantly suppressed the increased levels of etoposide-induced phosphorylated SRF to the control level in parental empty vector-transfected cells (Fig. 8C). Taken together, these results strongly suggest that etoposide-induced active JNK could specifically and directly phosphorylate the SRF protein on its N-terminal side.

The Suppression of cyr61 Induction Inhibits Neuronal Cell Death—The functional role of cyr61 activation during etoposide-induced neuronal cell death was further examined by using H19-7 cell line stably transfected with antisense cyr61. Stable H19-7 clonal cells were characterized by metabolic labeling with [35S]methionine followed by the immunoprecipitation of cultured media with anti-Cyr61 polyclonal antibodies. Autoradiographic analysis was performed to examine whether stable transformant of antisense cyr61 blocks the endogenous Cyr61 expression and its subsequent extracellular secretion upon the stimulation with etoposide. Culture media were prepared from stable transformant that was resistant to 300 μg/ml Zeocin, and the proteins in the medium were analyzed on an 8% PAGE (Fig. 2). As shown in Fig. 9A, the stimulation of stable H19-7 cell line to express antisense cyr61 with etoposide resulted in the complete blockade of cyr61 expression, compared with that in control cells transfected with parental vector only. In addition, the cells with antisense cyr61-transfected population had remarkably reduced levels of cell death (~19%, p < 0.001), whereas the addition of etoposide led to ~62% cell death in the parental empty vector-transfected cells. These results suggest that the cyr61 expression by etoposide can play an important role in the cell death of the embryonic hippocampal neural progenitor cells.

**FIG. 8. SRF phosphorylation by etoposide-induced JNK.** A, in vitro kinase assay. The H19-7 cells were treated with 85 μM etoposide for the indicated times. The cell extracts containing 200 μg of the proteins and 1 μg of the JNK antibodies prebound to the Protein A-Sepharose beads were incubated for overnight. After incubation, 30 μl of the protein-A beads were added to 50 μg of the bacterially recombinant wild type GST-SRF (SRF508), the N-terminal SRF peptide (residues 1–140: SRF140), or the C-terminal SRF peptide (residues 198–508: SRF198/508) as a substrate mixtures, and in vitro SRF phosphorylation was performed as described under “Experimental Procedures.” The final eluates from the beads were resolved by SDS-PAGE in 8.0%-gel and visualized by autoradiography. B, in vitro in-gel kinase assay. The H19-7 cells were untreated (C) or treated with 85 μM etoposide (E) for 60 min, as indicated. The cell extracts containing 100- to 150-μg proteins were immunoprecipitated against the anti-JNK antibodies, and the immunocomplex proteins were resolved by SDS-PAGE on a 10% gel containing either 50 μg of the bacterially expressed wild type GST-SRF (wSRF) or the C-terminal-deleted GST-SRF104 peptide (SRF-N) per milliliter. The in-gel kinase renaturation assay was performed as described under “Experimental Procedures.” JNK1 and JNK2 that were activated by etoposide are indicated by arrows. C, the blocking effect of JNK on SRF phosphorylation. The H19-7 cells were mock-transfected (No T, Cont, and JI), or transiently transfected with 3 μg of each pCMV5-mJNK1 and pSR-HA-JNK2 plasmid-encoding kinase-deficient JNK1 and JNK2, respectively (mJ12). Where indicated, the cells were pretreated with 300 ng/ml 1,9-pyrazoloanthrone, a cell-permeable and selective JNK inhibitor (JI) for 30 min prior to stimulation. The cells were then stimulated with 85 μM etoposide for 60 min, immunoprecipitations were performed by using JNK antibodies, and immunocomplex kinase assays were done with 50 μg of recombinant wild type GST-SRF (wSRF) as a substrate.

**DISCUSSION**

It has been known that, as members of the CCN and IEG families, both cyr61 and CTGF are induced by the serum, basic fibroblast growth factor, and the platelet-derived growth factor in fibroblasts and neuronal H19-7 cells (1, 6, 8, 33). Both cyr61 and CTGF are associated with the extracellular matrix, share a 45% amino acid sequence identity, and bind to heparin (6). Although CTGF is known to induce apoptosis in the breast cancer cell line by transforming growth factor-β (34), the physiological role of cyr61 during cell death is unknown. Cyr61 regulates cell adhesion, migration, proliferation, differentiation, and chondro-
with 85H9262) were either untreated (parental) or stimulated as Cyr61 using anti-Cyr61 antibodies, resolved by SDS-PAGE, and visualized by autoradiography. B, where indicated, H19-7 cells stably transfected with parental empty control vector (parental) or antisense Cyr61-expressing cells (asCyr61) were either untreated (Cont) or stimulated with 85 μM etoposide (Etop), and the extent of cell death was quantified. Data are expressed as mean ± S.E. from three independent experiments conducted in triplicates. Asterisks indicate significant differences compared with parental vector-transfected control values (p < 0.001).

Fig. 9. Effect of antisense Cyr61 expression on etoposide-induced neuronal cell death. A, while metabolically labeled with [35S]methionine, either stable H19-7 cell lines to express 300-nucleotide antisense Cyr61 to block the endogenous Cyr61 expression (asCyr61) or control cells transfected with parental empty expression vectors (parental) were stimulated with 85 μM etoposide for 24 h. Newly synthesized Cyr61 proteins in the cell culture media were immunoprecipitated using anti-Cyr61 antibodies, resolved by SDS-PAGE, and visualized by autoradiography. B, where indicated, H19-7 cells stably transfected with parental empty control vector (parental) or antisense Cyr61-expressing cells (asCyr61) were either untreated (Cont) or stimulated with 85 μM etoposide (Etop), and the extent of cell death was quantified. Data are expressed as mean ± S.E. from three independent experiments conducted in triplicates. Asterisks indicate significant differences compared with parental vector-transfected control values (p < 0.001).

Induction of IEG Cyr61 during Neuronal Cell Death

Many studies have revealed that Cyr61 is involved in tumor growth. For example, Cyr61 promotes angiogenesis and tumor growth in endothelial cells (10) and enhances DNA synthesis in umbilical vein endothelial cells (36).

Based on the high degree of homology between cyr61 and CTGF, it is believed that cyr61 could function through cell death. In support of this belief, a recent report (3) shows that cyr61 can provide a negative regulation of cell growth. The cyr61 mRNA expression was markedly reduced in human lung tumor samples, compared with their normal matched lung samples. Moreover, a considerable up-regulation of the p53 levels was detected in the cells stably transfected with cyr61 (3). The present study indicates that cyr61 plays an important role during the process of neuronal cell death. It was demonstrated that the expression of the angiogenic factor, Cyr61, occurs in response to various neurotoxic stimuli in H19-7 cells. The cyr61 mRNA levels increased after being exposed to etoposide, NMDA, and glutamate. In addition, the transcription and the protein levels of Cyr61 increased during etoposide-induced neuronal cell death.

Among the MAPK family, etoposide uniquely activates the JNK pathway in H19-7 cells. This finding is consistent with previous reports showing that the induction of apoptotic cell death and DNA damage by etoposide in U937 human monoblastic leukemia cells are closely associated with JNK activation (37). To investigate whether the JNK-dependent pathway is directly involved in the expression of cyr61 by etoposide, chemical inhibitors or kinase-inactive MAPK mutants were used to examine the blocking effect of the MAPK pathways. The results showed that the cyr61 mRNA levels in the presence of the specific p38 kinase inhibitor, SB203580, were similar to that in the control cells treated with etoposide. When a plasmid encoding kinase-deficient MEKK mutant, which is present upstream of JNK in the JNK signaling pathway, was transfected in a transient manner in the H19-7 cells, cyr61 expression was significantly reduced compared with the mock-transfected cells. In contrast, cyr61 induction during neuronal differentiation appears to be closely related to ERK activation in H19-7 cells (8), which suggests that the diverse signals of cyr61 are transmitted through different MAPK pathways.

Moreover, we also demonstrated that the JNK-dependent phosphorylation of the transcription factor, SRF, appears to be crucial for cyr61 expression. The cis-regulatory serum response element (SRE) is essential for the expression of several growth factor-inducible immediate early genes, such as c-fos and egr-1 (13, 38, 39). SRF binds to the SRE, which subsequently permits the recruitment of the SRF-accessory factors like the ternary complex factors (TCFs) in the c-fos gene. The MADS domain in the SRF binds to the CarG box, which has a consensus sequence C3(A/T)6G2. The ternary complex factors, namely Elk1, Sap-1, and Net-1, which binds to the Ets motif, can only bind to the SRE in c-fos after the adjacent SRE site has been occupied with the SRF. Interestingly, although Elk-1 is commonly phosphorylated by ERK during fibroblast growth factor-induced neuronal differentiation as well as by p38 and JNK during cell death in neurons and fibroblasts (27, 30), the molecular mechanism responsible for activating SRF and SRF-containing complexes is unclear. As an important nuclear-relay player between cellular signaling and gene activity, the SRF is believed to serve both as a direct and indirect target of the signaling cascades. The SRF contains multiple sites that can be phosphorylated by kinases, of which the Ser-103 residue has been extensively examined. For example, Ser-103 is phosphorylated by being stimulated by growth factors or increasing the intracellular calcium levels. This is because it is mediated, at least in vitro, by pp90ERK, CaM kinase II, CaM kinase IV, and MAPKAP kinase 2 (MK2) (17, 18). In this study, it was shown by using in vitro immunocomplex kinase assay that the activation of the JNK pathway by etoposide leads to SRF phosphorylation at its C-terminal portion, rather than the Ser-103 residue. Furthermore, the in-gel kinase assay showed that SRF is directly phosphorylated by active JNK.

The induction of cyr61 appears to play an important role during etoposide-induced neuronal cell death in the hippocampal H19-7 cells. The cyr61 gene encodes a secretory protein. In accord with this property, the maximum level of Cyr61 synthesis was also observed in the cell lysates after 5 h of etoposide-stimulation in H19-7 cells and was maintained for approximately until 10 h. Once synthesized inside the cells, the Cyr61 proteins were presumably translocated into the extracellular space, and were solely detected in the culture media 24 and 48 h after etoposide stimulation but not in cell lysates. In contrast to this study, the heparin-binding Cyr61 protein in mouse fibroblasts is associated with the cell surface and the extracellular matrix but not the culture media (40). These findings indicated that the localization of the synthesized Cyr61 during cell death is distinct from that in the serum-induced cell growth. The induction of another secretory protein, the secreted apoptosis-related proteins (SARPs), has been reported during cell death (41). The SARPs resemble growth factors and cytokines, and their tissue-specific expres-
In the upstream cyr61 function of how the secretory Cyr61 protein may cause or potentiate cell proteins. To the cysteine-rich domain in the transmembrane frizzled SARP family have a cysteine-rich domain that is homologous to the cysteine-rich domain in the transmembrane frizzled proteins.

We are currently investigating the molecular mechanisms of how the secretory Cyr61 protein may cause or potentiate cell death. This will provide new insights into another biological function of cyr61 during neuronal cell death. In summary, cyr61 is expressed during the etoposide-induced neuronal cell death in neuronal H19-7 cells, and the SRE-like CARG domain in the upstream cyr61 promoter is necessary for its induction by etoposide.

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Expression of Angiogenic Factor Cyr61 during Neuronal Cell Death via the Activation of c-Jun N-terminal Kinase and Serum Response Factor
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