Essential Roles of c-JUN and c-JUN N-Terminal Kinase (JNK) in Neuregulin-Increased Expression of the Acetylcholine Receptor ϵ -Subunit

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Neuregulin is a neural factor implicated in upregulation of acetylcholine receptor (AChR) synthesis at the neuromuscular junction. Previous studies have demonstrated that the extracellular signal-regulated kinase (ERK) subgroup of MAP kinases is required for neuregulin-induced AChR gene expression. We report here that the neuregulin-mediated increase in AChR ϵ -subunit mRNA was a delayed response in C2C12 muscle cells. Neuregulin induced expression of immediate early genes *c-jun* and *c-fos*, which followed and depended on the ERK activation. Treatment of muscle cells with cycloheximide to inhibit c-JUN synthesis at the protein level and suppression of c-JUN function by a dominant-negative mutant blocked neuregulin-induced expression of the ϵ -subunit gene, indicating an essential role of c-JUN in neuregulin signaling. Furthermore,

neuregulin activated c-JUN N-terminal kinase (JNK) in C2C12 muscle cells. Blockade of JNK activation by overexpressing dominant-negative MKK4 inhibited ϵ -promoter activation. Moreover, overexpression of the JNK dominant-negative mutant inhibited neuregulin-mediated expression of the ϵ -transgene and endogenous ϵ -mRNA. Taken together, our results demonstrate important roles of c-JUN and JNK in neuregulin-mediated expression of the AChR ϵ -subunit gene and suggest that neuregulin activates multiple signaling cascades that converge to regulate AChR ϵ -subunit gene expression.

Key words: neuregulin; AChR; c-JUN; JNK; neuromuscular junction; synapse; immediate early gene

Synaptic transmission at the neuromuscular junction is guaranteed by a high density of acetylcholine receptor (AChR) at the postsynaptic membrane. AChR expression is regulated both spatially and temporally during development. AChR synthesis is confined to the adult neuromuscular junction (Froehner, 1993; Hall and Sanes, 1993; Sanes and Lichtman, 1999). Studies using transgenic mice have shown that the regulatory elements of AChR genes can direct the expression of reporter genes at the neuromuscular junction (Klarsfeld et al., 1987; Sanes et al., 1991; Simon et al., 1992; Gundersen et al., 1993), indicating that transcription of AChR genes is most active in nuclei beneath the postsynaptic membrane, contributing to the high density of AChR at the neuromuscular junction.

Synaptic expression of AChR genes is believed to be mediated by neuregulin, a family of factors that have been originally identified as acetylcholine receptor-inducing activity (ARIA) that stimulates muscle AChR synthesis (Fischbach and Cohen, 1973; Jessell et al., 1979; Falls et al., 1993), as ligands of the receptor tyrosine kinase *erbB2* (neu differentiation factor and heregulin) (Wen et al., 1992), and as neuronal factors essential for the proliferation and survival of Schwann cells (glia growth factor) (Marchionni et al., 1993). They are products of the *nrg-1* gene

generated by alternative splicing. Four lines of evidence indicate that neuregulin is a trophic factor released from motoneurons to regulate AChR synthesis at the neuromuscular junction. First, neuregulin heterozygous mutant mice that produce a low amount of neuregulin have a decreased postsynaptic AChR density and are myasthenic (Sandrock et al., 1997). Second, neuregulin mRNA is expressed in motoneurons before the onset of the neuromuscular junction and persists through adulthood (Fischbach and Rosen, 1997; Sandrock et al., 1997). Third, neuregulin accumulates in synaptic basal lamina in adult skeletal muscle (Chu et al., 1995; Goodearl et al., 1995; Jo et al., 1995; Sandrock et al., 1995), and *ErbB* proteins, neuregulin's receptors, are localized at the neuromuscular junction (Altiok et al., 1995; Zhu et al., 1995). Last, in cultured muscle cells, neuregulin promotes AChR synthesis both at the protein level (Jessell et al., 1979; Usdin and Fischbach, 1986; Sandrock et al., 1997) and at the level of mRNA (Martinou et al., 1991; Tang et al., 1994; Chu et al., 1995).

Until recently, little was known about the signaling mechanism of neuregulin in the regulation of AChR gene expression. The *ErbB2* and *ErbB3* proteins become tyrosine phosphorylated in response to neuregulin (Corfas et al., 1993; Altiok et al., 1995; Jo et al., 1995; Si et al., 1996). Concomitantly, extracellular signal-regulated kinase (ERK) activity (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997) and phosphatidylinositol 3 (PI 3)-kinase activity (Tansey et al., 1996) are increased. Activation of ERK kinase is required for neuregulin regulation of AChR gene expression in cultured muscle cells (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997) and for synapse-specific expression of the ϵ -transgene *in vivo* (Si and Mei, 1999). Here we report that the neuregulin-mediated increase in AChR ϵ -subunit mRNA was a delayed response. Neuregulin induced expression of immediate

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early genes *c-jun* and *c-fos* and activated c-JUN N-terminal kinase (JNK), both of which were required for the ϵ -promoter transcription.

MATERIALS AND METHODS

Materials. Recombinant neuregulin (rHRG_{β1}177–244, a peptide of HRG_{β1} residues 177–244) was generously provided by Dr. M. Sliwkowski (Holmes et al., 1992). Anti-ERK kinase antibody was a gift from Dr. G. S. Feng. The mouse AChR ϵ -subunit cDNA was provided by Dr. R. Huganir. The *c-fos* and *c-jun* cDNAs were provided by Drs. T. Curran and A. Langley (Curran et al., 1987). Dr. R. Davis provided JNK1, MKK4, and p38 constructs (Whitmarsh et al., 1997), and Dr. M. Birrer provided the c-JUN mammalian expression constructs (Brown et al., 1994). PD98059, AG1478, and SB202190 were purchased from Calbiochem (La Jolla, CA). Rapamycin was from Research Biochemicals (Natick, MA). Cell culture media were purchased from Life Technologies (Gaithersburg, MD). Anti-FLAG antibody and all other chemicals were from Sigma (St. Louis, MO).

Cell culture and transfection procedures. Mouse muscle C2C12 cells were maintained as undifferentiated myoblasts in a nutrient-rich growth medium containing DMEM supplemented with 20% fetal bovine serum, 0.5% chicken embryo extract, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in an atmosphere of 5% CO₂ and 95% humidity. To induce differentiation, we cultured myoblasts at 50–70% confluence in the differentiation medium (DM), DMEM supplemented with 4% horse serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. For transient transfection, C2C12 myoblasts at ~50% confluence in six-well plates were cotransfected with the ϵ 416-Luc transgene (1 μ g of DNA) and p-cytomegalovirus β (pCMV β ; 0.1 μ g of DNA) with or without 4 μ g of either wild-type or mutant c-JUN, MKK4, or JNK by the calcium phosphate technique (Sambrook et al., 1989). Twenty-four hours later cells were switched to DM. Myotubes then were treated with neuregulin for 24 hr. Stable C2C12 cell lines carrying wild-type or mutant JNK were generated as described previously (Si et al., 1996).

Treatment of cells. Forty-eight hours after switching to DM, the C2C12 myoblasts formed myotubes and were then treated with neuregulin. In experiments studying inhibitory effects, chemicals were added into the culture medium 30 min before neuregulin treatment and remained in the medium for the entire neuregulin stimulation period. The volume of solvent vehicles was kept $\leq$ 0.1% of the culture medium and did not significantly change the biological outcome. Culture medium was changed every 24 hr to keep myotubes healthy.

Northern blot analysis. Total RNA was extracted from C2C12 muscle cells by the use of a single-step RNA-isolating method modified from that of Chomczynski and Sacchi (1987). Briefly, cells of a 10 cm dish were lysed in 10 ml of the RNA-extracting buffer containing 2 M guanidinium thiocyanate, 12.5 mM sodium citrate, pH 7.0, 50 mM β -mercaptoethanol, 0.25% *N*-lauroylsarcosine, 0.1 M sodium acetate, and 50% phenol. After the addition of 2 ml of chloroform, the lysate was mixed vigorously and subjected to a centrifugation at 8000 $\times$ g at 4°C for 20 min. The aqueous phase was mixed with an equal volume of isopropanol, and total RNA was isolated in the pellet after centrifugation at 8000 $\times$ g at 4°C for 20 min. Unless otherwise indicated, 20 μ g of total RNA was resolved on a 1% agarose gel by electrophoresis in 3-(*N*-morpholino)propanesulfonic acid-formaldehyde buffer as described by Lehrach et al. (1977). RNA was transferred to a nylon membrane in 20 $\times$ SSC by capillary transfer and cross-linked to the membrane by the use of a UV cross-linker. The membrane filters were probed with a cDNA fragment of the ϵ -subunit or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), both of which were labeled with [α -³²P]dCTP by a random-prime method. RNA blots were hybridized in a buffer containing 6 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.5% SDS, 50% formamide, and 200 μ g/ml salmon sperm DNA at 42°C for 48 hr. The membrane filters were washed with 0.1 $\times$ SSC and 0.5% SDS at 42°C four times, each for 15 min, and exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) at –80°C with an intensifying screen.

Quantitative image analysis. The quantification was performed by image analysis of radioautographic films by scanning the film with the Personal Densitometer (Molecular Dynamics, Sunnyvale, CA), and the captured image was analyzed with the ImageQuant software (Molecular Dynamics).

ERK kinase assay. The ERK kinase assay was performed essentially as described previously (Si et al., 1996).

JNK and p38 MAP kinase assays. C2C12 myotubes were cultured in serum-free DMEM medium for 24 hr before the stimulation with neuregulin. Myotubes were harvested in the lysis buffer containing 137 mM NaCl, 1% NP-40, 5 mM EDTA, 20 mM Tris-HCl, pH 7.5, 1 μ M pepstatin, 1 μ g/ml leupeptin, 0.2 mM PMSF, 2 μ g/ml aprotinin, and 2 mM sodium vanadate. JNK was purified as described previously (Whitmarsh et al., 1997). Briefly, an aliquot of lysate (400 μ g of protein) was incubated with 10 μ g of glutathione *S*-transferase (GST)–c-JUN (human c-JUN amino acids 1–79) fusion protein immobilized on agarose beads in a final volume of 0.4 ml at 4°C for 2 hr. After being washed three times with the lysis buffer and twice with a buffer containing 20 mM HEPES, pH 7.5, and 20 mM MgCl₂, the beads were then incubated in 30 μ l of the kinase assay buffer containing 20 mM HEPES, pH 7.5, 20 mM MgCl₂, 20 mM β -glycerophosphate, 0.1 mM sodium vanadate, 20 μ M ATP, 2 mM DTT,

Figure 1. Delayed increase in the AChR ϵ -subunit mRNA by neuregulin in C2C12 myotubes. Stimulation of C2C12 myotubes was started by the addition of neuregulin (NRG) to a final concentration of 1 nM or an equal volume of vehicle (as a control). Cells were harvested for RNA isolation at the indicated times. Twenty micrograms of total RNA were resolved on a 1% agarose gel, transferred to nitrocellulose, and hybridized with [³²P]-labeled DNA probes specific for the ϵ -subunit. The loading was uniform as evidenced by an equal amount of GAPDH mRNA. *A*, Representative Northern blot radiographs. *B*, Histograms showing mean $\pm$ SD of two different samples. The mRNA levels at 0 min were considered to be 100%. ***p* < 0.01.

Figure 2. Increase in the AChR ϵ -mRNA by transient stimulation with neuregulin. *A*, Stimulation protocol. Stimulation of myotubes with NRG defined in Figure 1 began at time 0; after an incubation for the indicated time, the cells were washed and incubated in neuregulin-free medium. At 24 hr, cells were lysed to isolate total RNA, which was then blotted for AChR mRNA expression. *B*, Activation of ERK kinase by neuregulin. *Top*, C2C12 myotubes stimulated continuously without (control) or with 1 nM neuregulin for the indicated times. *Bottom*, C2C12 myotubes stimulated with 1 nM neuregulin for 1 min and then incubated in neuregulin-free medium for the indicated times. Control C2C12 myotubes were treated with an equal volume of DMEM (vehicle) without neuregulin for 5 min. ERK kinase was immunoprecipitated and assayed using MBP as a substrate *in vitro* with [γ - 32 P]ATP. The phosphorylated MBP was resolved on a 15% SDS gel and exposed to x-ray film. *C*, Northern blot radiograms. C2C12 myotubes were stimulated as described in *A*. Control C2C12 myotubes were treated with the same volume of DMEM (without neuregulin) for 24 hr. Twenty micrograms of total RNA were probed with [32 P]-labeled ϵ -subunit probe. *D*, Histograms showing mean $\pm$ SD of three different samples. The mRNA levels in the absence of neuregulin were considered to be 100%. CON ST, Continuous stimulation; ST, stimulation.

and 5 μ Ci of [γ - 32 P]ATP at 30°C for 15 min. The p38 MAP kinase was purified from the cell lysate using 50 μ l of protein G-agarose beads (1:1 slurry) preloaded with goat anti-p38 MAP kinase antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and assayed using as a substrate 2 μ g of the GST-ATF2 fusion protein in the same phosphorylation buffer. The kinase reaction was stopped by the addition of 10 μ l of 4 $\times$ sample-loading buffer and subjected to electrophoresis on a 10% SDS-polyacrylamide gel that was then exposed to an x-ray film.

Luciferase and β -galactosidase assays. The luciferase assay was performed using a kit from Promega (Madison, WI) and following the manufacturer's instructions. Briefly, 100 μ l of cell lysate was mixed in an equal volume of luciferase substrate solution containing 20 mM Tricine, 1.07 mM (MgCO_3) $_4$ Mg(OH) $_2$ 5H $_2$ O, 2.67 mM MgSO $_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μ M coenzyme A, 470 μ M luciferin, and 530 μ M ATP and was placed in a micro β luminometer (Wallac, Turku, Finland) to measure light production for 10 sec. β -Galactosidase activity was determined as described previously (Si et al., 1996). Luciferase activity of transgenes was normalized to β -galactosidase activity to correct for variation in transfection.

Protein concentration determination. Protein concentration was measured by the Bradford method using a Coomassie Protein Assay Reagent (Pierce, Rockford, IL) with BSA as a standard (Bradford, 1976).

Statistical analysis. Values were shown as mean $\pm$ SD. Statistical differences were analyzed using the one-tailed Student's *t* test. A value of *p* < 0.05 is considered statistically significant.

RESULTS

Delayed increase in AChR ϵ -subunit mRNA in neuregulin-stimulated C2C12 myotubes

The basal level of the ϵ -mRNA in C2C12 myoblasts was barely detectable and remained low until 3–4 d after the cells were switched to DM (data not shown). Neuregulin stimulation did not increase the ϵ -subunit expression in C2C12 myoblasts (Si et al., 1996). We studied the time course of neuregulin in the increase of AChR gene expression in C2C12 myotubes. Forty-eight hours after the medium switch when myotube formation was complete, cells were stimulated with 1 nM neuregulin for various times. In the presence of neuregulin, AChR ϵ -mRNA remained unchanged until 8–12 hr later when an increase was detectable. The neuregulin-induced increase in the ϵ -mRNA became robust 24 hr after stimulation (Fig. 1). The increase in the ϵ -mRNA by neuregulin was specific because neuregulin had no effect on GAPDH mRNA (Fig. 1). The basal level of the ϵ -mRNA (in the absence of neuregulin) did not change significantly during the time of the experiment (Fig. 1*A*, *top*). Figure 1*B* shows the means of two separate experiments. The expression of other AChR subunit

Figure 3. Inhibition of the neuregulin-mediated increase in AChR ϵ -mRNA by actinomycin D. C2C12 myotubes were pretreated with 5 μ g/ml actinomycin D for 30 min before 1 nM neuregulin stimulation for 12 hr. In the late-addition experiments, C2C12 cells were stimulated with 1 nM neuregulin for 12 hr to increase the ϵ -mRNA. Actinomycin D (5 μ g/ml) was then added in the medium. After incubation for another 4 hr, C2C12 myotubes were collected for Northern blotting using 20 μ g of total RNA. *A*, Representative Northern blot radiograms. *B*, Histograms showing mean $\pm$ SD of two different samples. The mRNA levels in the absence of neuregulin were considered to be the control (100%). ** $p < 0.01$ in comparison with the neuregulin-mediated increase in the absence of actinomycin D.

genes in response to neuregulin followed a similar time course (data not shown).

Increase in the ϵ -mRNA by transient neuregulin stimulation

Neuregulin-induced expression of AChR genes requires activation of the ERK subgroup of MAP kinases in cultured muscle cells (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997). The increase in ERK kinase activity by neuregulin is transient with a peak at ~5–8 min after neuregulin stimulation (Fig. 2*B*) (Si et al., 1996). The ERK kinase activity returns to the basal level within 60 min of neuregulin stimulation. Yet the increase in the ϵ -mRNA was a delayed response (Fig. 1). These observations prompted us to determine the stimulation time sufficient for neuregulin to activate AChR gene expression. Previous experiments in the literature used a continuous stimulation protocol, in which neuregulin was present in the culture medium until cells were harvested.

To study this, we have designed the protocol depicted in Figure

2*A*. Neuregulin stimulation of myotubes began at time 0; after an incubation of the indicated time, cells were washed and incubated in neuregulin-free DM. At 24 hr, total RNA was isolated and assayed for AChR expression. As shown in Figure 2*B*, exposure of C2C12 myotubes to neuregulin for as short as 1 min was sufficient to activate ERK MAP kinases and subsequently increase the AChR ϵ -mRNA (Fig. 2*C*). The ϵ -mRNA level increased by transient neuregulin stimulation was comparable with that induced by the continuous stimulation (Fig. 2*D*). *In vivo* neuregulin binds to the extracellular matrix via the Ig-like domain in the N terminus (Loeb and Fischbach, 1995) and thus becomes concentrated at the neuromuscular junction. The recombinant neuregulin used in this study contained only the epidermal growth factor domain and should not bind to the extracellular matrix. Thus, ERK activation was more transient by neuregulin stimulation in the transient stimulation protocol. These results suggest that the initiation of the signal pathways required for neuregulin-increased AChR gene expression may be completed within 1 min. After being activated, AChR gene transcription machinery remained active even in the absence of neuregulin.

To determine whether the ϵ -mRNA elevation reflects an increase in transcription of the ϵ -subunit gene, we studied the effect of actinomycin D, an inhibitor of RNA polymerases, on neuregulin's action. Treatment of C2C12 myotubes with actinomycin D decreased the basal level of the ϵ -mRNA (data not shown). Pretreatment with actinomycin D prevented the neuregulin-mediated increase in the ϵ -mRNA in C2C12 myotubes, suggesting an important role of active transcription in increasing the AChR mRNA (Fig. 3*A*). Furthermore, the addition of actinomycin D after neuregulin stimulation decreased the ϵ -mRNA, suggesting that the sustained elevation also requires active transcription (Fig. 3*B*). These results demonstrated that both the initial increase and the maintenance of the ϵ -mRNA depended on active transcription in C2C12 myotubes.

Induction of *c-fos* and *c-jun* immediate early genes by neuregulin

To understand neuregulin's signaling mechanism further, we sought to identify genes whose expression was upregulated by neuregulin in C2C12 myotubes. We and others have demonstrated previously that neuregulin activates ERK MAP kinase that is required for AChR subunit gene expression (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997). Moreover, evidence is compelling that the Ras-ERK pathway regulates transcription of immediate early genes such as *c-jun* and *c-fos* in a variety of cells (Segal and Greenberg, 1996). We determine whether neuregulin regulates *c-fos* and *c-jun* expression in C2C12 cells. Neuregulin had no apparent effect on the level of *c-jun* and *c-fos* mRNAs in C2C12 myoblasts (Fig. 4*A*). However, both *c-jun* and *c-fos* mRNAs were increased by neuregulin within 5 min in C2C12 myotubes (Fig. 4*B*). These results indicate that, as observed with AChR gene expression in C2C12 cells (Si et al., 1996), the neuregulin induction of *c-jun* and *c-fos* genes depended on cell differentiation. The *c-jun* expression peaked at ~10 min, whereas the *c-fos* expression maximized at ~30 min after the stimulation. Both mRNAs returned to the basal level (i.e., before stimulation) within 90 min. The neuregulin-induced expression of *c-jun* and *c-fos* had a concentration–response relationship (Fig. 4*C*) similar to that of neuregulin-induced AChR gene expression (Si et al., 1996).

To determine the signaling mechanism of neuregulin in the regulation of the expression of *c-jun* and *c-fos* genes, we studied

Figure 4. ERK kinase-dependent increase in *c-jun* and *c-fos* mRNAs in C2C12 myotubes by neuregulin. **A**, Effect of neuregulin on *c-jun* and *c-fos* mRNA levels in myoblasts. **B**, Time course of NRG-induced expression of *c-jun* and *c-fos* in myotubes. **C**, Concentration–response relationship of neuregulin-induced expression of *c-jun* and *c-fos* in myotubes. The fold increase by neuregulin in C2C12 cells was 5.2 ± 0.28 for *c-fos* and 2.4 ± 0.11 for *c-jun* mRNAs. **D**, Dependence of neuregulin-induced expression of *c-jun* and *c-fos* on ERK kinase activation. C2C12 myoblasts were treated with 10 nM neuregulin, 10% serum, or vehicle (control) for 30 min (**A**). Myotubes were treated with 1 nM neuregulin for the indicated times (**B**) or with various concentrations of neuregulin for 30 min (**C**). C2C12 myotubes were pretreated with 40 or 80 μ M PD98059 (lower concentration of each treatment in the left-hand lane), 200 nM or 1 μ M rapamycin, 200 nM or 1 μ M wortmannin, or 10 nM or 1 μ M tyrphostin AG1478 for 30 min before stimulation with 1 nM neuregulin for 30 min (**D**). Twenty micrograms of total RNA isolated from neuregulin-treated myotubes were resolved on a 1% agarose gel, blotted to nitrocellulose, and probed with [32 P]-labeled *c-jun* or *c-fos* cDNA fragments.

the effect of several chemicals known to affect the activity of ErbB protein tyrosine kinases, ERK, and PI 3-kinase in C2C12 myotubes (J. Si and L. Mei, unpublished observation). Pretreatment with tyrphostin AG1478, a potent and selective inhibitor of ErbB protein kinases (Levitzki and Gazit, 1995), and PD98059, an inhibitor of MEK (Dudley et al., 1995), had no effect on the basal levels of *c-jun* and *c-fos* mRNAs (data not shown) yet blocked the neuregulin-induced increase in *c-jun* and *c-fos* mRNAs in a concentration-dependent manner (Fig. 4D). The inhibitory effect of tyrphostin AG1478 and PD98059 was specific because they had no effect on the GAPDH mRNA. In contrast, the PI 3-kinase inhibitor wortmannin (Carraway et al., 1995) and the S6 kinase inhibitor rapamycin (Chung et al., 1992) did not appear to affect the basal or neuregulin-induced *c-jun* and *c-fos* mRNA. These results demonstrated that neuregulin-induced expression of *c-jun* and *c-fos* genes in C2C12 myotubes, like neuregulin-mediated AChR subunit gene expression (Si et al., 1996; Altiok et al., 1997), requires the activation of the ERK subgroup of MAP kinases but not PI 3-kinase.

Inhibition of neuregulin-induced ϵ -mRNA expression by cycloheximide

The similar concentration–response curves of neuregulin in the induction of immediate early genes and in the upregulation of AChR genes (Fig. 4C) (Si et al., 1996) and their dependence on ERK activation (Fig. 4D) (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997) indicated a strong correlation between these two events. Immediate early gene expression peaked at 10–30 min, which was after ERK activation (5–10 min) but before the increase in AChR mRNAs (8–12 hr). These results raised the

possibility that AChR transcriptional activation may require the previous induction and expression of immediate early genes. To address this possibility, we determined whether neuregulin was capable of increasing AChR mRNA expression in the presence of cycloheximide, a protein synthesis inhibitor that is known to block expression of the proteins encoded by immediate early genes (Greenberg et al., 1986). Treatment of C2C12 myotubes with 10 μ g/ml cycloheximide had no effect on basal expression of the ϵ -subunit (Fig. 5A). However, pretreatment with cycloheximide completely inhibited the neuregulin-mediated increase in the ϵ -mRNA (Fig. 5A,B), suggesting that the ϵ -subunit gene transcription required *de novo* protein synthesis. The inhibitory effect of cycloheximide did not appear to result from its potential toxicity because GAPDH mRNA did not change in these cells. Because cycloheximide alone did not affect the basal level of the ϵ -mRNA, it is unlikely that the decrease in neuregulin-induced ϵ -mRNA by cycloheximide is attributable to inhibiting synthesis of an mRNA-stabilizing factor. In the latter case, treatment with cycloheximide should lead to an increase in ϵ -mRNA in the absence of neuregulin. In contrast, cycloheximide blocked the neuregulin-mediated increase in the ϵ -mRNA. These results suggest that products of immediate early genes may be involved in regulation of ϵ -mRNA expression. It is worth noting that neuregulin was still able to induce immediate early gene *c-fos* mRNA expression in the presence of cycloheximide (Fig. 5C) that does not require *de novo* protein synthesis. Furthermore, the *c-fos* mRNA was superinduced by neuregulin in the presence of cycloheximide (Fig. 5C). Cycloheximide has been shown to increase the stability of immediate early gene mRNAs and thus to lead to a superinduction (Greenberg and Ziff, 1984; Greenberg et al., 1986).

Figure 5. Inhibition by cycloheximide of the neuregulin-mediated increase in the ϵ -mRNA. C2C12 myotubes were treated with or without 10 μ g/ml cycloheximide (CH) for 30 min before neuregulin stimulation for the indicated times. Total RNA was isolated for Northern blot analysis using the respective probe for the ϵ -subunit or *c-fos*. The loading was uniform as evidenced by an equal amount of GAPDH mRNA. *A*, Representative Northern blot radiograms of the ϵ -mRNA. *B*, Histograms showing mean $\pm$ SD of three different samples. The mRNA levels in the absence of neuregulin were considered to be 100% for the indicated time treatment. $**p < 0.01$ in comparison with the neuregulin-mediated increase in the absence of cycloheximide. *C*, Representative Northern blot radiograms of the *c-fos* mRNA.

Requirement of c-JUN in neuregulin-induced ϵ -subunit gene expression

Because c-FOS has to form a heterodimer with c-JUN to be functional whereas c-JUN can form functional homodimers (Angel and Karin, 1991), inhibition of c-JUN function should affect the neuregulin induction of the ϵ -subunit gene if it depends on the function of immediate early gene products. Therefore, we next investigated the role of c-JUN in the regulation of AChR gene expression using a dominant-negative (D/N) approach. The c-JUN dominant-negative mutant TAM-67 lacks amino acids 3–122 and has been shown previously to function as a potent inhibitor of c-JUN-mediated transcriptional activation (Brown et al., 1994, 1996). Because of a low transient transfection efficiency in C2C12 cells, which made it difficult to study the effect of transfected c-JUN on expression of the endogenous AChR gene, we studied the effect of TAM-67 on the promoter-driven luciferase gene expression using the transgene ϵ 416-Luc. ϵ 416-Luc contains the 416 nucleotides of the 5'-flanking region of the ϵ -subunit gene fused with the luciferase gene downstream of the transcription initiation site (Si et al., 1997). The 416 nucleotides contain *cis*-elements required for neuregulin induction in C2C12 cells (Si et al., 1997) as well as synapse-specific expression (Duclet et al., 1993, 1996). C2C12 myoblasts were cotransfected with

the transgene ϵ 416-Luc with the parental vector pCMV, wild type (WT) c-JUN, or TAM-67. The parental vector pCMV or c-JUN wild type had no effect on the neuregulin-induced ϵ -transgene expression. In contrast, the D/N mutant TAM-67 blocked the neuregulin-induced ϵ -subunit gene expression in the C2C12 cells (Fig. 6). These results argue that c-JUN is required for neuregulin-induced expression of the ϵ -subunit gene. Coexpression of the c-JUN wild-type protein decreased the basal transgene expression, probably because of repressing transcriptional activation by myogenic transcription factors. There are two E box elements within the 416 nucleotide 5'-flanking region (Si et al., 1997). Mutation of the proximal E box decreased the basal but not the neuregulin-induced expression in Sol 8 muscle cells (Chu et al., 1995). c-JUN can interact physically with MyoD (Bengal et al., 1992) and thus inhibit *trans*-activation of the muscle creatine kinase enhancer by myogenic factors (Bengal et al., 1992; Li et al., 1992).

Activation of JNK by neuregulin is required for its induction of the ϵ -subunit gene expression

The function of c-JUN is regulated by phosphorylation of the N-terminal-transactivating domain by JNK, a subgroup of MAP kinases structurally related to ERK (Foletta, 1996). We deter-

Figure 6. Dependence of the neuregulin-induced expression of the AChR ϵ -transgene on c-JUN. C2C12 myoblasts were transiently cotransfected with ϵ 416-Luc and pCMV β without (CONTROL) or with pCMV (pCMV), pCMV-c-JUN [c-JUN(WT)], or pCMV-TAM-67 [c-JUN(D/N)]. Twenty-four hours after transfection, myoblasts were incubated with the DM to induce myotube formation. After another 48 hr, myotubes were stimulated with 1 nM NRG for 24 hr. Luciferase activity and β -galactosidase activity were assayed as described in Methods and Materials. The ratios of the relative luciferase or β -galactosidase activity in neuregulin-stimulated cells over that in control cells are shown. Histograms are mean $\pm$ SD of four different samples.

mined whether neuregulin activates JNK in muscle cells. JNK was purified from control and neuregulin-stimulated C2C12 cells and assayed using a GST-c-JUN fusion protein as a substrate. Stimulation of C2C12 myotubes with neuregulin indeed increased the JNK activity in manners dependent on both concentration and time (Fig. 7). The JNK activation by neuregulin peaked at 10 min after stimulation and at 100 pM, which were $\sim$ 2.2- to 2.6-fold above the basal (Fig. 7).

To explore the role of JNK in the regulation of AChR gene expression, we studied effects of a JNK mutant on ϵ 416-Luc expression. The JNK1(ALF) mutant is kinase deficient because its tripeptide dual phosphorylation motif Thr-Pro-Tyr has been mutated to Ala-Leu-Phe and thus cannot be activated by its upstream kinase (Gupta et al., 1995). The mutant and wild-type JNK were cotransfected in C2C12 cells with ϵ 416-Luc. As shown in Figure 8, the JNK kinase-deficient mutant blocked neuregulin-induced expression of the ϵ -transgene, whereas the wild type had no apparent effect, suggesting an essential role of JNK or a related kinase in neuregulin regulation of the ϵ -subunit gene. MKK4 is a kinase upstream of JNK that phosphorylates and thus activates JNK (Natali et al., 1992; Sanchez et al., 1994). To address further the importance of JNK or a related kinase in the regulation of AChR gene expression by neuregulin, a mutant MKK4 was introduced into C2C12 cells, and its effect on neuregulin-induced expression of ϵ 416-Luc was assessed. The MKK4(Ala) mutant, in which Ser257 and Thr261 are converted to Ala residues, cannot be phosphorylated or activated and thus functions as a dominant-negative mutant of MKK4 (Whitmarsh et al., 1995). Overexpression of MKK4 inhibits activation of JNK in mammalian cells (Whitmarsh et al., 1995). When it was cotransfected with ϵ 416-Luc, MKK4(Ala) effectively inhibited neuregulin-mediated induction of the ϵ -promoter activity (Fig. 8).

In addition to JNK, p38 MAP kinase is another subgroup of

Figure 7. Activation of JNK in C2C12 myotubes by neuregulin. C2C12 myotubes were stimulated with 1 nM neuregulin for the indicated times (A) or with various concentrations of neuregulin for 10 min (B) and then harvested in the lysis buffer. To purify JNK, we incubated an aliquot of lysate with agarose beads containing the GST-c-JUN fusion protein at 4°C for 2 hr. After washing, the beads were incubated in the kinase assay buffer containing [γ - 32 P]ATP at 30°C for 15 min. The kinase reaction was stopped by the addition of the sample-loading buffer and subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Shown are means $\pm$ SD of five different samples. The JNK activity levels in the absence of neuregulin were considered to be 100%. The inset of each panel shows a representative radiogram. * p < 0.05; ** p < 0.01.

MAP kinases whose activity is activated by stress, proinflammatory cytokines, and lipopolysaccharide (Han et al., 1994; Raingeaud et al., 1995). We tested whether p38 MAP kinase is a regulator of neuregulin-mediated expression of AChR genes. P38 kinase was measured in an immune complex kinase assay using ATF2 as a substrate. Neuregulin did not appear to activate the p38 MAP kinase in C2C12 myotubes (data not shown). Treatment of C2C12 myotubes with SB202190, an inhibitor of p38 (Lee et al., 1994), had no apparent effect on neuregulin-induced expression of the ϵ -mRNA (data not shown). Coexpression of the p38 dominant-negative mutant p38(AGF) had no apparent effect

Figure 8. Requirement of JNK in neuregulin-induced AChR ϵ -transgene expression. C2C12 myoblasts were transiently transfected with ϵ 416-Luc and pCMV β with pCMV, pCMV-JNK1(WT), pCMV-JNK1(ALF), pCMV-MKK4(Glu), pCMV-MKK4(Ala), pCMV-p38(WT), or pCMV-p38(AGF). Twenty-four hours after transfection, myoblasts were incubated with the DM to induce myotube formation. After another 48 hr, myotubes were stimulated with 1 nM neuregulin for 24 hr and then harvested for assays of luciferase and β -galactosidase activity. The fold of the relative luciferase or β -galactosidase activity in neuregulin-stimulated cells over that in control cells is shown. Histograms are mean $\pm$ SD of four different samples. * $p < 0.05$.

on the ϵ -transgene transcription activity (Fig. 8). These results suggest that p38 MAP kinase may not contribute to the regulation of AChR gene expression by neuregulin.

Having demonstrated the important role of JNK in the neuregulin-induced ϵ -transgene expression, we next asked whether JNK is also a critical regulator of the expression of the endogenous ϵ -subunit gene in response to neuregulin. To address this issue, the wild type or dominant-negative mutant JNK were introduced into C2C12 cells by transfection. Both constructs were tagged with a FLAG epitope. Neomycin-resistant clones were screened for expression of JNK proteins by Western blot analysis with anti-FLAG antibodies. We have obtained C2C12 cell lines that stably expressed the wild type or mutant of JNK1. Figure 9A shows a Western blot demonstrating expression of wild type and mutant JNK1 in two representative cell lines. Similar levels of expression were obtained in other stable cell lines (data not shown). No apparent morphological changes were observed when the JNK wild-type or mutant-expressing C2C12 cells were compared with the parental cells or the cells transfected with the empty vector. Stable expression of the JNK wild type or mutant did not appear to affect differentiation from myoblasts to myotubes. We examined neuregulin-induced ϵ -mRNA expression in two cell lines that stably expressed wild-type JNK1 and four cell lines that stably expressed mutant JNK1. As shown in Figure 9B, the wild-type JNK1 had no apparent effect on neuregulin's effect; however, the dominant-negative mutant inhibited the neuregulin-induced expression of the endogenous ϵ -mRNA. A summary of three independent Northern blot analyses of parental, control (pCMV-transfected), JNK1(WT)#2, and JNK1(ALF)#1 cells is presented in Figure 9C. These results were in agreement with those with the ϵ -transgene transcription in transient expression experiments, supporting the notion that activation of JNK is required for neuregulin-induced expression of the ϵ -subunit gene.

DISCUSSION

Neuregulin-mediated expression of the AChR ϵ -subunit gene in muscle cells requires c-JUN and JNK in addition to the previously documented ERK. First, neuregulin induced expression of immediate early genes *c-jun* and *c-fos*. Expression of *c-jun* and *c-fos* by neuregulin followed and depended on ERK activation, which has been demonstrated to be essential for neuregulin-upregulated AChR gene expression. Second, inhibition of protein synthesis or blockade of the c-JUN function by a c-JUN dominant-negative mutant attenuated or abolished neuregulin-mediated transcription from the ϵ -promoter. Third, neuregulin activated JNK in muscle cells. Blockade of JNK activation by overexpressing dominant-negative MKK4 inhibited ϵ -promoter activation. Moreover, overexpression of the JNK dominant-negative mutant inhibited neuregulin-mediated expression of the ϵ -transgene and endogenous ϵ -mRNA in muscle cells. These results suggest that neuregulin activates multiple signaling cascades that converge to regulate AChR subunit gene expression.

In contrast to the transient induction of immediate early genes like *c-fos* and *c-jun*, the induction of the ϵ -mRNA was delayed and persisted after neuregulin treatment in C2C12 myotubes. This time course is similar to that of other delayed-response gene products such as VGF (Levi et al., 1985; Salton et al., 1991), GAP43 (Federoff et al., 1988), transin (Machida et al., 1989), and COS-1 (Kaplan et al., 1997) whose expression is induced by NGF or basic FGF (bFGF) in pheochromocytoma 12 cells. Expression of these messages is also inhibited by cycloheximide. The mechanisms concerning how these delayed-response genes are regulated remain primarily unknown. NGF and bFGF both cause a prolonged activation of Ras resulting in a prolonged increase of ERK activity (Kaplan et al., 1997). The prolonged ERK activation leads to the phosphorylation of the cAMP regulatory element-binding protein for up to several hours that, along with immediate early gene products, leads to subsequent delayed-response gene expression (Bonni et al., 1995; Segal and Greenberg, 1996). The regulatory mechanisms of the AChR ϵ -subunit gene may be different from those induced by NGF or bFGF because neuregulin activated ERK transiently (Fig. 2).

The c-JUN and c-FOS proteins are the cellular homologs of viral oncogenes. The c-JUN proteins are able to dimerize among themselves or form a heterodimer with c-FOS, whereas c-FOS has to dimerize with c-JUN to form a dimer. These homo- or heterodimers are major components of the activator protein-1 (AP-1) transcription factor (Rauscher et al., 1989; Angel and Karin, 1991; Foletta, 1996). AP-1 plays a role in the regulation of both basal and inducible transcription of various genes in response to growth factors, cytokines, tumor promoters, and carcinogens. c-JUN may directly regulate neuregulin-induced AChR gene expression by binding to the promoter region of the ϵ -subunit gene. That c-JUN affects the neuregulin-induced ϵ 416-Luc transgene expression in C2C12 myotubes may suggest that the element(s) required for the c-JUN effects, either direct or indirect, lies within the 416 nucleotides in the 5'-flanking region of the ϵ -subunit gene. A careful examination that failed to identify the palindromic sequence 5'TGAg/cTCA, the consensus binding site for AP-1, in this region does not support the notion that c-JUN regulates AChR gene expression by binding to a typical AP-1 element in the 5'-flanking region. An alternative hypothesis is that c-JUN interacts with other transcription factors to regulate AChR gene expression and thus does not require a direct interaction with a *cis*-element.

Figure 9. Attenuation of neuregulin-induced expression of endogenous ϵ -mRNA by dominant-negative JNK1. C2C12 myoblasts were transfected with pCMV, pCMV-JNK1(WT), or pCMV-JNK1(ALF). All DNA constructs contained the G418-resistant gene *neo*. The FLAG epitope was tagged between the codons 1 and 2 of JNK. G418-resistant clones were isolated and characterized for JNK1 expression and AChR gene expression in response to NRG. **A**, Western blot analysis showing expression of wild-type and mutant JNK1 in the parental and stable cell lines JNK1(WT)#2 and JNK1(ALF)#1. Forty micrograms of proteins extracted from the indicated myotubes were resolved on a 10% SDS-PAGE gel, transferred, and blotted with anti-FLAG antibodies. Similar levels of JNK1 expression were observed in other stable cell lines described in **B**. **B**, Northern blot analysis. Twenty micrograms of total RNA were used. The loading was uniform as evidenced by an equal amount of GAPDH mRNA. **C**, Histograms showing mean $\pm$ SD of three different samples. The mRNA level of the parental cell line in the absence of neuregulin was considered to be 100%. $^{**}p < 0.01$ in comparison with the neuregulin-mediated increase in parental, pCMV, or JNK1(WT)#2 cells.

The ETS family of transcription factors may be candidate factors whose function is regulated by c-JUN. Recent studies indicate that ETS2 and GABP, both members of the ETS family, are required for neuregulin-induced and synapse-specific expression of the ϵ -subunit gene (Fromm and Burden, 1998; Sapru et al., 1998; Schaeffer et al., 1998). In a recent report, ETS2 has been found to associate strongly with the c-JUN–c-FOS dimer, and such an interaction is enhanced by promoter DNA containing the ets sites but does not require the AP-1 site (Basuyaux et al., 1997). Thus, c-JUN may regulate the ϵ -promoter transcription via interacting with the ETS family of proteins. It is worth noting that overexpression of the wild-type c-JUN or the MKK4 or JNK active mutant did not increase the basal level of ϵ -promoter transcription, suggesting that c-JUN, MKK4, or JNK is not sufficient to upregulate AChR gene expression. Previous studies have demonstrated that angiotensin II stimulates AP-1-driven transcription and c-JUN–c-FOS heterodimer DNA-binding activity in C2C12 cells (Puri et al., 1995). Treatment of C2C12 myotubes with angiotensin II had no effect on the basal or neuregulin-upregulated expression of the AChR ϵ -subunit (data not shown). These results suggest that c-JUN and/or c-FOS and JNK are required for but not sufficient to mediate the upregulation of AChR gene expression by neuregulin. It is also possible that c-JUN activates transcription of another factor that in turn activates ϵ -subunit gene expression.

Synaptic nuclei continue to express the AChR ϵ -subunit many weeks after the nerve has been removed (Brenner et al., 1990), suggesting an imprint signal capable of inducing AChR expression in the absence of the nerve. This imprint signal is believed to be neuregulin concentrated in the extracellular matrix at the neuromuscular junction. However, denervation decreases dramatically the neuregulin-like immunoreactivity at the neuromuscular junction (Sandrock et al., 1995). Yet the accumulation of AChR mRNA, especially the ϵ -mRNA, is not dependent on the continued presence of the nerve terminal (Goldman and Staple, 1989; Brenner et al., 1990). Our observation of the sustained increase in the AChR mRNA by a brief neuregulin stimulation supports the “imprinting” hypothesis. Clearly, the elevation was not caused by a continuous activation of the ErbB kinases because their activity, monitored by tyrosine phosphorylation, decreased within 15 min after and returned to the basal level within 60 min of neuregulin stimulation (Si et al., 1996). Concomitantly, the ERK kinase activation was transient by either continuous or transient stimulation of C2C12 cells with neuregulin. A simple explanation of this phenomenon is that the AChR mRNA may be stable and, after being synthesized, remains intact in the muscle cells for a longer period of time. Although this possibility cannot be ruled out in the present study, the fact that actinomycin D, an inhibitor of transcription, inhibited the neuregulin-elevated AChR mRNAs suggests a role of continuous active transcription

in the maintenance of the AChR mRNA. Presumably, the intracellular neuregulin pathway, after being activated, may remain functional without further stimulation from extracellular neuregulin. The prolonged transcription should be mediated by a covalent modulation of transcription regulators in nuclei because the activation of ERK by neuregulin was transient and the phosphorylated MAP kinases were quickly translocated into nuclei in C2C12 myotubes (Si and Mei, unpublished observation). Potential candidate transcription factors are members of the ETS family, whose phosphorylation regulates expression of AChR genes (Sapru et al., 1998; Schaeffer et al., 1998). Alternatively and/or probably in addition, neuregulin may activate novel signaling events to sustain the transcription of AChR genes. The finding that c-JUN and its phosphorylation are required for neuregulin-upregulated expression of the AChR ϵ -subunit gene suggests that the immediate early gene products play a role in maintenance of the AChR ϵ -mRNA in the absence of neuregulin.

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