Neuregulin-1-Stimulated Phosphorylation of GABP in Skeletal Muscle Cells†

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ABSTRACT: Localization of acetylcholine receptors (AChRs) to neuromuscular synapses is mediated, in part, through selective transcription of AChR genes in myofiber synaptic nuclei. Neuregulin-1 (NRG-1) is a good candidate for the extracellular signal that induces synapse-specific gene expression, since NRG-1 is concentrated at synaptic sites and activates AChR synthesis in cultured muscle cells. NRG-1-induced transcription requires activation of Erk and Jnk MAP kinases, but the downstream substrates that mediate this transcriptional response are not known. Previous studies have demonstrated that a consensus binding site for Ets proteins is required both for NRG-1-induced transcription and for synapse-specific transcription in transgenic mice. This regulatory element binds GABPα, an Ets protein, and GABPβ, a protein that dimerizes with GABPα, raising the possibility that phosphorylation of GABP by MAP kinases induces transcription of AChR genes. To determine whether MAP kinases might directly regulate the activity of GABP, we studied MAP kinase-catalyzed and NRG-1-induced phosphorylation of GABPα and GABPβ. We show that GABPα and GABPβ are phosphorylated in vitro by Erk and by Jnk. Using recombinant proteins containing mutated serine and threonine resides, we show that GABPα is phosphorylated predominantly at threonine 280, while serine 170 and threonine 180 are the major phosphorylation sites in GABPβ. We generated antibodies specific to the major phosphorylation site in GABPα and show that NRG-1 stimulates phosphorylation of GABPα at threonine 280 in vivo. These results suggest that GABPα is a target of MAP kinases in NRG-1-stimulated muscle cells and are consistent with the idea that phosphorylation of GABPα contributes to transcriptional activation of AChR genes by NRG-1.

Shortly after motor axons contact developing myotubes, signals are exchanged between nerve and muscle that initiate the formation and assembly of a highly differentiated presynaptic nerve terminal and a highly specialized postsynaptic apparatus (*1, 2*). Acetylcholine receptors (AChRs)[1] are among the proteins that become localized to this small patch of the muscle fiber membrane, and their localization to synaptic sites during development is a hallmark of the inductive events of synapse formation.

Current data suggest that postsynaptic differentiation, including clustering of AChRs, is initiated and maintained by two different ligands that stimulate distinct signaling pathways. Agrin, the signal for one pathway, stimulates the posttranslational reorganization of proteins, including AChRs, in the muscle cell membrane (*3*). The signal for the second pathway is not known, but this pathway leads to enhanced transcription of AChR genes in myofiber nuclei that are situated at synaptic sites.

Synapse-specific transcription was demonstrated in transgenic mice that carry gene fusions between regulatory regions of AChR subunit genes and reporter genes (*4*−*6*). These transgenes are transcribed at a higher rate in myofiber nuclei near the synaptic site than in nuclei elsewhere in the myofiber, suggesting that motor neurons supply a signal to myofibers that activates AChR transcription in synaptic nuclei. Synapse-specific transcription leads to accumulation of AChR mRNA at synaptic sites (*7*−*9*), resulting in increased AChR protein synthesis in the synaptic region of the myofiber. RNAs encoding other synaptic proteins, including AChE, MuSK, Rapsyn, S-laminin, N-CAM, Utrophin, and the regulatory subunit of Protein Kinase A, are also concentrated at synaptic sites (*10*−*14*), implying that synapse-specific transcription may be a general and important mechanism for clustering proteins at developing and adult neuromuscular synapses.

Neuregulin-1 (NRG-1), a widely expressed growth and differentiation factor that is structurally related to EGF, is currently the best candidate for the signal that activates synapse-specific transcription (*15*). NRG-1 was independently isolated as a growth factor that stimulates tyrosine phosphorylation of ErbB2 (epidermal growth factor receptor 2), as a ligand that regulates Schwann cell proliferation and survival, and as a factor that stimulates AChR synthesis in muscle cells (*16*−*19*). Three lines of evidence suggest that NRG-1 induces synapse-specific transcription. First, NRG-1 and its receptors, ErbB3 and ErbB4, are concentrated at synaptic sites (*20*−*24*). Second, NRG-1 activates AChR gene expression in cultured muscle cells (*21, 25*), and the same cis-acting region that confers NRG-1 responsiveness also confers synapse-specific transcription in transgenic mice (*26, 27*). Further, a 5 bp regulatory element within this cis-acting

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[1] Abbreviations: AChR, acetylcholine receptor; NRG, neuregulin; GST, glutathione-*S*-transferase.

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region is required both for NRG-1-induced and for synapse-specific transcription (*28*−*32*). Third, although mice lacking NRG-1, ErbB2, or ErbB4 die, owing to defects in cardiac development, several days prior to neuromuscular synapse formation (*33*−*35*), adult mice that are heterozygous for the Ig allele of NRG-1 (NRG-$1^{Ig+/-}$) have a mild deficiency in synaptic transmission and fewer AChRs at their neuromuscular synapses (*36*).

The receptors for NRG-1, ErbB3 and ErbB4, are members of the EGF receptor family of receptor tyrosine kinases (*37, 38*). NRG-1 stimulation of ErbB receptors leads to activation of PI3-kinase and two MAP kinases, Erk and Jnk (*39*−*42*). Because Erk and Jnk but not PI3-kinase activities are required for NRG-1 to induce AChR transcription [*39, 41, 42*; but see (*40*)], substrates for Erk and Jnk have a critical role in regulating AChR gene expression in response to NRG-1.

The critical cis-acting region of AChR genes contains a consensus binding site for Ets proteins that is required to respond to NRG-1 and to confer synapse-specific transcription (*28*−*32*). Mutation of this cis-acting element in the AChR epsilon subunit gene has been linked to certain congenital human myasthenic syndromes (*43, 44*), diseases that are characterized by reduced AChR expression, indicating that this regulatory element indeed has a critical role in regulating AChR gene expression. GABP, a heterodimer of GABPα, an Ets protein, and GABP*β*, a non-Ets protein that enhances the DNA-binding activity of GABPα (*45, 46*), are the predominant proteins in myotube nuclear extracts that bind this element (*30, 32*). Because NRG-1 stimulation does not increase the binding of GABP to DNA, NRG-1 signaling may stimulate transcription by increasing the transcriptional activity of GABP without affecting its DNA-binding activity (*30*).

Ets proteins are known targets of MAP kinase signaling (*47*), and in vitro studies have shown that Ets proteins, including GABPα, as well as GABP*β*, can be phosphorylated directly by MAP kinases (*48*). Because the transcriptional activity of other Ets proteins is stimulated by phosphorylation (*49*−*54*), NRG-1 may stimulate phosphorylation of GABP and increase its transcriptional activity. Consistent with this idea, total phosphorylation of GABPα increases ∼2-fold following NRG-1 stimulation (*32*).

To begin to address whether MAP kinases directly regulate the activity of GABP in response to NRG-1, we have studied MAP kinase-catalyzed and NRG-1-induced phosphorylation of GABP. We used in vitro kinase assays to identify the serine and threonine residues in GABPα and GABP*β* that are phosphorylated by Erk and Jnk, and we generated antibodies specific to the major in vitro phosphorylation sites in GABPα and GABP*β* to determine whether NRG-1 stimulates phosphorylation of these residues in vivo. We found that NRG-1 stimulates phosphorylation of GABPα at threonine 280, the major site for Erk- and Jnk-catalyzed phosphorylation in vitro. These results suggest that phosphorylation of GABPα may be important for NRG-1-induced transcription of AChR genes in skeletal muscle cells.

EXPERIMENTAL PROCEDURES

Plasmids. A plasmid expressing polyhistidine-tagged GABPα was constructed by inserting an *Eco*RI−*Kpn*I fragment containing the coding sequences of GABPα into the bacterial expression vector ProExHTa (Life Technologies). Bacterial expression plasmids encoding polyhistidine-tagged GABP*β*, glutathione-*S*-transferase (GST)-tagged GABPα, and GST-tagged GABP*β* were provided by Dr. Fred Stanley (NYU Medical School) (*55*). Mutations at consensus MAP kinase phosphorylation sites in polyhistidine-tagged GABPα and GABP*β* were introduced by site-directed mutagenesis (*56*). Plasmids expressing FLAG-tagged GABPα (MDAF-GABPα-FLAG) and FLAG-tagged GABP*β* (MDAF-GABP*β*-FLAG) were constructed by introducing a FLAG epitope at the carboxy terminus of GABPα and GABP*β*, respectively, and inserting the tagged cDNA into a MDAF expression vector (*57*). The structures of the plasmids were verified by DNA sequencing.

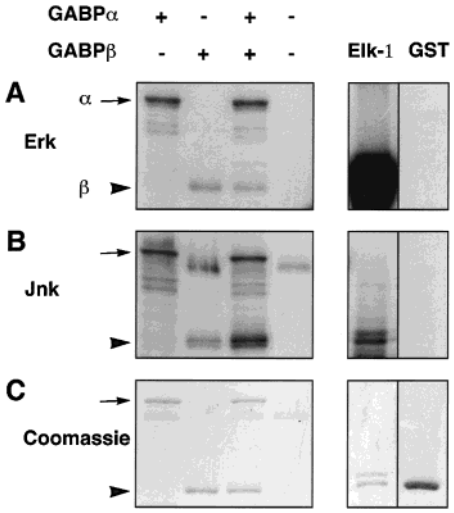

FIGURE 1: GABPα and GABP*β* are phosphorylated in vitro by Erk and Jnk. GST-tagged GABPα, polyhistidine-tagged GABP*β*, and GST-tagged Elk-1 were phosphorylated either by Erk or by Jnk, and the proteins were fractionated by SDS−PAGE. The gel was scanned in a Phosphorimager (A, B) and stained with Coomassie brilliant blue (C). Erk phosphorylates GABPα and GABP*β*, although less efficiently (100-fold) than Elk-1. Jnk phosphorylates GABPα, GABP*β*, and Elk-1 to a similar extent. Autophosphorylated Jnk appears as an additional radiolabeled protein. The positions of GABPα (arrow) and GABP*β* (arrowhead) are indicated.

Recombinant proteins were expressed in *E. coli* strain BL21 (DE3). Lysates containing GST-tagged proteins (in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 8 M urea) were dialyzed against buffer lacking urea and purified using a glutathione−agarose matrix (*58*). Polyhistidine-tagged proteins were purified in 8 M urea using a nickel−agarose matrix according to the manufacturer's instructions (Qiagen) and dialyzed against buffer without urea.

A plasmid expressing GST-tagged Jnk (*59*) was transfected into 293T cells using lipofectamine (Life Technologies). Two days following transfection, cells were stimulated for 30 min with 0.3 M sorbitol, and activated Jnk was purified using a glutathione−agarose matrix (*59*).

In Vitro Kinase Assay. Erk-mediated phosphorylation was assayed in a reaction containing 8−12 pmol of recombinant GABPα, GABP*β*, Elk-1 (NEB), or GST, 2 nmol of ATP, 2 μCi of [γ-^{32}P]ATP, and 25 units of Erk2 (p42 MAPK, NEB) in 28 μL of MAPK buffer (NEB). Jnk-mediated phosphorylation was assayed in a reaction containing 8−12 pmol of each recombinant protein, 2 nmol of ATP, 2 μCi of [γ-^{32}P]-

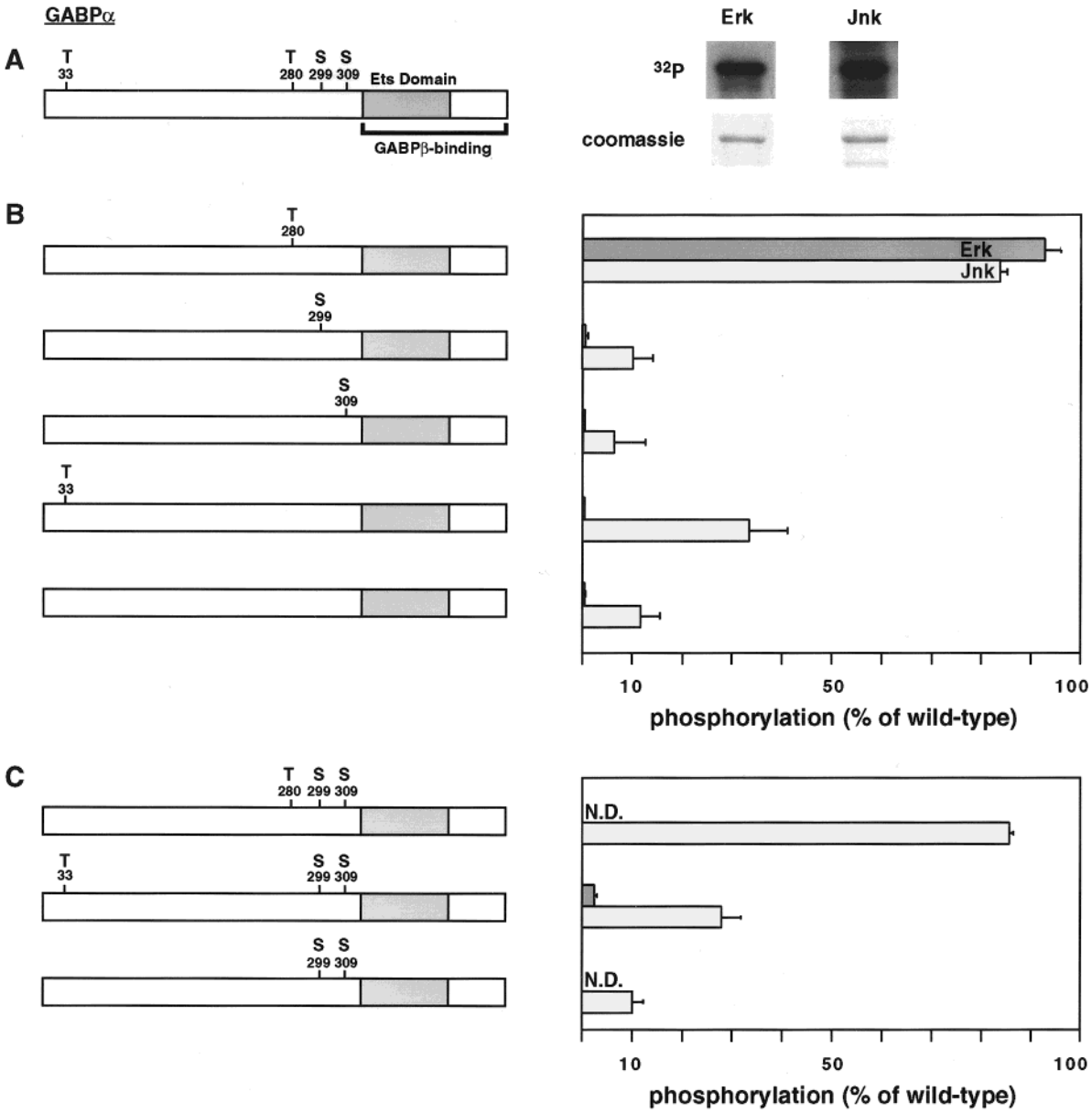

FIGURE 2: T280 is the major phosphorylation site in GABPα. Wild-type (A) and mutant (B, C) forms of polyhistidine-tagged GABPα, complexed with polyhistidine-tagged GABP*β*, were phosphorylated by Erk or Jnk, and the incorporation of ^{32}P (mean ± SEM) was determined as described in Figure 1. (B) A GABPα mutant retaining T280, but lacking T33, S299, and S309, is phosphorylated similar to wild-type. In contrast, GABPα mutants retaining only S299 or S309 fail to be phosphorylated by Erk and are phosphorylated poorly by Jnk. A GABPα mutant retaining only T33 is phosphorylated by Jnk but not by Erk. (C) Consistent with these data, a GABPα mutant lacking T280, but retaining T33, S299, and S309, is phosphorylated by Jnk but poorly by Erk.

ATP, 10 μL of activated Jnk, 20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 10 mM NaF, 0.2 mM Na_3VO_4, and 1 mM DTT in a total volume of 28 μL. Reactions were incubated for 30 min at 30 °C, and proteins were fractionated by SDS−PAGE. Incorporation of ^{32}P was quantitated using a Phosphorimager.

Antibodies. Antibodies to phospho-T280 GABPα and phospho-S170 GABP*β* were produced by Research Genetics. Rabbits were immunized with KLH coupled to a 13 amino acid phosphopeptide corresponding to the phosphorylated region of GABPα or GABP*β*; the phosphorylated residue was positioned at the center of the peptide. Monoclonal antibodies to GABPα were produced by immunizing mice with GST-tagged GABPα (7 mg in MPL + TDM adjuvant, Sigma). Following several boosts, spleen cells were fused with NS1 myeloma cells, and hybridoma cells secreting antibodies to GABPα were identified by ELISA and cloned by limiting dilution (*60*).

Immunoprecipitation. Sol8 myoblasts were transfected with the MDAF-GABPα-FLAG or the MDAF-GABP*β*-FLAG expression plasmid, and pools of stably transfected cells were selected and induced to differentiate into myotubes (*21*). Myotubes grown on 10 cm plates were serum-starved for 5 h, stimulated with NRG-1 (HRG*β*1, R&D Systems, 125 ng/mL) for 10−150 min, and lysed in 500 μL of HNTG buffer (1% Triton X-100, 150 mM NaCl, 20 mM Hepes, pH 7.5, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$) containing 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin, 100 μg/mL PMSF, 50 mM NaF, 30 mM $Na_4P_2O_7$, and 1 mM Na_3VO_4.

Phosphorylated GABPα was immunoprecipitated from in vitro kinase reactions (4 μL diluted to 200 μL with HNTG)

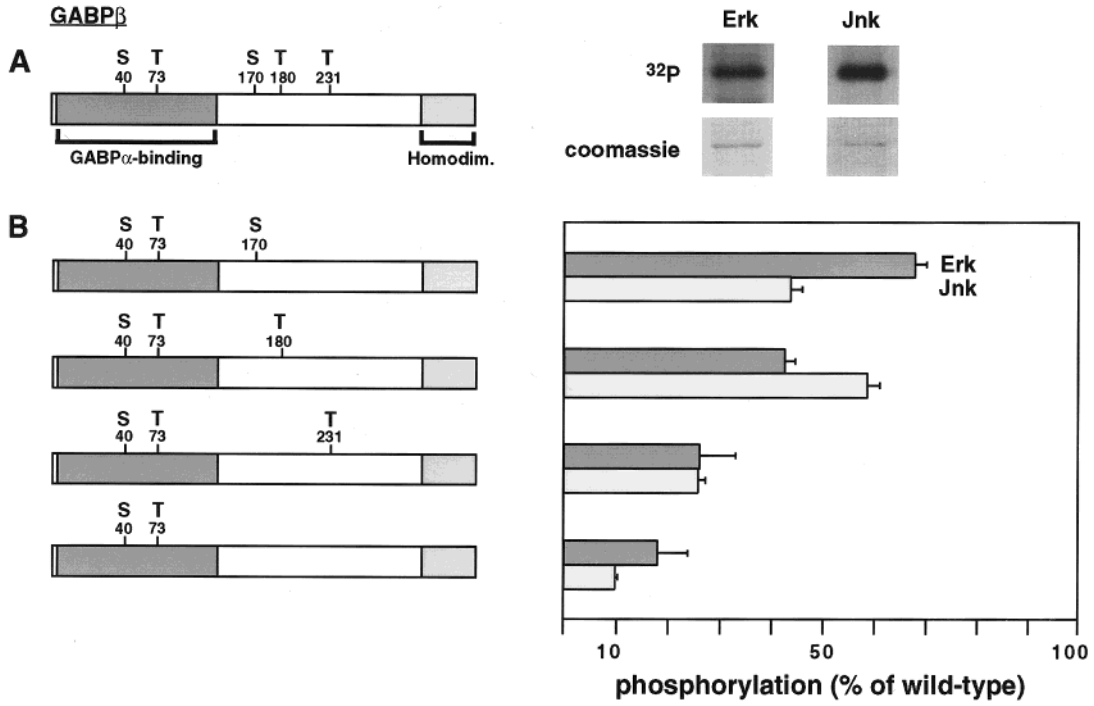

FIGURE 3: S170 and T180 are the major phosphorylation sites in GABPβ. Wild-type (A) and mutant (B) forms of polyhistidine-tagged GABPβ, complexed with GST-GABPα, were phosphorylated by Erk or Jnk, and the incorporation of ^{32}P (mean ± SEM) was determined as described in Figure 1. (B) Mutation of S170, T180, and T231 reduces Erk- and Jnk-catalyzed phosphorylation by 5−10-fold. Retaining S170 increases Erk-catalyzed phosphorylation by 4-fold and Jnk-catalyzed phosphorylation by 5-fold; retaining T180 increases Erk-catalyzed phosphorylation by 2.5-fold and Jnk-catalyzed phosphorylation by 6-fold; retaining T231 increases Erk-catalyzed phosphorylation by 1.5-fold and Jnk-catalyzed phosphorylation by 2.5-fold.

and myotube lysates (1 mg of protein in 200 μL) with antibodies (4 μL of antiserum) to phospho-T280 GABPα. Following a 2 h incubation at 4 °C, immune complexes were collected using Protein A−agarose and washed 3 times with 1 mL of HNTG. Immunoprecipitated proteins were eluted from the agarose beads and resolved by SDS−PAGE, and Western blots were probed with a monoclonal antibody (3-12D) to GABPα or an antibody to FLAG (M2, Sigma, diluted 1:1000).

FLAG-tagged GABPβ was immunoprecipitated from myotubes stably transfected with MDAF-GABPβ-FLAG. Myotube lysates (1 mg of protein) were incubated with 15 μL of agarose-coupled anti-FLAG (M2, Sigma) for 3 h at 4 °C, and immune complexes were washed 3 times with 1 mL of HNTG. Proteins eluted from the agarose beads were resolved by SDS−PAGE, and Western blots were probed with antibodies to phospho-S170 GABPβ (1:1000 dilution of antiserum). Antisera were preadsorbed by incubating undiluted antisera with peptides (5 mg/mL) for 1 h at room temperature. Stripped Western blots were reprobed with a mouse antiserum (diluted 1:1000) to recombinant, full-length GABPβ.

RESULTS

Ets proteins are known targets of MAP kinase signaling, and in vitro studies have shown that Ets proteins can be phosphorylated directly by MAP kinases (*47*). We studied the ability of Erk and Jnk to phosphorylate GABPα, an Ets protein, and GABPβ, a protein that dimerizes with GABPα. Figure 1 shows that GABPα and GABPβ are phosphorylated by Erk and Jnk in vitro. Although these MAP kinases phosphorylate monomers of GABPα and GABPβ, Jnk phosphorylates GABPβ to a greater extent (~3-fold) when GABPβ is complexed with GABPα (Figure 1). Elk-1 is an Ets protein that is a known substrate for Erk and Jnk (*61*). Jnk phosphorylates GABP and Elk-1 to a similar extent, whereas Erk phosphorylates Elk-1 to a far greater extent (~100-fold) than GABP (Figure 1). Thus, both GABP subunits appear to be favorable substrates for Jnk and poor substrates for Erk.

GABPα and GABPβ contain multiple consensus sites (S/T-P) for Erk and Jnk phosphorylation (*62*). To determine the extent of phosphorylation at each of these sites, we constructed mutant forms of GABPα and GABPβ in which serine or threonine residues were mutated to alanine, and we compared the extent of phosphorylation of wild-type and mutated proteins.

GABPα contains four potential MAP kinase phosphorylation sites. We analyzed a series of GABPα mutants that retain only a single consensus phosphorylation site. Wild-type GABPα and a mutant form of GABPα, retaining T280 as the only consensus site, are phosphorylated to a similar extent by Erk (Figure 2). GABPα mutants lacking T280 but retaining any one of the other three consensus sites are not phosphorylated by Erk. Thus, T280 is the major phosphorylation site for Erk.

The GABPα mutant retaining only T280 is phosphorylated, like wild-type, by Jnk (Figure 2). Jnk, unlike Erk, can phosphorylate the GABPα mutant retaining T33 as the only consensus site. To further analyze the contribution of T33 and T280 to GABPα phosphorylation, we mutated T33 or T280 alone or together. Mutation of T280, as expected, resulted in a substantial decrease in Erk- (40-fold) and Jnk-mediated (4-fold) phosphorylation. In contrast, mutation of

T33 resulted in only a 15% decrease in Jnk-mediated phosphorylation (Figure 2). Consistent with these findings, T33 is not conserved in human GABPα (*63*), indicating that T33 is unlikely to be critical for regulating the transcriptional activity of GABP. These data indicate that T280 is the only site phosphorylated by Erk and the predominant site phosphorylated by Jnk, while T33 contributes in part to Jnk-mediated phosphorylation.

GABP*β* contains five potential MAP kinase phosphorylation sites. S40 and T73 are located within the ankyrin-like repeats that form an interface with GABPα. Because mutation of S40 or T73 disrupts association of GABP*β* with GABPα (data not presented), we did not study phosphorylation of GABP*β* containing mutations in these residues. Figure 3 shows that phosphorylation of a mutant lacking S170, T180, and T231 is reduced 5−10-fold, indicating that these three sites are the major sites for Erk- and Jnk-mediated phosphorylation. We analyzed the individual contribution of these three sites by studying phosphorylation of GABP*β* constructs retaining only one of these sites. Retaining S170 increases Erk-mediated phosphorylation by 4-fold and Jnk-mediated phosphorylation by 5-fold; retaining T180 increases Erk-mediated phosphorylation by 2.5-fold and Jnk-mediated phosphorylation by 6-fold; retaining T231 increases Erk-mediated phosphorylation by only ∼1.5-fold and Jnk-mediated phosphorylation by 2.5-fold (Figure 3). These results indicate that although T231 contributes to Erk- and Jnk-mediated phosphorylation, S170 and T180 are the major sites for phosphorylation by these MAP kinases. S170 and T180 are phosphorylated by either Erk or Jnk, but Erk has a weak preference for S170 and Jnk has a weak preference for T180.

NRG-1 stimulation of muscle cells results in activation of both Erk and Jnk, and these kinases are required for NRG-1-induced transcription of AChR genes (*39*−*42*). Because Erk and Jnk phosphorylate GABPα and GABP*β* in vitro, NRG-1 stimulation may induce GABP phosphorylation in vivo. To study phosphorylation of GABP in muscle cells, we generated antibodies that are specific to major phosphorylation sites in GABPα and GABP*β*. Figure 4 shows that antibodies to phosphorylated T280 in GABPα, the major phosphorylation site for both Erk and Jnk, specifically recognize recombinant GABPα phosphorylated in vitro. We treated muscle cells with NRG-1, immunoprecipitated phospho-T280 GABPα, and probed Western blots with a monoclonal antibody to GABPα. We found that NRG-1 stimulates phosphorylation of T280 in vivo (Figure 4). Maximal T280 phosphorylation is detectable 10 min following NRG-1 stimulation and gradually decreases to the levels found in unstimulated cells during the following few hours.

We produced antibodies to phosphopeptides in GABP*β* that include the two major phosphorylation sites (S170 and T180) for Erk and Jnk. Because antibodies to phospho-T180 GABP*β* do not distinguish between nonphosphorylated GABP*β* and GABP*β* that was phosphorylated in vitro (data not presented), we were unable to use these antibodies to determine whether NRG-1 stimulates phosphorylation of T180 in vivo. In contrast, antibodies to phospho-S170 GABP*β* specifically recognize phosphorylated GABP*β*, since they react with GABP*β* phosphorylated in vitro by Erk and not with nonphosphorylated GABP*β* (Figure 5). We stimulated myotubes, stably expressing FLAG-tagged GABP*β*, with NRG-1, immunoprecipitated FLAG-tagged GABP*β*, and probed Western blots with antibodies to phospho-S170 GABP*β*. We found that S170 is phosphorylated in nonstimulated cells and that NRG-1 does not alter the level of phosphorylation at this site (Figure 5). Thus, NRG-1 fails to stimulate phosphorylation of GABP*β* at S170 in vivo, indicating that phosphorylation at this site does not directly mediate transcriptional activation in response to NRG-1.

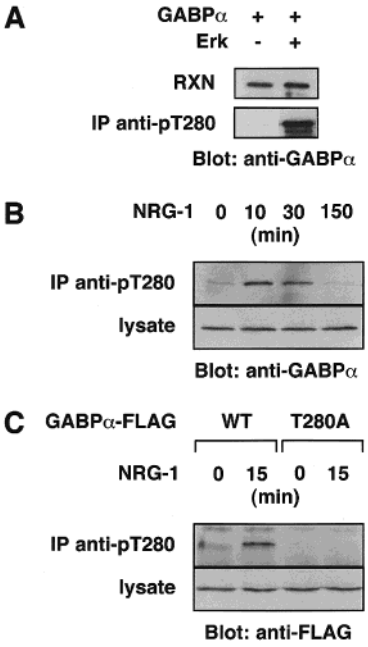

FIGURE 4: NRG-1 induces phosphorylation of T280 in GABPα. (A) GABPα was phosphorylated by Erk in vitro and immunoprecipitated with antibodies to phospho-T280 in GABPα. Proteins in the in vitro kinase assay (RXN) and in the immunoprecipitate (IP) were resolved by SDS−PAGE, and Western blots were probed with a monoclonal antibody (3-12D) to GABPα. Antibodies to phospho-T280 specifically recognize phosphorylated GABPα, since they react with GABPα phosphorylated in vitro by Erk and not with nonphosphorylated GABPα. (B) Myotubes were stimulated with NRG-1, and phospho-T280 GABPα was immunoprecipitated from the lysate. Proteins in the lysate and in the immunoprecipitate were resolved by SDS−PAGE, and Western blots were probed with a monoclonal antibody to GABPα. T280 phosphorylation is maximal 10 min following NRG-1 stimulation and decreases to basal levels by 150 min despite the continual presence of NRG-1. (C) Myotubes, stably transfected with wild-type GABPα or GABPα T280A, each containing a FLAG tag, were stimulated with NRG-1, and phosphorylated GABPα was immunoprecipitated with antibodies to phospho-T280. Western blots were probed with antibodies to FLAG. The phosphopeptide antibodies are specific for T280 in GABPα since they recognize wild-type but not GABPα T280A.

DISCUSSION

We previously identified a cis-acting regulatory element that is required for NRG-induced transcription of AChR genes in cultured muscle cells and synapse-specific transcription in transgenic mice (*30*, *31*). We found that GABP is the predominant protein in myotube nuclear extracts that binds this cis-acting element, suggesting that GABP is required for synapse-specific transcription and for activating transcription in response to NRG-1 (*30*, *32*). A dominant negative form of GABP*β* lacking sequences for transcriptional activation interferes with AChR expression in adult muscle expressing ectopic agrin, consistent with the idea that

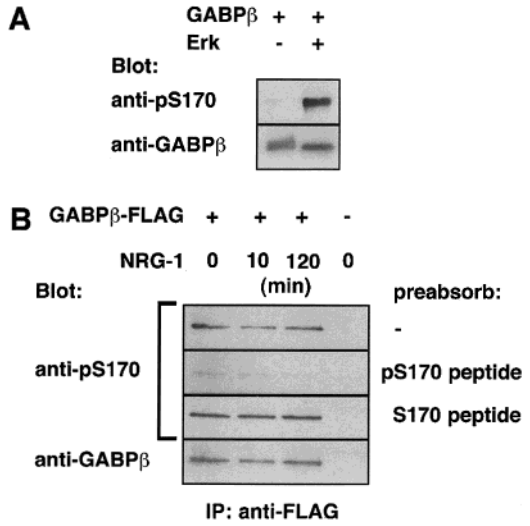

FIGURE 5: GABPβ is phosphorylated at S170 independent of NRG-1. (A) GABPβ was phosphorylated in vitro by Erk. Phosphorylated and nonphosphorylated GABPβ were resolved by SDS−PAGE, and Western blots were probed with antibodies to phospho-S170 GABPβ or with antibodies to GABPβ. The phospho-S170 antibodies specifically recognize phosphorylated GABPβ. (B) Myotubes, stably transfected with FLAG-tagged GABPβ, were stimulated with NRG-1, and FLAG-tagged GABPβ was immunoprecipitated with antibodies to FLAG. Proteins in the immunoprecipitate were resolved by SDS−PAGE, and Western blots were probed either with antibodies to phospho-S170 GABPβ or with antibodies to GABPβ. GABPβ is phosphorylated at S170 in nonstimulated cells, and the amount of phosphorylation at this site does not change following NRG-1 stimulation. Binding of antibodies to phospho-S170 GABPβ is specific, because binding is inhibited by preabsorbing the antibodies with the phosphorylated and not with the nonphosphorylated S170 peptide.

GABP is required for synapse-specific gene expression (*64*). Since NRG-1 signaling does not increase binding of GABP to DNA, we suggested that NRG-1 signaling might lead to posttranslational modifications of GABPα that increase GABP transcriptional activity (*30*). We show here that T280 in GABPα and S170 and T180 in GABPβ are phosphorylated by Erk and Jnk in vitro. Further, we demonstrate that NRG-1 stimulates phosphorylation of T280 in GABPα without leading to phosphorylation of S170 in GABPβ. Thus, our results indicate that T280 in GABPα is phosphorylated by MAP kinases in NRG-1-stimulated muscle cells and raise the possibility that phosphorylation of GABPα at this site regulates its transcriptional activity.

Indeed, MAP kinases are known to increase the transcriptional activity of other Ets proteins, including Ets1, Ets2, Pointed-P2, Elk-1, and ER81, by phosphorylating residues located within their transcriptional activation domains (*49*−*54*). PEA3 and Erm, which are highly related to ER81, can mediate transcriptional induction by constitutively activated forms of ErbB2 and Raf, respectively, suggesting that these Ets proteins may also be regulated by phosphorylation (*65*, *66*). Although it is not known whether MAP kinase phosphorylation increases the transcriptional activity of GABPα in skeletal muscle, GABP-mediated transcription is regulated by MAP kinase signaling in other cell types. For example, induction of interleukin-2 in T cells requires both a GABP-binding site and activation of Erk and Jnk (*67*). Likewise, Erk activation can stimulate transcription from the LTR in HIV through a GABP-binding site (*48*).

Phosphorylation of GABP may not be sufficient to activate AChR transcription in response to NRG-1. Although T280 in GABPα is the major site for both Erk- and Jnk-mediated phosphorylation, inhibition of either Erk or Jnk blocks NRG-1-induced AChR transcription (*39*−*42*). These results indicate that Erk and Jnk are not redundant and suggest that Erk or Jnk phosphorylate proteins in addition to GABPα that are required for NRG-1 to induce AChR genes.

NRG-1-induced phosphorylation of GABPα is transient, since phosphorylation at T280 peaks at 10 min following stimulation but thereafter declines to basal levels. In contrast, AChR transcription is detectable only hours after NRG-1 stimulation (*42*, data not presented). Further, NRG-1-induced AChR transcription requires de novo protein synthesis (*42*), raising the possibility that NRG-1 activation of MAP kinases induces expression of upstream genes that regulate GABP activity. Future studies will address whether NRG-1-induced AChR transcription requires direct phosphorylation of GABPα and/or phosphorylation of other proteins that indirectly regulate GABP transcriptional activity.

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