Neuronal Injury-inducible Gene Is Synergistically Regulated by ATF3, c-Jun, and STAT3 through the Interaction with Sp1 in Damaged Neurons*

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Nerve injury requires the expression of large ensembles of genes. The key molecular mechanism for this gene transcription regulation in injured neurons is poorly understood. Among many nerve injury-inducible genes, the gene encoding damage-induced neuronal endopeptidase (DINE) showed most marked expression response to various kinds of nerve injuries in central and peripheral nervous system neurons. This unique feature led us to examine the promoter region of the DINE gene and clarify both the injury-responsive element within the promoter and its related transcriptional machinery. This study showed that DINE promoter was activated by leukemia inhibitory factor and nerve growth factor withdrawal, which were pivotal for the up-regulation of DINE mRNA after nerve injury. The injury-inducible transcription factors such as activating transcription factor 3 (ATF3), c-Jun, and STAT3, which were located at the downstream of leukemia inhibitory factor and nerve growth factor withdrawal, seemed to be involved in the activation of the DINE promoter. Surprisingly, these transcription factors did not bind to the DINE promoter directly. Instead, the general transcription factor, Sp1, bound to a GC box within the promoter. ATF3, c-Jun, and STAT3 interacted with Sp1 and are associated with the GC box region of the DINE gene in injured neurons. These findings suggested that Sp1 recruit ATF3, c-Jun, and STAT3 to obtain the requisite synergistic effect. Of these transcription factors, ATF3 may be the most critical, because ATF3 is specifically expressed after nerve injury.

Nerve injury induces the expression of large ensembles of genes, required to elicit the intrinsic regeneration responses in the injured neurons (1). It was previously established that the 5′-flanking sequences of some nerve injury-associated genes such as GAP43, peripherin, and tubulin-α, have injury-responsive characteristics (2–5). However, both the detailed transcriptional machinery and the injury-responsive element within those promoters, which are pivotal for the appropriate gene responses to neuronal injury, have not been clarified.

Damage-induced neuronal endopeptidase (DINE2 in rats or ECEL1 in humans) is a neuron-specific membrane-bound metalloprotease, which we originally identified as a nerve injury-associated gene (6). The most striking feature of DINE is its remarkable induction of the transcript in response to various types of nerve damage in both central and peripheral nervous system neurons (6). This feature seemed to be useful for understanding transcriptional mechanism in damaged neurons. Therefore, we have attempted to clarify the gene regulation of DINE after nerve injury. Although the precise functions of DINE/ECEL1, including the substrates, have not been identified (6, 7) and are being studied, DINE might be important for neuropeptide processing, which may play a neuroprotective role as a consequence of adaptive response of neuronal injury. Our previous study showed that, in ischemic insult, DINE mRNA was expressed in neurons projecting their axons to or through the core region of the infarction (8). The DINE mRNA inductions were always accompanied by the simultaneous induction of the injury-inducible transcription factor, Activating transcription factor 3 (ATF3). In addition, both in vitro and in vivo study showed that the expression of DINE mRNA in dorsal root ganglion (DRG) neurons after sciatic nerve injury was significantly induced by leukemia inhibitory factor (LIF) treatment and withdrawal of nerve growth factor (NGF) (9). In sympathetic injury system, where it has been also established that LIF and NGF withdrawal are important for injury-induced change in gene expression (10, 11), DINE mRNA was up-regulated as well (12). These findings suggest that the transcription factors downstream of the LIF-gp130 pathway and the Jun N-terminal kinases (JNKs) pathway are involved in the gene regulation of DINE. Taken together, injury-induced expression of DINE is assumed to be regulated by ATF3, c-Jun, and signal transducers and activators of transcription 3 (STAT3), which are located downstream of the LIF-gp130 and the JNK signaling pathways.

ATF3, c-Jun, and STAT3 are the most commonly up-regulated transcription factors responding to various types of nerve injury. The on-line version of this article (available at http://www.jbc.org) contains supplemental text and Figs. S1–S3.

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The abbreviations used are: DINE, damage-induced neuronal endopeptidase; ATF3, activating transcription factor 3; DRG, dorsal root ganglion; LIF, leukemia inhibitory factor; NGF, nerve growth factor; JNK, Jun N-terminal kinase; STAT3, signal transducers and activators of transcription 3; HA, hemagglutinin; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; WT, wild type.

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injuries (13–18). In particular, ATF3, a member of the ATF/cAMP-response element-binding protein family of transcription factors, is regarded as a marker for nerve injury because of its specific expression in response to nerve injury (17, 19, 20). ATF3 is assumed to contribute to nerve regeneration (21). Several studies have reported that ATF3 expression promotes neurite elongation and cell survival by combination with an AP1 transcription factor, c-Jun, following the activation of INK pathways or the activation of p38 (18, 22). Concurrent with ATF3 up-regulation, c-Jun is also activated after axotomy. The interaction of ATF3 and c-Jun has not been reported in physiological conditions to date. However, it has been suggested that the heterodimer of ATF3/c-Jun functions as an activator of specific promoters for various nerve regeneration-associated genes following nerve injury. Apart from ATF3/c-Jun, several cytokines, such as the ciliary neurotrophic factor, interleukin-6, and LIF, which are released from surrounding glial cells after nerve injury, activate STAT3. This activation is elicited by their specific receptor complexes, including gp130, as a common receptor sub-chain and Janus kinase family members (23). Accumulating evidence has established that the gp130-STAT3 signaling pathway has an important role in regenerating neurons (24–26). Taken together, these injury-inducible transcription factors, ATF3, c-Jun, and STAT3, might be associated with the up-regulation of various kinds of genes to promote regeneration. However, the detailed transcriptional machinery in injured neurons has not been clarified.

In this study, general transcription factor, Sp1, is involved in the expression of the DINE gene as well. Sp1 has been classically assumed to regulate the constitutive expression of numerous housekeeping genes. However, recent reports have demonstrated that Sp1 has multifunctional transcriptional activities through its interaction with other transcription factors and is required for adaptive responses to differentiation, proliferation, and oxidative stress (27–29).

This study has demonstrated that the injury-inducible transcription factors (ATF3, c-Jun, and STAT3) are actually responsible for the expression of DINE gene regulation by existing at the Sp1 binding site after axotomy. It suggested that Sp1 function as a scaffolding protein that recruits ATF3, c-Jun, and STAT3 to elicit their synergistic transactivation activity. This possible mechanism may be important not only for DINE but also potentially for other nerve injury-associated genes to be induced simultaneously in damaged neurons.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mouse DINE Gene**—A mouse genomic library (AFIXII mouse 129 Library, Stratagene, La Jolla, CA) was screened to obtain the DINE mouse genome. The probes were labeled with [α-32P]dCTP (Amersham Biosciences) employing a multiprime DNA labeling system (Amersham Biosciences). Approximately 1 ¥ 10^6 plaques were plated at a density of 1 ¥ 10^4 plaque forming units/150-mm dish and screened by using the following conditions for hybridization: 1 ¥ 10^6 cpm/ml in 6 ¥ SSC, 5 ¥ Denhardt’s, and 0.5% SDS for hybridization at 65°C, and then washed at 65°C in 2 ¥ SSC containing 0.5% SDS and in 0.1 ¥ SSC containing 0.5% SDS. Five positive plaques were obtained, purified, and partially sequenced.

**Plasmids**—Various lengths of the DINE promoter fragments were generated by restriction enzyme digestion or PCR amplification of genomic DNA isolated from the 129 strain of mice. The fragments were then cloned into a luciferase reporter pGL3 basic vector (Promega, Madison, WI). Mutations were generated by using the QuikChange II site-directed mutagenesis kit (Stratagene). Plasmids encoding ATF3, c-Jun and ATF tethered to ATF3 (ATF3:ATF) and ATF3 tethered to c-Jun (ATF3c-cJun), were cloned into a pME18S expression vector. ATF3 and ATF3 cDNAs or ATF3 and c-Jun cDNAs were fused in-frame via a glycine-rich polypeptide tether of 18 amino acids. HA-STAT3delN-(162–700) and HA-STAT3delC-(1–605) were inserted into pCDNA3. HA-STAT3, HA-STAT3D, HA-STAT3Y705F, and STAT3β cloned in pCAGGS were kindly provided by K. Nakajima (Osaka City University, Japan). FLAG-STAT3 and FLAG-STAT3Y705F in pEF-BOS were gifts from T. Taga (Kumamoto University, Japan). STAT3C cloned in pME18S was a gift from A. Miyajima (Tokyo University, Japan). Sp1 in pCDNA3 was a gift from W. C. Wang (National Cheng Kung University, Taiwan). DINE (accession number AB026293, 1287–1761) in pBluescript was used for in situ hybridization as previously described (6).

**Adenovirus Preparation**—The fragment containing the DINE promoter region (–2765/+1) followed by firefly luciferase gene was cloned into the expression cassette pAx cosmid vector. STAT3, ATF3, c-Jun, and ATF3c-cJun were cloned into the Cre-inducible expression cassette pAxCALNLW, which usually is not expressed because of the neomycin resistance gene. The recombinant viruses were generated according to a previously described method (30).

**Cell Culture**—SK-N-SH cells were cultured in 5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium (Invitrogen) containing penicillin and streptomycin, supplemented with 10% fetal bovine serum. Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Sp1 and control small interference RNAs were purchased from Invitrogen and transfected according to the manufacturer’s protocol. After 36 h of transfection, the cells were harvested into reporter lysis buffer (Promega), and the luciferase intensity was measured using the Promega luciferase assay system, according to the manufacturer’s instructions in a TD luminometer.

For the preparation of neuron-glia mixed culture, DRGs were isolated from 1-day-old Wistar rats and collected into ice-cold L15 medium (Invitrogen). DRGs were dissociated with 2 mg/ml collagenase at 37°C for 1 h and then seeded onto 24-well polystyrene-coated plates. The feeding medium was a Dulbecco’s modified Eagle’s medium (Invitrogen), to which 0.5% penicillin-streptomycin (Sigma), 5 μM uridine (Sigma), 5 μM fluorodeoxyuridine (Sigma), and 50 ng/ml NGF (Promega) were added. DRGs were cultured in the presence of NGF at 37°C in a 5% CO₂ atmosphere. The treatment of ligands and the gp130 antibody (R&D Systems) was performed as described previously (9). The gp130 antibody treatment was performed at 5 days after plating, and the adenoviruses were added. At 24 h after infection, ligands were applied to each well. At 36 h after ligand treatments, cells were lysed and luciferase intensity was measured.
For immunostaining, cells on glass coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with anti-c-Jun antibody (Santa Cruz Biotechnology). For competition assays, a 100- or 200-fold excess of unlabeled oligonucleotides or mutant oligonucleotides was incubated with the labeled probe in binding buffer composed of 10 mM HEPES (pH 7.8), 1 mM EDTA, 150 mM NaCl, 5% glycerol, 0.1% Nonidet P-40, 5 mg/ml bovine serum albumin, and 0.5 µg of poly(dI-dC). The reaction mixture was incubated for 30 min at room temperature. For competition assays, a 100- or 200-fold excess of unlabeled oligonucleotides or mutant oligonucleotides was incubated for 15 min before the addition of the labeled probe. Anti-Sp1 antibody (1.5 µg) was also added to the reaction mixture to examine the supershift of the DNA probe. The DNA-protein complexes were resolved on 4% polyacrylamide gels in 0.5% TBE (89 mM Tris-borate, 2 mM EDTA) and 1% glycerol.

**In Situ Hybridization and Immunohistochemistry—** Mice were decapitated after the surgery, and the brain was removed and rapidly frozen in powdered dry ice. Then, 18-µm-thick sections were cut on a cryostat, thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides, and stored at −80 °C until use. All procedures for in situ hybridization were performed as previously described (31).

For ATF3 immunohistochemistry, animals were perfused with 4% paraformaldehyde containing 0.21% picric acid in 0.1 M phosphate buffer. The sections were air-dried, rinsed with PBS, permeabilized with 0.2% Triton X-100, and incubated in PBS containing 1% bovine serum albumin for 10 min at room temperature followed by incubation with anti-ATF3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. For c-Jun immunostaining, the freshly frozen sections were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked in 1% bovine serum albumin, and incubated with anti-c-Jun antibody (Santa Cruz Biotechnology). For STAT3 and Sp1 immunostaining, the freshly frozen sections were fixed with methanol for 20 min at −20 °C, incubated with PBS containing 1% bovine serum albumin for 15 min at room temperature, and incubated with anti-STAT3 (Santa Cruz Biotechnology) and anti-Sp1 (Santa Cruz Biotechnology) antibodies. These sections were subsequently incubated with anti-rabbit IgG conjugated with Alexa Fluor 488 (1:500, Molecular Probes, Eugene, OR).

**Electromobility Supershift Assays—** SK-N-SH cells from the upper strand) were also synthesized. Both were 5′-end-labeled with [γ-32P]ATP (Amersham Biosciences) and T4 polynucleotide kinase. For reaction of DNA binding to proteins, 3 µg of the nuclear extracts of SK-N-SH cells was incubated with the labeled probe in binding buffer composed of 10 mM HEPES (pH 7.8), 1 mM EDTA, 150 mM NaCl, 5% glycerol, 0.1% Nonidet P-40, 5 mg/ml bovine serum albumin, and 0.5 µg of poly(dI-dC). The reaction mixture was incubated for 30 min at room temperature. For competition assays, a 100- or 200-fold excess of unlabeled oligonucleotides or mutant oligonucleotides was incubated for 15 min before the addition of the labeled probe. Anti-Sp1 antibody (1.5 µg) was also added to the reaction mixture to examine the supershift of the DNA probe. The DNA-protein complexes were resolved on 4% polyacrylamide gels in 0.5% TBE (89 mM Tris-borate, 2 mM EDTA) and 1% glycerol.

**Immunoprecipitation—** SK-N-SH cells were infected with ATF3, c-Jun, ATFT3:c-Jun, and STAT3 adenosviruses with Cre recombinase. After 36 h the cells were washed with PBS twice and lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 5 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml leupeptin. After centrifugation for 15 min at 15,000 rpm, the supernatant was incubated with anti-Sp1 (Upstate) antibodies with constant agitation at 4 °C. The immunocomplex was precipitated with protein G-Sepharose (Zymed Laboratories Inc.). The pellet was washed four times and prepared for immunoblotting using primary antibodies for ATF3 (Santa Cruz Biotechnology), c-Jun (Upstate), and STAT3 (BD Transduction Laboratories).

**Chromatin Immunoprecipitation Assays—** Control and injured hypoglossal nuclei were collected from 20 mice 5 days after axotomy. Tissues were fixed with freshly prepared 1% formaldehyde in PBS at 37 °C for 10 min, rinsed in ice-cold PBS, and homogenized in SDS lysis buffer composed of 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 150 mM NaCl, and protease inhibitors, and precleared by incubating with a salmon sperm DNA-Protein A-agarose slurry for 1 h at 4 °C (Upstate Biotechnology). Immunoprecipitation was performed overnight at 4 °C using anti-ATF3 (Santa Cruz Biotechnology), anti-c-Jun (Santa Cruz Biotechnology), anti-STAT3 (Upstate), anti-Sp1 (Upstate) antibodies followed by additional incubation with a protein A-agarose slurry for 1 h at 4 °C. Immune complexes were washed with low salt wash buffer containing 0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 150 mM NaCl, and protease inhibitors, and precleared by incubating with a salmon sperm DNA-Protein A-agarose slurry for 1 h at 4 °C. Immune complexes were washed with low salt wash buffer containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl and a high salt wash buffer composed of 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl, and a LiCl buffer composed of 0.25 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 1 mM Tris-HCl (pH 8.1) and a TE buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA. Immune complexes were eluted twice with an elution buffer containing 1% SDS, 0.1 mM NaHCO3, 1 mM dithiothreitol. Cross-links were reversed by incubation with 0.3 M NaCl at 65 °C for 4 h. DNA was purified by phenol-chloroform extraction and ethanol precipitation. Immunoprecipitated DNA was analyzed by PCR. The primer sequences for
The results of the promoter assay showed that neurotrophic factors such as NT-3, BDNF, GDNF, and IGF, did not significantly affect the activity of the promoter, but LIF and NGF withdrawal additively increased the promoter activity by 4- to 5-fold (Fig. 1A). The luciferase activity induced by LIF was antagonized by anti-gp130 antibody (Fig. 1B). In addition, immunostaining showed that the expression of luciferase was up-regulated in neurons after LIF treatment and NGF withdrawal, because it was observed only in cells displaying the characteristic neuronal cell marker MAP-2 (Fig. 1B). Other Hoechst-stained cells were luciferase-negative, including the Schwann cells and fibroblasts. These results were consistent with the response of endogenous DINE mRNA after treatment with these factors. This region of the DINE promoter is sufficient to reflect the expression of endogenous DINE gene in response to LIF treatment and NGF withdrawal, suggesting that it might contain an injury-responsive element.

The Combination of ATF3:c-Jun Heterodimer and STAT3 Synergistically Enhanced DINE Promoter Activity—Considering both the Janus kinase-STAT signaling pathway and the JNK signaling pathway are activated by NGF deprivation and the LIF stimulus, STAT3, c-Jun, and ATF3, which are located downstream in the signaling pathways, seemed to be the most potent transcription factors for DINE gene regulation. To confirm this possibility, the co-expression of these transcription factors was investigated to determine if they would mutually up- or down-regulate their separate effects on the transcription from the DINE promoter. The plasmids carrying the transcription factors were transfected into SK-N-SH neuroblastoma cells together with the DINE promoter (nucleotides +2765 to +1) driven luciferase reporter vector (Fig. 2A). Overexpression of individual STAT3, ATF3, or c-Jun did not change the luciferase activity. Co-expression of ATF3 and c-Jun or co-expression of ATF3, c-Jun, and STAT3 increased the luciferase activity by 4- to 5-fold, suggesting that the dimerization of ATF3 and c-Jun may be important for stimulating DINE promoter activity. Although previous studies (18, 32) demonstrated in vivo that ATF3 and c-Jun interacted each other, ATF3 and c-Jun interaction has not been reported in any physiological settings to date. Because it is still unclear about the mechanism for effective dimerization of ATF3 and c-Jun, a forced dimmer construct of ATF3:c-Jun was designed in which ATF3 and c-Jun cDNAs were fused in-frame through a glycine-rich polypeptide tether of 18 amino acids. As expected, transfection of the forced ATF3:c-Jun heterodimer significantly enhanced transcriptional activity by 20-fold. Because co-transfection with a forced homodimer of ATF3 and ATF3 (ATF3:ATF3) did not produce an apparent increase in promoter activity, it is assumed that the

**RESULTS**

**Characteristics of the DINE Promoter**—Genomic clones were isolated from a mouse (129 strain) genome library to examine injury-responsive expression of the DINE gene. The nucleotide sequence of 2.8 kb of the 5'-flanking region of the DINE gene was determined. Similar to many neuronal genes, the upstream portion of the mouse DINE gene is GC-rich and lacks a consensus TATA box (supplemental Fig. S1A). Although endogenous DINE mRNA is specifically expressed in neurons, there was no apparent neuron-restrictive silencer element sequence in this promoter region. However, the 5'-flanking region of the DINE gene promoter induced gene expression preferentially in neuronal cell lines rather than in non-neuronal cell lines (supplemental Fig. S1B).

**DINE Promoter Is Activated in DRG Neurons in Response to LIF Stimulus and NGF Deprivation**—To determine if the 5'-flanking region of DINE gene contains the injury-responsive element, a promoter assay was carried out using DRG cultures. It has been shown that the injury responsiveness of the endogenous DINE mRNA of DRG neurons both in vivo and in vitro was partially regulated by signaling pathways after LIF treatment and NGF withdrawal (9). Therefore, we attempted to learn if the promoter region of the DINE gene was activated by LIF treatment and NGF withdrawal in a similar manner to the endogenous DINE gene. DRGs were infected with an adenovirus containing the firefly luciferase regulated by a 2.8-kb fragment of DINE promoter. At 24 h after infection, DRGs were treated with various trophic factors and cytokines such as NGF, NT-3, GDNF, ciliary neurotrophic factor, and LIF, which are typically released from surrounding glial cells after nerve injury.

**FIGURE 1.** Application of LIF together with NGF withdrawal increases the activity of DINE promoter in cultured DRG. A, luciferase activity of DINE promoter in DRG cultures. After infection of DINE promoter-driven luciferase adenovirus, various factors were added. Luciferase activity is shown as the fold increased activity compared with non-treated cultures. Values of luciferase activity are plotted as mean ± S.D. of three or more independent experiments in quadruplicate wells. B, addition of anti-gp130 antibody suppressed LIF-induced promoter activity. C, after infection of DINE-luciferase adenovirus, cells were treated with LIF together with NGF deprivation and then immunostained with anti-luciferase, anti-MAP2 antibodies. Bar = 40 μm.

**PCR** were as follows: forward, 5'-GGTGGCTCCAAGTGCTGATCTCAGAAGG-3', and backward, 5'-AGCTGGGC-TGGCTGGCGCTCTTTGTG-3'.

**DINE Promoter**—It was previously shown (33) that the 5'-flanking region of the mouse DINE gene contains an apparent neuron-restrictive silencer element. However, the 5'-flanking region of the DINE gene promoter induced gene expression preferentially in neuronal cell lines rather than in non-neuronal cell lines (supplemental Fig. S1B). Although endogenous DINE mRNA is specifically expressed in neurons, there was no apparent neuron-restrictive silencer element sequence in this promoter region. However, the 5'-flanking region of the DINE gene promoter induced gene expression preferentially in neuronal cell lines rather than in non-neuronal cell lines (supplemental Fig. S1B).

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glycine-rich polypeptide tether sequences have no apparent effect on luciferase activity. Furthermore, co-transfection of the ATF3:c-Jun heterodimer with STAT3 greatly enhanced the DINE promoter activity by 60-fold.

**STAT3 Functions without Phosphorylation and DNA Binding**—To further elucidate the details of the mechanism of transcriptional regulation of the DINE gene, an ATF3:c-Jun plasmid was co-transfected with various types of mutated STAT3 plasmids, including STAT3 Y705F, having a mutation of a phosphorylation site, STAT3D, having a mutation of a DNA binding site, and a constitutive active form of STAT3C obtained by forced dimerization. Because transfection of the STAT3 plasmid alone had no effect on the DINE promoter activity (Fig. 2A), various STAT3 mutants were co-transfected together with the ATF3:c-Jun heterodimer. Most of the STAT3 mutants as well as the wild-type STAT3 did not cause a significant suppression of transcriptional activity as shown in Fig. 2B. Only the N-terminal deletion mutant of STAT3 suppressed the DINE promoter activity, even in the presence of the ATF3:c-Jun heterodimer. The N-terminal deleted STAT3 was localized only in the cytoplasm, whereas other types of STAT3 mutants were localized in both the nucleus and cytoplasm (supplemental Fig. S2). Consistent with the present data, a recent publication showed that the activation and nuclear translocation of STAT3 was regulated by the N-terminal domain of STAT3, independently from the phosphorylation of the SH2 domain (33). Thus, STAT3, upon translocation to the nucleus, can function in combination with the ATF3:c-Jun heterodimer without DNA binding activity and without phosphorylation. This suggests that STAT3 might function as a part of the complex regulating the DINE gene without binding to the DNA.

**ATF3:c-Jun Heterodimer Is Necessary and Functions without Binding to the Putative DNA Binding Elements**—A commonly used dominant-negative form of c-Jun, TAM-67, was tethered to ATF3 (ATF3:c-JunTAM). TAM-67 lacks the N-terminal transactivation domain of c-Jun and has no transcriptional activity. DINE promoter activity was not increased by co-transfection of ATF3:c-JunTAM, with STAT3 as shown in Fig. 2C. These findings implied that the ATF3:c-Jun heterodimer might bind to a specific CRE/ATF or an AP1 site within the DINE promoter. When the cells were transfected with the shorter DINE promoter (nucleotide −2765 to −1010 containing the upstream region from the transcription start site)-driven reporter construct, the response pattern was similar to that observed with the previously described constructs shown in Fig. 2 (A–C) (data not shown).
Sp1 Directly Binds to the DINE Promoter and Recruits the ATF3:c-Jun Heterodimer and STAT3—To examine the functional importance of the Sp1 binding to the regions ranging from −2050 to −2015 and from −1235 to −1010, the mutations, in which critical G and C were changed to A and T, were introduced into the Sp1 sites within −2050 to −2040 (mut1), within −1226 to −1216 (mut2), within −1181 to −1158 (mut3), and within −1145 to −1135 (mut4). A series of mutated DINE promoter-luciferase plasmids were co-transfected with the ATF3:c-Jun heterodimer into SK-N-SH cells, and their luciferase activity was determined (Fig. 3A). The mut1 or mut2 DINE promoter-driven luciferase activity was induced by the ATF3:c-Jun heterodimer as well as in the wild type. However, the mut3 DINE promoter could not effectively drive luciferase activity in response to expression of the ATF3:c-Jun heterodimer. Considering these observations along with the results of the other double or triple mutated promoter-luciferase activity, it appears that the Sp1 GC binding box between −1181 and −1158 is critical for induction by ATF3:c-Jun.

To further examine the functional relevance of the GC box ranging from −1181 to −1158 in DINE regulation, an EMSA was performed using SK-N-SH cell extracts (Fig. 3B). An oligonucleotide from −1181 to −1158 (WT) was used as a probe. When nuclear extracts from SK-N-SH cells were incubated with this radiolabeled WT probe, the mobility of WT oligonucleotide was reduced. To determine if the DNA-protein interactions were specific, EMSA using competitor oligonucleotides were performed. Excess unlabeled WT oligonucleotides competed the formation of a DNA-protein complex with labeled probe, but excess unlabeled mutant oligonucleotides had no effect on complex formation. In addition, an antibody supershift experiment confirmed that these complexes contain Sp1.

The induction of DINE promoter activity by the ATF3:c-Jun heterodimer, and STAT3 was enhanced when Sp1 was co-transfected (Fig. 3C). In contrast, the suppression of endogenous Sp1 using small interference RNA did not up-regulate DINE promoter activity even though ATF3:c-Jun heterodimer was co-transfected (Fig. 3D). These results suggest that Sp1 functions as a scaffolding protein, which pro-

To identify the DNA element that contributes to the enhancement of promoter activity of DINE in response to the ATF3:c-Jun heterodimer, deletions of the DINE promoter reporter constructs at the upstream region from −1010 were generated (Fig. 2D). Promoter analysis showed that deletion of the DINE promoter regions ranging from −2045 to −2015 and from −1235 to −1010 significantly reduced the luciferase activity in response to expression of ATF3:c-Jun. These results suggest that DNA elements within these regions are responsible for the induction of the ATF3:c-Jun heterodimer. However, to the best of our knowledge, these promoter regions contain neither an ATF/CRE element nor an AP1 element. Instead, the regions contain the GC-rich Sp1 binding sites. Considering that Sp1 protein is endogenously abundant in SK-N-SH cells, it is likely that Sp1 would bind to the DINE promoter and associate with the ATF3:c-Jun heterodimer and STAT3.

To examine the DNA element that contributes to the transcriptional activation of DINE promoter in response to ATF3:c-Jun and STAT3. A, DINE promoter luciferase reporter plasmids with wild-type or GC box mutant sequences were transfected into SK-N-SH cells together with control or ATF3:c-Jun plasmids. Data are expressed as -fold increase in response to ATF3:c-Jun. Closed circles represent Sp1 binding sites where mutations are inserted (mut1−4). β, EMSA using a radiolabeled wild-type (WT) DINE probe. Association of SK-N-SH nuclear protein with Sp1 causes retardation in the migration of the labeled probe (arrow). The reactions were blocked with an excess of unlabeled WT probe. In the presence of an anti-Sp1 antibody, the DNA-protein complex was supershifted (arrowhead). C, ATF3:c-Jun- and STAT3-induced luciferase activity of DINE promoter was further enhanced by co-expression of Sp1. D, Sp1 knockdown by Sp1 small interference RNA suppressed DINE promoter activity in response to ATF3:c-Jun. Data is shown as percent inhibition of the -fold increase by ATF3:c-Jun.

FIGURE 3. Sp1 is pivotal for the transcriptional activation of DINE promoter in response to ATF3:c-Jun and STAT3. A, DINE promoter luciferase reporter plasmids with wild-type or GC box mutant sequences were transfected into SK-N-SH cells together with control or ATF3:c-Jun plasmids. Data are expressed as -fold increase in response to ATF3:c-Jun. Closed circles represent Sp1 binding sites where mutations are inserted (mut1−4). B, EMSA using a radiolabeled wild-type (WT) DINE probe. Association of SK-N-SH nuclear protein with Sp1 causes retardation in the migration of the labeled probe (arrow). The reactions were blocked with an excess of unlabeled WT probe. In the presence of an anti-Sp1 antibody, the DNA-protein complex was supershifted (arrowhead). C, ATF3:c-Jun- and STAT3-induced luciferase activity of DINE promoter was further enhanced by co-expression of Sp1. D, Sp1 knockdown by Sp1 small interference RNA suppressed DINE promoter activity in response to ATF3:c-Jun. Data is shown as percent inhibition of the -fold increase by ATF3:c-Jun.
provides ATF3, c-Jun, and STAT3 with a platform for their assembly.

To test this hypothesis, the possibility that Sp1 is able to interact with ATF3:c-Jun and STAT3 was examined. SK-N-SH cells were infected with adenoviruses expressing ATF3, ATF3:c-Jun, c-Jun, and STAT3. The interaction with endogenous Sp1 was investigated 36 h after infection employing a co-immunoprecipitation procedure (Fig. 4). Immunoblot analysis demonstrated that ATF3, c-Jun, ATF3:c-Jun, and STAT3 specifically interacted with Sp1 (Fig. 4). These results strongly suggest that Sp1 is a scaffolding transcription factor for assembling ATF3, c-Jun, and STAT3.

**FIGURE 4.** Sp1 interacts with ATF3, c-Jun, and STAT3. SK-N-SH cells were infected with ATF3, ATF3:c-Jun, c-Jun, and STAT3 adenoviruses. After 48 h of infection, whole cell lysates (WCL) were collected, subjected to co-immunoprecipitation using anti-Sp1 antibody (IP: Sp1), and blotted (WB) with anti-Myc, anti-c-Jun, and anti-STAT3 antibodies.

**FIGURE 5.** Sp1-recruited ATF3, c-Jun, and STAT3 may activate DINE gene after hypoglossal nerve injury. A, in situ hybridization and immunohistochemical analysis showed that expression of DINE mRNA and injury-inducible transcription factors proteins 5 days after hypoglossal nerve injury. XII, hypoglossal nucleus; cc, central canal. Bar = 40 μm. B, protein extracted from control (cont) and injured (inj) hypoglossal nuclei were subjected to co-immunoprecipitation using anti-ATF3 (IP: ATF3) or anti-Sp1 antibody (IP: Sp1) and immunoblotted (WB). WCL, whole cell lysates. C, control (cont) and injured (inj) hypoglossal nuclei were subjected to chromatin immunoprecipitation analysis with anti-ATF3, anti-c-Jun, anti-STAT3, or anti-Sp1 antibodies and primers targeted to the sequence flanking the Sp1 binding site of DINE promoter.

DINE mRNA was up-regulated in all the motor neurons of nerve-injured hypoglossal nucleus by 5- to 7-fold (Fig. 5A). The increase of DINE mRNA after nerve injury seemed to be reasonable, considering the increase of the DINE promoter activity in response to LIF treatment and NGF withdrawal in cultured DRG (Fig. 1A). Consistent with previous reports (18), the expression of the ATF3 and c-Jun proteins was up-regulated in injured hypoglossal motor neurons. Although the expression level of STAT3 protein did not appreciably change before and after axotomy, STAT3 was accumulated in the nucleus after injury. Sp1 remained unchanged before and after the insult. These observations indicated that all the factors mentioned above accumulated in the nucleus of motor neurons in response to nerve injury. Co-immunoprecipitation studies were performed using extracts from control and injured hypoglossal nuclei to determine if Sp1 interacts with the injury-inducible transcription factors in the injured neurons (Fig. 5B). These co-immunoprecipitations showed that Sp1 interacted with ATF3, c-Jun, and STAT3, suggesting that the complex is able to associate with the DINE promoter region. To further clarify the recruitment of Sp1, c-Jun, ATF3, and STAT3 to the Sp1 binding sites in the promoter region of the DINE gene. Chromatin immunoprecipitation assays were carried out using control and injured hypoglossal nuclei. The recruitment of ATF3 and c-Jun to the gene promoter DINE significantly increased after axotomy in the animals, but there was no change in the binding of STAT3 and Sp1 to the promoter as shown in Fig. 5C.

**DISCUSSION**

This study elucidated that the 5’-flanking region of nerve injury-associated gene, DINE, contained potential injury-responsive element and that injury-inducible transcription factors ATF3, c-Jun, and STAT3 participated in the expression of DINE. Although it is still unclear about the interaction of ATF3 and c-Jun under physiological condition, forced heterodimer of ATF3 and c-Jun activated DINE promoter together with STAT3. ATF3, c-Jun, and STAT3 did not directly bind to the DINE promoter. Instead, Sp1 directly bound to the GC-rich region within DINE promoter. ATF3, c-Jun, and STAT3 inter-
acted with Sp1 and associated with the GC box within the DINE promoter in damaged neurons. These findings suggested that ATF3, c-Jun, and STAT3 assemble on Sp1 to elicit their substantial synergistic promoter activity in DINE expression after nerve injury.

**Injury-inducible Transcription Factors ATF3, c-Jun, and STAT3 Participate in the Activation of DINE Gene**—It has been suggested that the dimerization of ATF3 with c-Jun is necessary to promote regeneration responses using promoters for other nerve-injury associated genes (18, 22). However, there is no evidence to show the interaction in physiological condition to date. This study demonstrated that the promoter activity of the nerve injury-associated gene, DINE, was increased by the forced heterodimer of ATF3 and c-Jun (ATF3:c-Jun). The combined expression of non-tethered ATF3 and c-Jun had a lower effect on promoter activity than the forced heterodimer ATF3:c-Jun. This ineffectiveness of ATF3 and c-Jun individually may be a result of their inability to dimerize without some additional signal. It is likely that some previously unidentified signaling pathway or associated proteins, which are activated by nerve injury, are involved in facilitating the dimerization of ATF3 and c-Jun. Supporting this possibility is the observation that ATF3:c-JunTAM, which lacks the N-terminal transactivation domain of c-Jun, could not activate the DINE promoter. Therefore it is likely that the N-terminal domain of c-Jun is also required for transactivation as previously described (34).

Another significant finding in this study is that STAT3 seems to have an additional role distinct from the ordinary DNA binding transcription factor for the following reasons. First, the transfection of STAT3 alone did not enhance DINE promoter activity, and its co-transfection together with ATF3:c-Jun did substantially enhance DINE promoter activity. Second, two commonly used dominant negative forms of STAT3, STAT3Y705 and STAT3D, which are mutated at the phosphorylation site and DNA binding site, respectively (35), increased DINE promoter activity as did the constitutive active form STAT3C (36) and the wild-type STAT3. Only the N-terminal deleted STAT3 failed to induce DINE promoter activity. In general, it is assumed that the phosphorylated STAT3 is capable of translocating into the nucleus. However, Liu et al. (33) demonstrated that the nuclear translocation of STAT3 is independent of the tyrosine phosphorylation and that the N-terminal region contains a nuclear localization signal with which importin α3 interacts. Indeed, only the N-terminal deleted STAT3 did not translocate into nucleus in this study. Another report mentioned that the unphosphorylated STAT3 influenced some gene expressions (37). It could be concluded that the translocation of STAT3 into the nucleus was necessary for the expression of DINE, but its phosphorylation and DNA binding activity are unnecessary. Considering that STAT3 can interact with numerous transcription factors, including CBP/p300 (38), STAT3 could function as a part of complex to bridge other transcriptional components or to change the chromosomal structure in promoting nerve regeneration.

**Sp1 May Provide ATF3/c-Jun/STAT3 with a Platform to Elicit Their Functional Synergy**—The present study revealed that Sp1 bound directly to the target DNA element of DINE gene, probably to function as a scaffolding protein for the recruited injury-inducible transcription factors such as ATF3/c-Jun/STAT3. This result was unexpected, because ATF3, c-Jun, and STAT3 were believed to bind to their own target DNA elements. Consistent with the present finding, it has been reported that gene promoters without the AP1 site or without the STAT3 binding site are activated by c-Jun or STAT3 through an interaction with Sp1 (39–42). Sp1 interacts with different transcription factors as well as with basal transcriptional factors producing synergistic effects leading to the activation of flexible pathways for gene expression (43). Of those transcription factors reported, many transcription factors such as cAMP-response element-binding protein and Smad are involved in nerve regeneration (44–46). These additional transcription factors may participate in the Sp1/ATF3/c-Jun/STAT3 complex and to yield maximum effects in injured neurons. Alternatively, the partner proteins interacting with Sp1 might be selected by the state of Sp1 modification such as acetylation or phosphorylation (47).

**The Sp1-mediated Transcriptional Complex May Be Common among Several Nerve Regeneration-associated Genes**—The present findings may provide one potential to explain how nerve injury-induced transcription factors participate in the dynamic alteration of the expression a group of genes during nerve regeneration. This possible Sp1-mediated mechanism would be practical and effective when increased gene expressions are required in the event of a fatal emergency such as neuronal injury. Many neuronal genes contain the general Sp1 binding site, suggesting that the nerve injury-inducible transcription factors will be capable of accessing many genes without any specific injury-responsive elements. For instance, another member of nerve regeneration-associated genes are manganese superoxide dismutase and p21, both contain a Sp1 binding GC box in their promoter (40, 48). Our preliminary experiment also demonstrated that the firefly luciferase carrying manganese superoxide dismutase promoter was also activated by the combination of ATF3:c-Jun and STAT3 (supplemental Fig. S3). However, there still remain ambiguous points about detailed transcriptional machinery, including the physiological dimerization of ATF3/c-Jun and the actual significance of Sp1. We anticipate further study to clarify the mechanism. In addition, we could not rule out a possibility that the inducible transcription factors regulate the DINE gene by binding to their individual binding sites separately in different enhancer region and that a combination of these proposed transcriptional mechanisms regulate the expression of DINE. Recently, Bacon et al. (44) reported that individual Ets, STAT, and Smad binding sites confer the responsiveness of the galanin gene to axotomy. The expression pattern of DINE mRNA in DRG after injury was quite similar to that of galanin (9). Further study might be required to examine the involvement of these transcription factors as well.

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Transcriptional Regulation of Nerve Injury-associated Gene

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