Neuregulin1 signaling targets SRF and CREB and activates the muscle spindle-specific gene Egr3 through a composite SRF–CREB-binding site

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**Abstract**

Muscle spindles are sensory receptors embedded within muscle that detect changes in muscle length. Each spindle is composed of specialized muscle fibers, known as intrafusal muscle fibers, along with the endings of axons from sensory neurons that innervate these muscle fibers. Formation of muscle spindles requires neuregulin1 (NRG1), which is released by sensory axons, activating ErbB receptors in muscle cells that are contacted. In muscle cells, the transcription factor Egr3 is transcriptionally induced by NRG1, which in turn activates various target genes involved in forming the intrafusal fibers of muscle spindles. The signaling relay within the NRG1–ErbB pathway that acts to induce Egr3 is presumably critical for muscle spindle formation but for the most part has not been determined. In the current studies, we examined, using cultured muscle cells, transcriptional regulatory mechanisms by which Egr3 responds to NRG1. We identified a composite regulatory element for the Egr3 gene, consisting adjacent sites that bind cAMP response element binding protein (CREB) and serum response factor (SRF), with a role in NRG1 responsiveness. The SRF element also influences Egr3 basal expression in unstimulated myotubes, and in the absence of the SRF element, the CREB element influences basal expression. We show that NRG1 signaling, to target SRF, acts on the SRF coactivators myocardian-related transcription factor (MRTF)-A and MRTF-B, which are known to activate SRF-mediated transcription, by stimulating their translocation from the cytoplasm to the nucleus. CREB is phosphorylated, which is known to contribute to its activation, in response to NRG1. These results suggest that NRG1 induces expression of the muscle spindle-specific gene Egr3 by stimulating the transcriptional activity of CREB and SRF.

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**Introduction**

Muscle spindles are sensory receptors embedded within muscle that detect changes in muscle length. Muscle spindles convey length information to the CNS through their associated sensory neurons. This information is processed in the brain to help sense positions of limbs and other body parts. Muscle spindles also participate in stretch reflexes, which involves the direct action of their innervating sensory neurons on motor neurons to resist muscle stretch.
Each muscle spindle is composed of specialized muscle fibers, known as intrafusal muscle fibers, along with the endings of axons from proprioceptive sensory neurons that innervate these fibers. Within the spindle, several intrafusal fibers are arranged in parallel with other muscle fibers and are encapsulated by connective tissue. These fibers have distinctive characteristics, including their morphology and gene expression pattern, which includes having distinctive isoforms of myosin heavy chain [1]. Intrafusal muscle fibers are innervated by axons, also known as afferent fibers, of sensory neurons. The endings of these fibers spiral around and terminate on central portions of intrafusal fibers and contain stretch-sensitive ion channels for performing sensory function. Because intrafusal muscle fibers are embedded in parallel to other muscle fibers, the length of intrafusal fibers changes in conjunction with the entire muscle.

Formation of muscle spindles involves reciprocal interactions between developing muscle fibers and proprioceptive sensory afferents that contact them [2]. Specification of a muscle fiber to develop into an intrafusal fiber of a muscle spindle requires inductive interactions of sensory afferent endings with developing muscle fibers [3]. Indeed, in mice lacking sensory neurons due to loss of signaling by particular neurotrophic factors, muscle spindles fail to develop [4,5].

According to mouse genetic approaches, the NRG1–ErbB signaling pathway is a critical component of the inductive interactions between sensory axons and muscle. NRG1, a secreted signaling protein, is produced by various neurons including proprioceptive sensory neurons and released from axons. In mice in which NRG1 is inactivated in a variety of neurons including sensory neurons that innervate muscle spindles, muscle spindles fail to develop even though sensory neurons form normally and contact muscle fibers [6]. ErbB2, which is a key component of the NRG1 receptor, is present in muscle fibers. Likewise, in mice lacking ErbB2 in skeletal muscle, muscle spindles fail to form [7,8]. Further, in cultured muscle cells, NRG1 stimulates expression of various genes associated with intrafusal fibers including the characteristic myosin heavy chain isoforms [9]. Activated ErbB receptors are likely directly linked to ShcA, a docking protein that interacts directly with activated receptor tyrosine kinases, including ErbB receptors, based on mouse genetic studies showing that spindle formation requires ShcA in skeletal muscle [10]. Several specific domains of ShcA were found to be required, including those that interact with activated receptors and one that interacts with the adaptor Grb2, suggesting that in developing spindles, signaling by activated ErbB receptors is linked to recruitment of ShcA, which in turn binds to Grb2. Based on these various studies on NRG1, ErbB2, and ShcA, when a proprioceptive sensory axon contacts a developing muscle fiber, NRG1 that is released by the axon activates ErbB receptors in the muscle fiber that is contacted. Activation of ErbB receptors, likely through ShcA and Grb2 to which they are linked, then initiates an intracellular signaling pathway that ultimately contributes to that muscle fiber forming into an intrafusal fiber of a muscle spindle.

A critical component of the intracellular pathway that acts downstream of ErbBs is the transcription factor Egr3, which is transcriptionally induced as part of NRG1–ErbB signaling and in turn activates various target genes involved in formation of muscle spindles. Egr3 is a member of a family of transcription factors, whose original member was identified as an immediate early gene that is induced upon growth factor stimulation [11]. Expression of Egr3 is induced in developing intrafusal fibers following innervation by sensory axons but is not detected in extrafusal fibers, which do not receive sensory innervation [12]. Induction of Egr3 occurs as part of the NRG1–ErbB pathway because Egr3 expression is induced in cultured primary human myotubes following stimulation with NRG1 [9], and in mice in which either NRG1 or ErbB2 is disrupted, Egr3 induction in muscle fails to occur [6,8]. Egr3 is required to form muscle spindles because in mice lacking Egr3, muscle spindles fail to develop [12]. Further, forced expression of Egr3 throughout the muscle induces expression of intrafusal fiber-specific genes and converts all muscle fibers to intrafusal fibers [13]. Thus, NRG1 acts, at least in part, by inducing transcription of Egr3, which in turn activates various target genes involved in formation of muscle spindles.

The signaling relay within the NRG1–ErbB pathway that acts to induce Egr3 is presumably critical for muscle spindle formation but for the most part has not been determined. In the current studies, we have examined, using cultured muscle cells, the signaling relay that acts upstream of Egr3 by identifying transcriptional regulatory elements that are required for inducing transcription of Egr3 in response to NRG1 and determining how the transcription factors that bind to these regulatory elements are acted upon by the NRG1–ErbB pathway. We demonstrate that adjacent binding sites for CREB and for SRF are each involved with transcriptional induction of the Egr3 gene by NRG1. We show that CREB and SRF are each targeted during NRG1 signaling in ways that presumably could stimulate their transcriptional activity, suggesting that NRG1 induces expression of the muscle spindle-specific gene Egr3 by stimulating the transcriptional activity of CREB and SRF.

Material and methods

Cell culture

C2C12 myoblasts were propagated using growth media (DMEM, 10% bovine growth serum (Hyclone), 50 µg/ml gentamycin). To induce differentiation into myotubes, cells were grown to confluency and the media was replaced with differentiation media (DMEM, 4% horse serum, 50 µg/ml gentamycin).

Transcriptional reporter assay

An Egr3 (+/−2173/+87)-human growth hormone (hGH) gene fusion was produced by inserting Egr3 sequences from a mouse Egr3 genomic clone (pJD2786, from Jeff Milbrandt) into the plasmid p0GH [14]. 5’ deletions were generated by cutting Egr3 (+/−2173/+87)-hGH with HindIII and either EagI, HincII, NgoMIV, or BstElI, followed by blunt-ending with klenow and circularizing plasmid by self-ligation. PCR-based mutagenesis was used to introduce point mutations into Egr3 gene sequences: CREF-56, TACGCTA→TACctaaA; CREB-105, TACACGT→TttaGTC; SRE-71, CCATATGG→CcagCATAGG; Ets-145, −228, and −457, TTCTC→TTggT; Ets-443 and −30, AGGAA→AccAA.

C2C12 cells in growth media were seeded at 4 × 10⁵ cells onto 35 mm dishes and transacted 24 h later using Lipofectamine 2000 (Invitrogen). Twenty-four hours following transfection,
cells were placed in differentiation media and re-fed every 1–2 days. For experiments in which induction of the reporter by NRG1 would be measured, each dish was transfected using 1 μg Egr3-hGH plasmid and 2 μl Lipofectamine. Three days following initial switch to differentiation media, media was removed and subsequently used for measuring hGH levels by ELISA (Roche). The relative hGH levels at this time point, within each set of dishes transfected with the same Egr3-hGH plasmid, provide a measure of transfection efficiency. For experiments in which induction of hGH protein by NRG1 would be measured, three days following initial switch to differentiation media, cells were placed in differentiation media containing 1% horse serum either with or without NRG1 (HRGβ1, R&D Systems, 100 ng/ml), and 20 h later media was removed and subsequently used for measuring hGH levels. For experiments in which induction of hGH mRNA by NRG1 would be measured, four days following initial switch to differentiation media, cells were serum-starved for 2 h and either stimulated with NRG1 or left untreated. Two hours later, RNA was isolated from cells using RNA Stat-60 (Tel-Test) and subsequently used for measuring hGH mRNA levels by quantitative reverse-transcriptase PCR (qRT-PCR). The fold-difference in hGH protein or mRNA expression between NRG1-stimulated and untreated dishes was normalized to transfection efficiency. For experiments in which basal expression of the reporter would be measured, each dish was transfected using 3 μg Egr3-hGH plasmid, 1 μg secreted alkaline phosphatase-expressing plasmid, RSV-SEAP [15], and 8 μl Lipofectamine. Three days following initial switch to differentiation media, cells were placed in differentiation media containing 1% horse serum, and 20 h later media was removed and subsequently used for measuring SEAP levels by chemiluminescence substrate kit (Clontech) and hGH levels. hGH expression from each of the Egr3-hGH plasmids was normalized to levels of SEAP.

**qRT-PCR**

For experiments in which endogenous Egr3 expression would be analyzed, C2C12 myotubes that had been in differentiation media for four days were serum starved for 2 h and either stimulated with NRG1 or left untreated, and 2 h later, RNA was isolated from cells using RNA Stat-60 (Tel-Test). DD-A-CREB-containing C2C12 myotubes [16], after three days in differentiation media, were either stimulated with Shld1 (Clontech, 1 μM) for 24 h or left untreated. For experiments in which AChR ε subunit expression would be analyzed, C2C12 myotubes that had been in differentiation media for four days cells were placed in differentiation media containing 1% horse serum either with or without NRG1, and 24 h later RNA was isolated from cells. Cycloheximide (Sigma) at 10 μg/ml was added 30 min prior to NRG-1 stimulation and remained throughout NRG-1 treatment.

Reverse transcription reactions were performed using AffinityScript cDNA Synthesis Kit (Stratagene) with total RNA (5 μg from cells that were transfected with hGH reporter, 2 μg from cells that were not transfected) and random primers. qPCR was performed using Brilliant SYBR Green QPCR Core Reagent Kit (Stratagene) [17], with reverse transcription reactions (1.67 μl for Egr3 analysis, 2.5 μl for hGH mRNA analysis), and primers for Egr3: GCCCATACATACAGATGGCT (forward), GCTGGAATAAGAGTTCGGA (reverse) or for hGH: CACCTACAGGAGTGGTGGAA (forward), GGTGTCCGAATAGACTTCAGA (reverse). qPCR was performed as described [17] for analysis of x1 actin, which was used to normalize expression of other genes, and of AChR ε subunit.

**Electrophoretic mobility shift assay (EMSA)**

Nuclear extracts were prepared [18] from C2C12 myotubes that had been in differentiation media for four days. For isolation of whole-cell extracts, C2C12 myotubes that had been in differentiation media for four days were serum starved for 2 h and either stimulated with NRG1 or left untreated, and myotubes were lysed using 1% Triton X-100, 150 mM NaCl, 20 mM Heps, (pH 7.5), 10% Glycerol, 0.2 mM EDTA, 1.5 mM MgCl2, 1% protease inhibitor cocktail (Sigma), 10 mM NaF, 30 mM Na2P2O7, 1 mM Na3VO4. Binding reactions contained 5 μg nuclear extract or 12 μg whole-cell extract, 25 mM Heps (pH 7.5), 50 mM KCl, 4 mM MgCl2, 5% glycerol, 0.1% Igepal CA-630, 3.0 mM DTT, 0.25% Tween 20, 75 μg/ml poly(dI–dC), and 50 fmol double-stranded oligonucleotide probe labeled with IRDye 700 (Integrated DNA Technologies) in a total volume of 20 μl. Some reactions also contained unlabeled competitor DNA. Reactions were initially set up that contained all components except probe and placed at room temperature for 10 min. Probe was then added and the reactions were placed for an additional 15 min at room temperature and placed on ice for 5 min. For binding reactions containing antibodies, an antibody to CREB, 4 μg (Santa Cruz Biotechnology, sc-271), SRF, 2 μg (Santa Cruz Biotechnology, sc335), or Egr1, 4 μg (Santa Cruz Biotechnology, sc-189), or rabbit IgG, 1 μg was incubated with nuclear extract for 1 h on ice prior to addition of other components. Complexes were resolved by electrophoresis (1.5 h at 150 V) in a 5% polyacrylamide gel (0.5 × Tris–borate–EDTA), and gels were imaged using the Odyssey Infrared Imaging System (Li-Cor).

**Chromatin immunoprecipitation (ChIP)**

C2C12 myotubes that had been in differentiation for four days were used. Formaldehyde was added to media to 1% with 10 min incubation, followed by addition of glycine to 125 mM with 5 min incubation. Cells were scraped in PBS, spun down, and resuspended in lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS) containing 1% protease inhibitor cocktail (Sigma) at 1.25 ml per 15 cm dish. Cell extract was sonicated for approximately 10 min using Branson Sonifier 450 with microtip, and cell debris was removed by brief centrifugation. Cell extract was pre-cleared using protein A-agarose that had been washed with lysis buffer and absorbed with single stranded herring sperm DNA to 75 ng/μl and BSA to 0.1 μg/ml. Antibodies to CREB, 10 μl (Cell Signaling Technology, 9197), SRF, 12 μg (Santa Cruz Biotechnology, sc335), or HA-Tag, 10 μl (Cell Signaling Technology, 3724) were added to 250 μl cell extract and incubated overnight at 4 °C. Absorbed protein A-agarose was added (40 μl of 50% slurry) and incubated for 1–2 h at 4 °C with rotation. Protein A-agarose beads were spun down, washed (for 5 min each using 1 ml) three times with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl pH 8.0 followed by two times with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris–HCl pH 8.0, and eluted with 400 μl 1% SDS, 100 mM NaHCO3 at 37 °C for 15 min with rotation. Proteinase K (0.1 mg) was added for 10 min at 50 °C, and the immune complex was purified using magnetic beads (Dynal).
added with overnight incubation at 65 °C. Samples were phenol:
chloroform extracted, ethanol precipitated in the presence
of 50 μg glycogen, and resuspended in 100 μl 10 mM Tris–HCl,
pH 8.0, 1 mM EDTA. qPCR was performed using Brilliant II SYBR
Green PCR Master Mix (Stratagene) with 5 μl sample and
primers spanning —151 to —46 of Egr3 gene: GCCCGCTTCTC-
TGTTTCTTAAT (forward), TCCGTGACGTAGCTGCCCATAT (reverse)
or 107 bp of an intergenic region: GCTGTGGATGAGCAATGGTTG
TGCTTTCTAAT (forward), TCCGTGACGTAGCTGCCCATAT (reverse).

Western blotting

C2C12 myotubes that had been in differentiation media for four
days were serum starved for 2 h and either stimulated with
NRG1 for 10 min or left untreated. DD-A-CREB-containing C2C12
myotubes [16] that had been in differentiation media for three
days were either stimulated with Shld1 (Clontech, 1 μM) for 24 h
or left untreated. Myotubes were lysed using 50 mM Tris–HCl,
(pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium
deoxycholate, 0.4 mM EDTA, 1% protease inhibitor cocktail
(Sigma), and for myotubes being used to analyze phosphory-
lated CREB, phosophatase inhibitors (10 mM NaF, 30 mM Na3P04,
1 mM Na2VO4). Proteins from cell extracts were analyzed by
Western blotting (Fromm and Rhodes, 2004) using an antibody
to serine 133 phosphorylated CREB (Cell Signaling Technology
4276), diluted 1:1000 or FLAG (Sigma, M2) at 2.5 μg/ml, along
with peroxidase-conjugated secondary antibodies, or by staining
with 0.1% Coomassie Brilliant Blue in 50% methanol, destaining
with 50% methanol, 10% acetic acid.

Immunofluorescence staining of cells

C2C12 myotubes that had been in differentiation media for four
days were serum starved for 2 h and either stimulated with
NRG1 for 10 min or left untreated. Cells were fixed with 4%
paraformaldehyde in PBS for 10 min, permeabilized with 0.2%
 Triton X-100 in PBS for 10 min, and blocked with 1% bovine
serum albumin in PBS for 30 min. Cells were probed with
antibodies to MRTF-A (Santa Cruz Biotechnology, sc-21558) or
MRTF-B (Santa Cruz Biotechnology, sc-47282), each diluted
1:100, for 1 h, followed by Alexa Fluor 555 anti-goat IgG (Life
Technologies, A21428), diluted 1:500, for 30 min, and counter-
stained with 300 nM DAPI for 5 min. Stained cells were visua-
lized using a LSM 5 Pa Confocal Microscope (Zeiss).

Results

SRF and CREB elements are involved in NRG1-induced
and basal Egr3 transcription

We used C2C12 cells, a skeletal muscle cell line in which Egr3
expression is induced by NRG1, to identify gene regulatory
elements and associated transcription factors involved in NRG1
responsiveness of Egr3. To identify transcriptional regulatory
elements that are functional, we used a transcriptional reporter
assay with C2C12 cells. For this assay, we generated Egr3-hGH
gene fusions using potential Egr3 transcriptional regulatory
sequences. We then transfected C2C12 muscle cells with these
gene fusions and measured the amount of hGH expression from
untreated or NRG1-stimulated myotubes. We initially performed
a transcriptional reporter assay using 2.2 kb of upstream flanking
sequence from the Egr3 gene to determine if this portion of the 5’
flanking region is able to confer NRG1 responsiveness, or if other
distal regions are required. As shown in Fig. 1, NRG1
induces expression of the hGH transcriptional reporter driven by
Egr3 upstream sequences (4-fold), indicating that regulatory
elements contained within the 2.2 kb upstream region confer a
transcriptional response. To more precisely define the location of
regulatory elements that confer NRG1 responsiveness, we gen-

Fig. 1 – In the Egr3 gene, a region extending to 0.5 kb
upstream from the transcriptional start site confers NRG1
responsiveness. C2C12 myotubes, which had been transfect-
ected with gene fusions consisting of the indicated sequences from
the Egr3 gene and hGH coding sequences, were treated with
NRG1 and the levels of hGH in the culture media were
measured. For each type of transfected DNA, the fold-
induction of hGH levels from NRG1 treatment, after
normalizing to transfection efficiency, is shown relative to
untreated sample (mean induction ± SEM, n ≥ 3 for each).
themselves being transcriptionally induced in response to NRG1. We examined the transcriptional response of Egr3 to NRG1 in myotubes that had been treated with cyclohexamide, an inhibitor of protein synthesis. As shown in Fig. 2, when new protein synthesis is inhibited, NRG1 still induces transcription of Egr3. Thus, the mechanism for inducing Egr3 transcription presumably is through activation of existing latent factors by postranslational modification.

Among all characterized transcription factors, a limited subset is known to be activated primarily by a postranslational process and a subset of these in response to extracellular ligands that activate signaling pathways, including receptor tyrosine kinase pathways [20]. For transcription factors thought to be able to function in this way, we examined the Egr3 gene 469 bp upstream region for any potential binding sites using two independent sequence analysis tools [21,22]. Within the 469 bp upstream region, we found the strongest evidence for the presence of binding sites for three classes of transcription factors: SRF, the Ets family, and the CREB family. To determine the roles of these potential binding sites in mediating a transcriptional response to NRG1, we generated Egr3-hGH gene fusions containing point mutations within these elements and used these gene fusions in transcriptional reporter assays. We introduced mutations within the context of the 2.2 kb upstream region because among the upstream segments that we tested, this region exhibited the strongest response to NRG1. In these transcriptional reporter assays, in addition to measuring hGH secreted protein, in some cases we further examined hGH mRNA, with the idea being that if a particular mutation does indeed reduce the relative rate of transcription between NRG1-stimulated and untreated cells, hGH mRNA, being less stable than protein, might more robustly reflect reduced transcription.

A single element that conforms to a binding site for SRF, also referred to as a CARG box, is present within the 469 bp upstream region, at −71, which is within the highly conserved upstream region. As shown in Fig. 3, mutation to this element caused the transcriptional response of the reporter to NRG1 to be reduced. Based on hGH mRNA levels, NRG1 responsiveness was decreased by approximately 55% (Fig. 3A). A reduction, albeit more modest, was also seen by examining hGH protein levels (Fig. 3B). Distal to the 469 bp upstream region, another element conforming to an SRF binding site is present, at −2028. Because the effects of mutations were being examined within the 2.2 kb upstream context, we considered whether this element might function redundantly along with the −71 element. Mutation to the −2028 SRF element, together with the −71 element, did not further reduce NRG1 responsiveness (data not shown), suggesting that for SRF elements, only the proximal one at −71 is involved in transcriptional induction of Egr3 by NRG1.

Within the 469 bp upstream region, several potential binding sites for Ets proteins are present. One element, which is present within the highly conserved upstream region, at −145, conforms to an Ets site, based on the sequence analysis tools that we used. Four additional sequence motifs, located at −228, −443, −457, and −30, each of which conforms to the core Ets site consensus sequence but was not recognized using the sequence analysis tools, possibly because the sequences surrounding the core consensus at these locations are less favorable, may represent potential sites, although the evidence is not as strong. In order to perform the most comprehensive assessment of the role for Ets proteins, we mutated together all five potential sites. As shown in Fig. 3C, mutation of these elements in the 2.2 kb upstream context did not reduce the transcriptional response of the reporter to NRG1, and instead increased NRG1 responsiveness by about 30%. In the 2.2 kb upstream region, many additional potential Ets sites are present distal to the 469 bp upstream region [21 based on conforming to minimal core sequence], some of which could in principle function redundantly along with the more proximal ones. Because it would be very time consuming to mutate all these elements to address their possible redundancy, we instead performed transcriptional reporter assays with mutated Ets sites in the 469 bp upstream context, whereby all five potential Ets sites in this region were mutated together. As shown in Fig. 3D, mutation of these elements in the 469 bp upstream context did not reduce the transcriptional response of the reporter to NRG1, confirming that transcriptional induction of Egr3 by NRG1 does not involve Ets binding sites.

Two elements that conform to binding sites for the CREB family are present within the 469 bp upstream region, at −56 and −105, both of which are within the highly conserved upstream region. As shown in Fig. 3, mutation of these two elements together caused the transcriptional response of the reporter to NRG1 to be reduced. Based on hGH mRNA levels,
NRG1 responsiveness was decreased by approximately 45% (Fig. 3A). By examining hGH protein levels, a reduction from mutating both these elements, albeit more modest, was also seen (Fig. 3B). We found, with hGH protein levels, that the reduction in NRG1 responsiveness caused by mutation of the potential CREB sites was more pronounced when the normal and mutated sites were compared in a context in which functional Ets elements were lacking, which, as discussed above, by itself increases NRG1 responsiveness. As shown in Fig. 3C, in this context, mutating the potential CREB sites caused a decrease in NRG1 responsiveness of approximately 45%. We examined the roles of individual CREB elements and, as shown in Fig. 3B, found that mutation of only the CREB-56 element caused a reduction in NRG1 responsiveness that was the same as having both CREB elements mutated, suggesting that for the CREB elements, only CREB-56 is involved in NRG1 responsiveness. Thus, two adjacent elements, SRE-71 and CREB-56, are each involved with inducing Egr3 transcription in response to NRG1.
Fig. 4 – The SRF element influences Egr3 basal expression in unstimulated myotubes, and in the absence of the SRF element, the CREB element influences basal expression. In C2C12 myotubes, which had been transfected with Egr3-2173/+87 hGH gene fusions containing mutations within the indicated elements of the Egr3 gene, the levels of hGH in the culture media were measured. For each type of transfected DNA, the hGH level, after normalizing to transfection efficiency, is shown relative to transfected DNA with normal Egr3 sequence (mean expression ± SEM, n = 2). Mutation of the SRF element reduces basal expression by 30-fold. While mutation of CREB elements by themselves actually increases basal expression, mutation of CREB elements, when SRF is also mutated, reduces expression by an additional 14–38 fold. In contrast, mutation of five potential Ets sites together, located at −145, −228, −443, −457, +30, reduces basal expression by only 2.5-fold.

Although we were primarily interested in determining from transcriptional reporter assays if mutations to particular regulatory elements affect the amount by which treatment with NRG1 increases Egr3 transcription, we noticed that SRF and CREB elements substantially influence absolute, basal Egr3 expression level in muscle cells. As shown in Fig. 4A, mutation of the SRF −71 element caused a 30-fold reduction of expression in untreated myotubes. In the presence of normal SRF-71, the CREB-56 and −105 elements are not required for basal expression, with mutation of them actually increasing basal expression approximately 2.5 fold (Fig. 4A). In contrast, when the SRF-71 element was mutated, the reduced expression that remains was dependent on CREB elements, especially the CREB-56 element. As shown in Fig. 4A in the SRF mutated situation, further mutation of CREB-56 caused an additional 14-fold reduction in basal expression level. Further mutation of CREB −105 together with −56 reduced basal expression by an additional 2–3-fold beyond mutating −56. In contrast to effects of mutating SRF and CREB elements, mutation of all five Ets elements that we examined reduced basal expression by a relatively modest 2.5-fold (Fig. 4B). Thus, the adjacent SRF-71 and CREB-56 elements in the Egr3 gene, in addition to having roles in NRG1 responsiveness, also are involved with basal muscle expression. When both SRF-71 and CREB-56 were mutated together, the very low basal expression level, which was barely detectable, precluded determining how mutating these sites together might affect NRG1 responsiveness because the overall expression level was too low to be able to reliably access the degree to which NRG1 treatment might increase expression.

SRF and CREB bind to adjacent sites in the regulatory region of the Egr3 gene

We used an EMSA to identify proteins in muscle cells that might interact with the regulatory region in the Egr3 gene that contains adjacent SRF and CREB elements. For these experiments, we prepared nuclear extracts from myotubes and evaluated protein binding to a labeled oligonucleotide probe corresponding to nucleotides −80 to −41 of the Egr3 gene, which encompasses the adjacent CREB and SRF elements. As shown in Fig. 5, proteins from myotube nuclear extracts bind to the −80 to −41 region. Multiple protein-DNA complexes were detected, visualized as four distinct bands. The bands migrated as two clusters, with each cluster containing a doublet of closely-migrating bands.

To evaluate the DNA binding specificity of proteins in the various complexes, we included excess unlabeled competitor oligonucleotides in the EMSA. We used normal −80 to −41 as a competitor and mutated versions containing various nucleotide substitutions in the CREB and SRF elements that we found to affect NRG1 responsiveness and basal muscle expression. For the complexes represented by the lower migrating cluster of bands, a competitor containing normal −80 to −41 sequence or one mutated specifically at the SRF element each efficiently competed for binding. In contrast, competitors mutated at the CREB element failed to compete for binding, indicating that proteins in these complexes are specific for the CREB element (Fig. 5). For the complexes represented by the upper migrating cluster of
bands, the lower band in the doublet appears to represent binding that was not specific for either the CREB or SRF elements, because normal and various mutated competitors all competed for binding with equal effectiveness (Fig. 5). Specific binding was seen with the upper band in the doublet. For the complex(es) represented by this band, a competitor containing normal −80 to −41 sequences efficiently competed for binding. Competitors mutated individually at either the SRF or CREB element each competed for binding less effectively than a competitor of normal sequence, with the competitor mutated at the SRF element appearing to inhibit with lesser effectiveness. A competitor mutated at both SRF and CREB elements failed to compete for binding (Fig. 5). Thus, this complex(es) contains proteins that are specific for the SRF and for the CREB elements, with proteins specific for the SRF element appearing to predominate.

We evaluated the protein-DNA complexes for the presence of particular transcription factors with known specificity for the CREB and SRF elements. We incubated myotube nuclear extracts with antibodies to these transcription factors and determined whether these antibodies inhibit and/or super-shift any complex. The CREB family of transcription factors is comprised of CREB, ATF1, and CREM, all of which bind to the same conserved sequence. Because CREB had been reported to be highly expressed in myotubes [23], we evaluated protein-DNA complexes for the presence of CREB. As shown in Fig. 6A, antibodies that are specific for CREB almost entirely inhibited formation of the complexes represented by the lower migrating cluster of bands and super-shifted these complexes. Thus, CREB appears to be the major, if not the only member of its family present in these protein-DNA complexes. Although competitor experiments suggested that the complex(es) represented by the upper-most band also contained proteins specific for the CREB element, CREB antibodies did not alter this complex. We also evaluated protein-DNA complexes for the presence of SRF. As shown in Fig. 6A, antibodies that are specific for SRF substantially inhibited formation of the complex(es) represented by the upper-most band, indicating that this complex(es) does indeed contain SRF. A minor portion of the upper-most band remained in the presence of SRF antibodies, suggesting the presence in smaller amounts of other proteins, which according to the competitor experiments, might be specific for the CREB element.

According to our EMSA analysis, CREB and SRF interact with a NRG1-responsive regulatory element that is located in the proximal upstream region of the Egr3 gene. To determine if CREB and SRF are binding to this region of the Egr3 gene in myotubes, we analyzed CREB and SRF binding in myotubes by ChIP. For these experiments, myotubes were fixed with formaldehyde, and chromatin in myotube cell extracts was fragmented by sonication. Antibodies specific for CREB or SRF were used for ChIP, and chromatin fragments corresponding to the Egr3 proximal upstream region were quantified by real-time PCR using primers to this region. As shown in Fig. 6B, CREB and SRF bind to the proximal upstream region of the Egr3 gene in myotubes. Because this region contains a NRG1-responsive element that binds CREB and SRF, occupancy of this region demonstrated by ChIP suggests that CREB and SRF are binding to the NRG1-responsive element in myotubes.

**NRG1 signaling targets SRF and CREB**

Based on our EMSA and ChIP analyses, CREB and SRF bind to regulatory elements that function in the transcriptional response of Egr3 to NRG1, suggesting that CREB and SRF are targeted during NRG1 signaling in a way that causes them to induce transcription. Targeting CREB or SRF potentially could either affect their DNA binding activity or their transcriptional activity.
While CREB and SRF are most often regulated at the level of their transcriptional activity, regulation of their DNA binding activity also has been shown to occur. To determine if NRG1 stimulates the DNA binding activity of CREB or SRF, we isolated whole cell extracts from NRG1-stimulated and untreated myotubes and used an EMSA to measure protein binding. As shown in Fig. 6C, NRG1 stimulation does not appear to alter the capacity of CREB and SRF to bind to their regulatory elements in the Egr3 gene, suggesting that instead NRG1 signaling targets CREB and SRF to stimulate their transcriptional activity. The transcriptional activity of both CREB and SRF can be regulated through known mechanisms in response to various signals. We examined if CREB and SRF were being regulated through such mechanisms during NRG1 signaling.

A key mechanism by which the transcriptional activity of CREB is induced in response to various signals is through phosphorylation of serine 133, which facilitates recruitment of the CREB coactivators CBP and p300, thereby enhancing transcription [24]. To determine if CREB is phosphorylated in response to NRG1, we treated myotubes with NRG1 and probed Western blots with an antibody to serine 133-phosphorylated CREB. As shown in Fig. 7A, NRG1 stimulates phosphorylation of CREB. In addition, phosphorylation of the closely related protein ATF-1, which the antibody also recognizes, is induced to a similar degree as CREB, although its absolute levels are much lower. Thus, NRG1 signaling in myotubes targets the CREB family, of which CREB is the predominant member, in a way that facilitates its transcriptional activity.

To determine directly whether the CREB family is involved in induction of Egr3 in response to NRG1, we examined NRG1 responsiveness in myotubes in which CREB family proteins were inactivated. For these experiments, we used a previously generated C2C12 cell line with an integrated, dominant negative version of CREB known as A-CREB, which heterodimerizes with endogenous CREB family proteins and prevents their binding to DNA [16]. A-CREB expression in these cells is inducible due to being fused to a ligand-regulated destabilizing domain (DD) that rapidly degrades proteins. A synthetic ligand known as Shld1 induces expression of DD-containing A-CREB (Fig. 7B) by binding to the DD and preventing degradation. As shown in Fig. 7C, A-CREB expression in these cells causes a decrease in induction of Egr3 by NRG1. The loss of NRG1 responsiveness in this system (approximately 35% decrease) is not as strong as from mutation of the CREB element in transcriptional reporter assays. This less robust effect reflects a level of Egr3 transcriptional induction in DD-A-CREB-containing C2C12 cells in the absence of Shld1 (2-fold) that is less than in normal C2C12 cells (6-fold), which might be caused by residual A-CREB expression in the absence of Shld1 (Fig. 7B) partially inhibiting CREB function or might be an artifact from the stable selection of these cells. Nevertheless, inactivation of CREB family proteins by turning on expression A-CREB causes a significant reduction in NRG1 responsiveness, indicating a role for the CREB family in NRG1 responsiveness.

The transcriptional activity of SRF is regulated in response to various signals through targeting certain coactivators that interact specifically with SRF. Signal-regulated SRF coactivators belong to two families, p62TCF and myocardian/MRTF [25,26]. The p62TCF family is not likely to be involved in Egr3 transcriptional induction because interaction of p62TCF with SRF requires DNA-binding of p62TCF to an Ets site adjacent to the SRF site, and no nearby potential Ets binding sites are recognizable (see discussion). Myocardian/MRTF family coactivators are thought to interact with SRF independently of surrounding DNA sequences. Within the myocardian/MRTF family, MRTF-A and MRTF-B are widely expressed and known to be targeted by signaling pathways. A key mechanism by which certain signals activate MRTFs is by stimulating their translocation from the cytoplasm to the nucleus [27]. To determine if NRG1 signaling targets MRTFs by this mechanism, we examined by immunofluorescence the subcellular localization of MRTF-A and MRTF-B in myotubes that had either been untreated or stimulated with NRG1. As shown in Fig. 8, in untreated myotubes, MRTF-A and MRTF-B were found largely in the cytoplasm, while in most...
nuclei, no MRTF-A or MRTF-B was found, although some nuclei had staining of similar intensity to that of the cytoplasm. The cells in which MRTF-A and MRTF-B was detected consisted mainly of fused, multinucleated cells morphologically identifiable as myotubes and generally not mononucleated cells resembling myoblasts that were also present in the culture. In myotubes stimulated with NRG1, both MRTF-A and MRTF-B were clearly concentrated in nuclei. While cytoplasmic staining was also seen, nearly every myotube nuclei that we examined in NRG1-stimulated cells exhibited staining relative to the cytoplasm that was more intense (Fig. 8). Thus, NRG1 stimulates the translocation of MRTF-A and MRTF-B to the nucleus, where presumably they could facilitate SRF-mediated transcription.

Discussion

Our results demonstrate that a composite regulatory element consisting of adjacent binding sites for CREB and for SRF is involved with transcriptional induction of the Egr3 gene by NRG1. We show that CREB and SRF are each targeted during NRG1 signaling in ways that presumably could stimulate their transcriptional activity. These results suggest that NRG1 induces expression of the muscle spindle-specific gene Egr3 by stimulating the transcriptional activity of CREB and SRF.

Although various extracellular signals have been found to act on the SRF coactivators MRTF-A and MRTF-B by stimulating their translocation from the cytoplasm to the nucleus, no previous reports have specifically described NRG1 signaling through MRTFs. Translocation of MRTFs to the nucleus in response to certain other extracellular signals occurs through activation of Rho GTPases and the resulting actin filament polymerization [27]. In these pathways, receptors interact with Rho guanine nucleotide exchange factors (GEF) to activate Rho GTPases. Rho GTPases, through several effectors, then stimulate assembly of globular G actin subunits into filamentous F actin polymers. In the non-polymerized state, G actin interacts with MRTFs, causing MRTFs to be retained in or exported to the cytoplasm. The reduction in G actin concentration resulting from Rho-mediated assembly into F actin allows MRTFs to enter the nucleus. Future experiments will address whether NRG1 stimulates MRTF translocation to the nucleus in muscle cells through a pathway involving Rho GTPases and actin filament polymerization. In support of NRG1 being able to signal via such a pathway, direct binding and activation of Rho GEF proteins by ErbB receptors has been found in other cell types [28].

In addition to the myocardian/MRTF family, SRF coactivators of the p62TCF family, which comprises the Ets proteins Elk1, Elk3, and Elk4, are also activated in response to certain signals [26]. Unlike myocardian/MRTF coactivators, which are thought to interact with DNA-bound SRF independently of surrounding DNA sequences, interaction of p62TCF with SRF requires specific DNA sequences that bind p62TCF. This interaction occurs at elements known as serum response elements (SREs), which are comprised of an Ets site that binds p62TCF adjacent to an SRF site. None of the potential Ets binding sites that we studied, which comprise the sites that are most obviously recognizable, are adjacent to the functional SRF site that we identified, and in any case, these Ets sites were found not functional for NRG1 responsiveness. Other, suboptimal Ets sites might be functional in SREs because p62TCF binding to DNA is enhanced by interaction with SRF [29]. Any suboptimal Ets sites that might bind p62TCF would presumably at a minimum contain the sequence GGA, which is invariant within the consensus sequence for binding Ets proteins, because mutations in SREs within the

![Fig. 8 - NRG1 stimulates translocation of the SRF coactivitors MRTF-A and MRTF-B from the cytoplasm to the nucleus. Myotubes that had been untreated (A,C,E,G) or stimulated with NRG1 (B,D,F,H) were fixed, permeabilized, and stained by immunofluorescence with antibodies to MRTF-A (A,B) and MRTF-B (E,F). Myotube nuclei were identified by staining with DAPI (C,D,G,H). Representative fields of cells are shown from three experiments each for MRTF-A and MRTF-B staining. Proportion is indicated of myotube nuclei that exhibit MRTF staining relative to the cytoplasm that is more intense (at least 80 nuclei examined from each condition).](image-url)
Phosphorylation of CREB at serine 133 is known, along with other events, to facilitate recruitment of the CREB coactivators CBP and p300, thereby enhancing transcription. A large number of diverse stimuli are known to induce CREB phosphorylation. In the case of signaling by receptor tyrosine kinases, a MAP kinase pathway typically acts upstream of CREB. In these pathways, the MAP kinase Erk phosphorylates and activate Mk-type kinases that in turn directly phosphorylate CREB at serine 133 [24]. NRG1-induced phosphorylation of CREB in muscle cells requires activation of Erk (manuscript in preparation), so likely CREB is being phosphorylated through the action of Erk on Msks kinases.

Because mutation of binding sites for SRF or for CREB, when each is mutated individually, reduces induction of Egr3 transcription by NRG1 and because these two sites are located adjacent to each other in the Egr3 gene, SRF and CREB might be working cooperatively together to induce transcription in response to NRG1. Other studies have supported cooperativity between SRF and CREB in genes in which they occupy adjacent binding sites, which includes fos, Egr1, and Egr2. For example, in PC12 cells, in which NGF phosphorylates CREB and induces fos transcription, phosphorylation of CREB, while necessary for transcriptional induction, was not sufficient. A binding site for SRF adjacent to the CREB site was also required [31]. Similarly, transcriptional induction of the Egr1 gene by a GM-CSF/IL-3 fusion protein in myeloid leukemic cells involves adjacent binding sites for CREB and SRF [32].

The requirement for having SRF cooperating with CREB at adjacent binding sites to respond to certain signals might be a consequence of phosphorylation of CREB not being sufficient for transcriptional induction. Because a CREB binding site is sufficient to respond to certain signals, particularly those that use cAMP as an intracellular mediator, but not to other signals, such as growth factor and stress signals, even in cases in which the different signals induce CREB phosphorylation similarly, an event in addition to CREB phosphorylation has been presumed to be necessary for CREB-mediated transcription. Gene profiling and functional studies have pointed to two distinct mechanisms for inducing CREB-mediated transcription, depending on whether or not CREB is targeted using cAMP [33]. In the case of cAMP signaling, activation is required of the CREB coactivator TORC, which associates with CBP/p300 and increases its recruitment to phosphorylated CREB. In the case of signals that do not use cAMP as an intracellular mediator, however, TORC is generally not activated. CREB target genes being induced by these signals consist mostly of genes that also contain an SRF site, which was not found on target genes induced using cAMP. For CREB-mediated transcription without cAMP, SRF binding to the SRF sites is involved in activation of target genes, although how SRF might cooperate with phosphorylated CREB is not known. NRG1 signaling is not thought to involve cAMP, so presumably TORC would not be activated, which could explain why activation of Egr3 by NRG1 involves a site for SRF in addition to CREB. Future studies will address how SRF cooperates with phosphorylated CREB in Egr3 and in other genes to induce transcription, including whether SRF facilitates recruitment of CBP/p300 or other coactivators to phosphorylated CREB. In support of the idea that SRF might be able to facilitate recruitment of CBP/p300, SRF has been found in protein complexes in association with CBP [34,35].

While mutation of the binding sites for either SRF or CREB reduces induction of Egr3 transcription by NRG1, some NR1 responsiveness, albeit at a reduced level, remains, suggesting that in the absence of SRF function or in the absence of CREB function, weaker NR1 responsiveness can be mediated by other transcriptional regulatory proteins. Additional transcriptional regulatory proteins that bind to elements outside of the composite SRF-CREB binding site might be involved in activating Egr3 transcription in response to NRG1. Alternatively, the functions of SRF and CREB in inducing Egr3 transcription in response to NRG1 might be partially redundant, such that loss of either SRF or CREB function individually only partially blocks NRG1 responsiveness.

The role of the SRF element in Egr3 basal expression is consistent with a known role of SRF in regulating gene expression in muscle, where SRF binding sites have been found to regulate a variety of other genes, including some muscle-specific genes. The CREB element only exhibits a role in regulating Egr3 basal transcription if the adjacent SRF site is mutated, while in the presence of a normal SRF element, the CREB element is not required for basal expression, with mutation actually somewhat increasing basal expression. One explanation for the interdependence of mutations to the SRF and CREB sites on Egr3 basal expression is that both SRF and CREB can recruit proteins in unstimulated myotubes that enhance Egr3 basal expression but that the recruitment facilitated by SRF is much more effective at promoting transcription than that of CREB. In this scenario, whatever interactions CREB facilitates actually seem to somewhat compete with the ability of SRF to promote transcription. When the SRF element is mutated, however, the proteins that CREB recruits are able to promote Egr3 basal expression, although to a much lesser extent than SRF.

The mechanisms by which SRF and CREB might regulate NR1-induced transcription and basal transcription of Egr3 are likely to be different because of different protein interactions. Following stimulation with NR1, MRTF-A and MRTF-B are translocated to the nucleus where they presumably can interact with SRF, while CREB becomes phosphorylated, which presumably facilitates recruitment of CBP/p300. In unstimulated myotubes, MRTF-A and MRTF-B presumably are not interacting with SRF, while CREB is in an unphosphorylated state and presumably not recruiting CBP/p300, yet binding sites for SRF and CREB are influencing expression. Thus, other protein interactions with SRF and CREB presumably are occurring in unstimulated myotubes. Both SRF and CREB have been found to exhibit interactions, which are not known to depend on their activation by extracellular signals, with a variety of other proteins. SRF can interact with several widely-used transcriptional coactivators, including CBP/p300, activating signal cointegrator, and steroid receptor coactivator [34–37]. CREB contains a constitutive activation domain, which is distinct from the activation domain that is regulated by phosphorylation, that can interact with certain general
transcription factors and mediate recruitment of components of the transcription initiation complex [38]. Future studies will compare proteins that associate with SRF and CREB in unstimulated and in NRG1 treated myotubes.

Our studies pointing to roles for SRF and CREB in activating Egr3 transcription in cultured myotubes by NRG1 suggest that SRF and CREB might function in developing muscle spindles by inducing expression of Egr3 in intrafusal muscle fibers. In nascent intrafusal fibers, activation of Egr3 by NRG1 begins during embryonic development when proprioceptive sensory afferents that release NRG1 contact certain myotubes. Our studies in which NRG1 is added to cultured C2C12 myotubes were designed to model this developmental process, but it is not clear precisely how well the cultured myotubes that we used mimic the embryonic myotubes that are being induced by NRG1 in vivo. While C2C12 myotubes certainly have many molecular and morphological similarities to myotubes in vivo, it is nonetheless possible that the molecular mechanisms by which NRG1 induces Egr3 transcription could differ between myotubes of the C2C12 line and those being induced by NRG1 during development. Future in vivo studies might determine if SRF and CREB actually function in developing muscle spindles.

Mouse genetic experiments have revealed functions of SRF and CREB in skeletal muscle but so far have not shown definitively if SRF or CREB have roles in muscle spindle formation. Mice with conditional knockout of SRF in skeletal muscle exhibit severe muscle hypoplasia [39]. Although no specific defects in muscle spindles were reported, presumably SRF deficiency would substantially abolish growth of all muscle fibers, including intrafusal fibers, which could mask any additional function SRF might be having on intrafusal fibers. Because we found that NRG1 can activate MRTF-A and MRTF-B in myotubes, a specific function of SRF in developing intrafusal fibers might result from its interaction with MRTF-A and MRTF-B. For MRTF-A and MRTF-B, double knockout mice die too early during development to evaluate a role in muscle spindles [40,41], and conditional double knockout in skeletal muscle has not been reported. Mice expressing a muscle-specific dominant-negative CREB transgene specifically in skeletal muscle exhibit a dystrophic phenotype [16]. No muscle spindle defects were reported in these mice, although these mice might not be suitable for making a definitive determination on the role of CREB in muscle spindles because the transgene is thought to be induced shortly after birth, whereas Egr3 induction in nascent intrafusal fibers begins around E15 when sensory axons contact these fibers. For studies on spindle formation, inactivating CREB during muscle development prior to when Egr3 is induced might be more informative. Also, inactivating CREB along with SRF or MRTFs would address any redundant function that CREB or SRF might have for inducing Egr3 in developing muscle spindles.

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