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Novel Downstream Targets of EphA4
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THE REGULATION OF
ACETYLCHOLINESTERASE
EXPRESSION**

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Identification of the Jak/Stat Proteins as Novel Downstream Targets of EphA4 Signaling in Muscle

IMPLICATIONS IN THE REGULATION OF ACETYLCHOLINESTERASE EXPRESSION*

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Eph receptors and their cognate ligands ephrins are important players in axon guidance and neural patterning during development of the nervous system. Much of our knowledge about the signal transduction pathways triggered by Eph receptors has been related to the modulation of actin cytoskeleton, which is fundamental in mediating the cellular responses in growth cone navigation, cell adhesion, and cell migration. In contrast, little was known about whether long term activation of Eph receptor would regulate gene expression. Here we report a novel signaling pathway of EphA4, which involves activation of the tyrosine kinase Jak2 and the transcriptional activator Stat3. Transfection of COS7 cells with EphA4, but not the kinase-dead mutant, induced tyrosine phosphorylation of Jak2, Stat1, and Stat3. Treatment of cultured C2C12 myotubes with ephrin-A1 also induced tyrosine phosphorylation of Stat3, which was abolished by the Jak2 inhibitor AG490. Moreover, Jak2 was co-immunoprecipitated with EphA4 in muscle, and both proteins were concentrated at the neuromuscular junction (NMJ) of adult muscle. By using microarray analysis, we have identified acetylcholinesterase, the critical enzyme that hydrolyzed the neurotransmitter acetylcholine at the NMJ, as a downstream target gene of the Jak/Stat pathway in muscle. More importantly, ephrin-A1 increased the expression of acetylcholinesterase protein in C2C12 myotubes, which was abolished by AG490. In contrast, ephrin-A1 reduced the expression of fibronectin mRNA in C2C12 myotubes independently of Jak2. Finally, the expression level of acetylcholinesterase in limb muscle of EphA4 null mice was significantly reduced compared with the wild-type control. Taken together, these results have identified Jak/Stat proteins as the novel downstream targets of EphA4 signaling. In addition, the present study provides the first demonstration of a potential function of Eph receptors and Jak/Stat proteins at the NMJ.

Eph receptors, the largest family of receptor tyrosine kinases (RTK),¹ and their cognate ligands, ephrins, play critical roles in

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¹ The abbreviations used are: RTK, receptor tyrosine kinase; NMJ,

diverse processes in both developing and mature nervous system. Initial studies mainly focused on the roles of ephrins as a repulsive primary messenger in axon guidance, topographic mapping, and neural patterning during early development of the nervous system (1, 2). More recent studies revealed the unexpected functions of Eph receptors in synapse formation and synaptic plasticity (3–5). Outside the nervous system, Eph receptors are also involved in the regulation of fluid homeostasis and vascular development (6).

The biological functions of Eph receptors are therefore quite different from most other RTKs, which are involved in cell proliferation, survival, and differentiation. This can be attributed to the unusual properties of the ephrin ligands, as well as the unique bi-directional signaling of the ephrins/Eph receptors. Unlike the ligands of conventional RTK, ephrins are membrane-bound proteins. Eight ephrins have been identified in mammals, which are classified into A and B subclasses based on their modes of membrane anchorage. The ephrin-A ligands (ephrin-A1 to A5) are glycosylphosphatidylinositol-anchored, whereas the ephrin-B ligands (ephrin-B1 to B3) anchor to the membrane via a transmembrane domain. Receptor specificity has been reported for the ephrin/Eph interactions, such that the A class ephrins activate the EphA receptors, whereas the ephrin-B ligands activate EphB receptors, with the notable exception of EphA4 (7). The membrane anchorage of ephrins is essential for the activation of Eph receptors, which can be mimicked by artificial clustering of tagged soluble ephrins (8). In addition, the surface density of ephrins can determine the resulting signaling pathways and cellular responses (9).

Unlike most RTK, activation of Eph receptors does not lead to cell proliferation (10, 11), suggesting that Eph receptors trigger distinct signaling pathways when activated by their ligands. In addition, upon binding to the Eph receptors, both the glycosylphosphatidylinositol-linked and transmembrane ephrins are capable of transducing intracellular signals to elicit biological responses (12). Rapid advances have been made in recent years in elucidating the bi-directional signal transduction of ephrins and Eph receptors (6, 13). The Rho family of GTPases, for example, has been identified to link the Eph receptor signaling to the re-organization of actin cytoskeleton. Ephrin-A1 induces the ephexin-mediated exchange of GDP to GTP on Rho, resulting in the activation of RhoA and Rho kinase. On the other hand, activation of EphA receptors inhib-

neuromuscular junction; AChE, acetylcholinesterase; LIF, leukemia inhibitory factor; CBD2, cytokine-binding domain 2; JH, Jak homology; Me₂SO, dimethyl sulfoxide; GST, glutathione S-transferase; AChR, acetylcholine receptor; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; SH, Src homology; Stat, signal transducers and activators of transcription; Jak, Janus kinase.

its Rac/Cdc42 and the downstream effector p21-activated kinase (14, 15). Together, these lead to the initiation of growth cone collapse. Other guanine nucleotide exchange factors are also associated with EphB receptors, and they have been implicated as crucial downstream mediators of EphB signaling in dendritic spine morphogenesis (16, 17).

Besides the Rho family of GTPases, activation of Eph receptors also regulates the activity of other kinases such as phosphatidylinositol 3-kinase (18) and the mitogen-activated protein kinases ERK and c-Jun N-terminal kinase (19–23). In addition, several non-receptor tyrosine kinases have been implicated as downstream mediators of Eph receptor signaling. The tyrosine phosphorylation of focal adhesion kinase (FAK), a critical component of the integrin signaling pathway, is modulated after activation of EphA receptors (24, 25). Various members of the Src family kinases have also been shown to interact with activated Eph receptors through the SH2 domain (26–28). Moreover, Src is critical in the EphB2-induced tyrosine phosphorylation of *N*-methyl-D-aspartic acid receptor in cortical neurons (29), as well as the activation of ERK in response to EphB1 signaling (23). Finally, the tyrosine kinases Abl and Arg can interact with activated EphB2, and the kinase activity of Abl decreases upon treatment with ephrin-B1 (30). The downstream targets of Abl and Arg in the Eph signaling pathway remain to be elucidated.

Virtually all studies on the signaling mechanisms of Eph receptors focus on the potential link to actin dynamics, which is crucial in cellular responses such as growth cone collapse, cell adhesion, and cell migration. Much less is known about whether Eph signaling can regulate gene expression. It is unlikely that gene transcription is involved in some of the ephrin-mediated responses such as growth cone collapse, which happens within minutes. However, recent findings that Eph receptors are involved in synapse formation and synaptic plasticity have raised the possibility that long term activation of Eph receptors may produce long lasting effects. For example, long term infusion of ephrin-A5-IgG or EphA5-IgG into the hippocampus, which affects the learning behavior of mice, regulates the transcript level of tubulin and microtubule-associated protein-2 (31). Furthermore, the long lasting form of long term potentiation, which depends on protein synthesis and is thought to involve structural remodeling at the synapses, is impaired in mice lacking EphB2 (32). Finally, ephrin-B2 can enhance the *N*-methyl-D-aspartic acid-dependent gene transcription in cultured neurons, which may be important in the development and remodeling of neuronal synapses (29). These studies therefore suggest that regulation of gene transcription may represent an important mechanism underlying long term responses to ephrins, particularly at the synapses.

In the present study, the possibility of regulating gene expression by ephrin/Eph signaling and the underlying mechanism were explored. Here we report that activation of EphA4 induces the tyrosine phosphorylation of the transcriptional regulators Stat1 and Stat3, which belong to the signal transducers and activators of transcription (Stat) family. The tyrosine phosphorylation is mediated by the non-receptor tyrosine kinase Jak2, which is constitutively associated with EphA4. Consistent with our previous findings that EphA4 is concentrated at the neuromuscular junction (NMJ) (33), both Jak2 and Stat proteins are also localized at the NMJ of adult muscle. Finally, we have identified acetylcholinesterase (AChE) as a target gene of the Jak/Stat pathway in muscle, and ephrin-A1 increases the expression of AChE protein in cultured myotubes in a Jak2-dependent manner.

EXPERIMENTAL PROCEDURES

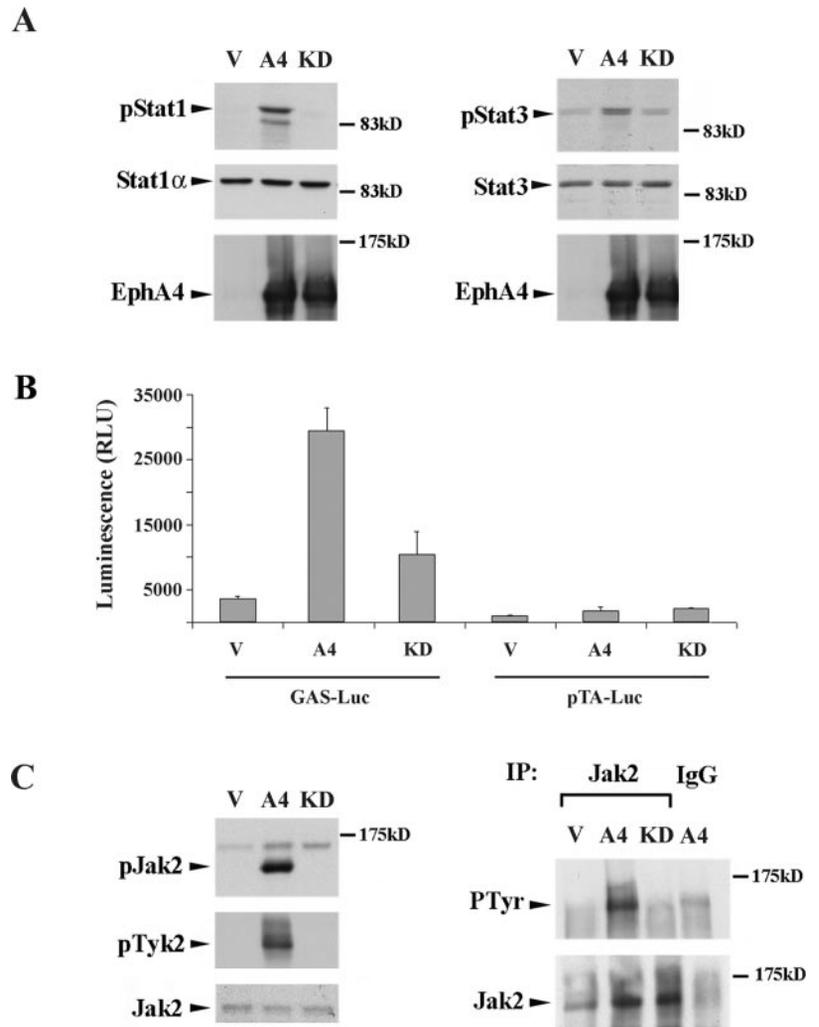
Antibodies and Reagents—The antibodies against tyrosine-phosphorylated Stat1, Stat3, Tyk2, FAK, and threonine/tyrosine-phosphorylated ERK-1/2 were purchased from Cell Signaling Inc., and the antibody against tyrosine-phosphorylated Jak2 was obtained from Upstate Biotechnology, Inc. Rabbit polyclonal antibodies that recognized Stat1 (sc-346), Stat3 (sc-7179), Jak2 (sc-278), and EphA4 (sc-921) were purchased from Santa Cruz Biotechnology, and the monoclonal antibodies that recognized AChE and the α -isoform of Stat1 were purchased from Transduction Laboratories and Zymed Laboratories Inc., respectively. The anti-EphA4 antibody that was used for Western blotting in the co-immunoprecipitation experiments was a kind gift from Dr. David Wilkinson (National Institute for Medical Research, UK). The Fc-tagged ephrin-A1 recombinant chimeric protein was purchased from R&D Systems, and the Fc fragment of human IgG and the anti-Fc antibody were obtained from Jackson ImmunoResearch Laboratories. The Jak2 inhibitor AG490 was purchased from Calbiochem. The EphA4 null mice were kindly provided by Dr. Perry Bartlett (Walter and Eliza Hall Institute of Medical Research, Australia). The expression constructs of EphA4 and kinase-dead EphA4 were subcloned into the expression vector pMT21 as described previously (33). The luciferase construct that was linked to the Stat1-responsive enhancer (pGAS-Luc) was a kind gift by Dr. Zilong Wen (Institute of Molecular and Cell Biology, Singapore). The full-length construct of AChE was kindly provided by Dr. Karl Tsim (The Hong Kong University of Science and Technology).

Transfection, Western Blot Analysis, and Luciferase Assay—COS7 cells (1.5×10^6) were plated onto a 100-mm culture dish in DMEM supplemented with 10% fetal bovine serum and were transfected the next day by 16 μ g of DNA using LipofectAMINE Plus according to the manufacturer's instructions (Invitrogen). Two days after transfection, the cells were serum-starved for 6 h before being lysed by RIPA at 4 °C for 1 h. The lysate was then analyzed in SDS-PAGE and immunoblotted by antibodies against phospho-Stat1, phospho-Stat3, phospho-Jak2, and phospho-Tyk2. To examine the role of Jak2, the transfected cells were serum-starved in the presence of Me₂SO or AG490 at various concentrations. For luciferase assay, COS7 cells (5×10^5) were plated on a 60-mm dish and co-transfected the next day by pGAS-Luc and EphA4, EphA4KD, or empty vector in a ratio of 1:9. pRL-CMV (25 ng), which encoded the *Renilla* luciferase, was included in the transfection mix for normalization. Two days after transfection, the cells were serum-starved overnight and lysed by the Reporter Lysis Buffer (Promega). The luciferase activity was measured by the Dual Luciferase Assay according to the manufacturer's instructions (Promega).

Immunoprecipitation and GST Pull-down—Transfected COS7 cells were lysed by Tris buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF) that contained 0.5% Nonidet P-40 plus 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamide, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml trypsin inhibitor, 2 μ g/ml antipain, and 1 mM sodium orthovanadate. The cell lysate (500 μ g) was then incubated with anti-Jak2 or anti-EphA4 (sc-921) antibodies (2 μ g) at 4 °C overnight, followed by incubation with protein A-Sepharose for 1 h at 4 °C. Likewise, cortical neurons that were cultured for 15 days were lysed by the same Tris buffer that contained 0.1% Nonidet P-40 plus all the protease inhibitors, and the lysate (800 μ g) was subjected to immunoprecipitation by anti-Jak2 or anti-EphA4 antibodies (3 μ g). To examine the interaction between EphA4 and Jak2 *in vivo*, frozen brain or muscle tissues were homogenized in the Tris lysis buffer (without Nonidet P-40) plus all the protease inhibitors. To obtain the membrane fraction, the samples were then centrifuged at 35,000 rpm for 1 h, and the pellet was solubilized with the homogenization buffer that contained all the protease inhibitors plus 0.5% Nonidet P-40. For co-immunoprecipitation, the membrane fraction (1.5 mg) was incubated overnight with anti-EphA4 or anti-Jak2 antibodies (4 μ g) at 4 °C. For the pull-down assay, the N- and C-terminal halves of Jak2, which contained JH domains 3–7 and JH domains 1–3, respectively (34), were amplified from the full-length construct of Jak2 (a gift from Dr. Zilong Wen) by Vent polymerase and subcloned into the GST vector. The GST-tagged cytokine-binding domain 2 (CBD2) of LIF receptor (35) was used as a negative control. The GST fusion proteins were purified and incubated with glutathione beads for 1 h (1 μ g of protein/10 μ l of beads per reaction). The immobilized protein was then incubated with lysate of COS7 cells (100 μ g) transfected with EphA4 for 1 h. Beads were washed six times by phosphate-buffered saline plus 0.1% Nonidet P-40, and the proteins were eluted by 2 \times SDS sample buffer (20 μ l).

Treatment of C2C12 Myotubes with Ephrin-A1—The muscle cell line C2C12 was cultured in DMEM supplemented with 20% fetal bovine

FIG. 1. Tyrosine phosphorylation of Jaks and Stat proteins by activated EphA4. **A**, COS7 cells were transfected with vector (V), EphA4 (A4), or kinase-dead (KD) mutant of EphA4, and the cell lysate was immunoblotted by anti-phospho-Stat1 and anti-phospho-Stat3 antibodies. Tyrosine phosphorylation of Stat1 (left panel) and Stat3 (right panel) was induced in COS7 cells that overexpressed EphA4 but not the kinase-dead mutant (upper panels). The membrane was stripped and re-probed with antibody against Stat3 or the α -isoform of Stat1 to indicate similar loading (middle panels). Both the wild-type and kinase-dead mutant was expressed at comparable level, as illustrated by Western blot with anti-EphA4 antibody (lower panels). **B**, induction of Stat1 transcriptional activity by EphA4. COS7 cells were transfected with vector (V), kinase-dead (KD) mutant of EphA4 or EphA4 (A4), together with a luciferase reporter construct that was linked to Stat1-responsive enhancer element (*GAS-Luc*) or control construct that lacked the enhancer (*pTA-Luc*). The level of luminescence, which corresponded to the activity of luciferase, was measured (mean \pm S.E., $n = 3$). **C**, Western blot using antibodies against phospho-Jak2 and phospho-Tyk2 (left panel) showed that tyrosine phosphorylation of Jak2 and Tyk2 was induced in COS7 cells that overexpressed EphA4 but not EphA4KD. Similar loading was indicated by re-probing the membrane with anti-Jak2 antibody. The induction of Jak2 phosphorylation by EphA4 was further verified by immunoprecipitating (IP) Jak2 from the cell lysate of transfected COS7 cells and immunoblotted with anti-phosphotyrosine antibody (right panel). Similar loading was indicated by re-probing the membrane with anti-Jak2 antibody.



serum and was differentiated into myotubes in 60-mm plates by culturing in DMEM supplemented with 2% horse serum for 3 days. The Fc fragment of IgG (negative control) or ephrin-A1-Fc recombinant protein (200 μ g/ml) was pre-clustered in phosphate-buffered saline by anti-Fc antibody in a ratio (w/w) of 10:1 at room temperature for 2 h. To examine the induction of Stat3 tyrosine phosphorylation, the myotubes were serum-starved for 6–8 h prior to treatment with ephrin-A1. They were then aspirated and treated with pre-clustered Fc or ephrin-A1-Fc, which was diluted in DMEM to 2 μ g/ml. To examine the role of Jak2, the C2C12 myotubes were starved for 6 h in the presence of Me₂SO or AG490 (75 μ M) before the addition of Fc or ephrin-A1-Fc. To study the regulation of AChE protein level by ephrin-A1, C2C12 myotubes were pre-incubated with Me₂SO or AG490 (75 μ M) for 3 h. The cells were then treated with pre-clustered Fc or ephrin-A1-Fc in the presence of Me₂SO or AG490 for 24 h, after which the cells were lysed by RIPA for 1 h at 4 °C. The protein samples (12 μ g) were analyzed by SDS-PAGE and immunoblotted by antibody against AChE (1:8000). The membrane was then stripped and re-blotted with antibody against ERK-1/2 (Cell Signaling Inc.), and the intensities of the bands corresponding to AChE and ERK-2 were quantified by Photoshop 7.0.

Immunohistochemistry, Microarray Analysis, RNA Extraction, and Northern Hybridization.—Gastrocnemius muscle sections from adult rat were immunostained using rabbit polyclonal antibodies against Stat1 (1:300), Jak2 (1:100), or Stat3 (1:100) as described previously (33). To confirm the specificity of staining, the antibody was pre-incubated at 4 °C overnight with an equivalent amount of the peptide against which the antibody was raised. To test the effect of AG490 in regulating gene expression, C2C12 myotubes that were differentiated for 3 days were treated with Me₂SO or AG490 (75 μ M) for 48 h, and total RNA was extracted by the RNeasy kit (Qiagen) and analyzed by formaldehyde RNA gel as described previously (36). An equal amount of total RNA (15 μ g) was then reverse-transcribed to cDNA and hybridized with microar-

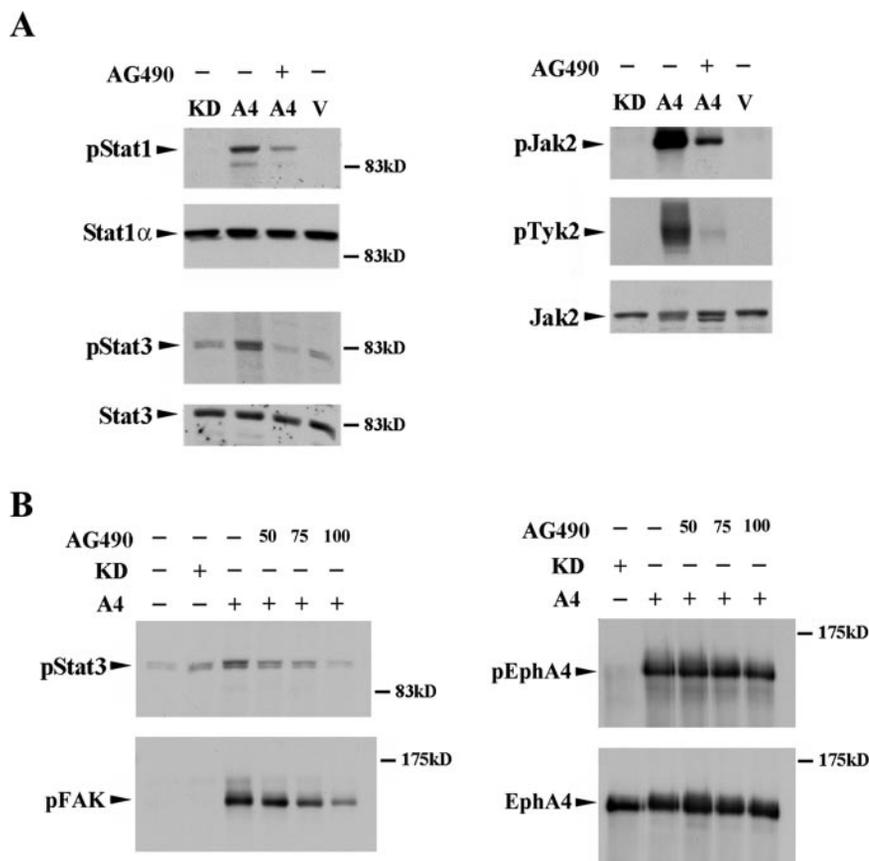
ray chip (Affymetrix chip set 230). Of the 6256 genes that were expressed in C2C12 myotubes, 186 genes were identified to be down-regulated in the presence of AG490 by 2-fold or more. To confirm the regulation of AChE transcript, differentiated C2C12 myotubes were treated with Me₂SO, AG490 (75 μ M), Fc, or ephrin-A1 (2 μ g/ml) for 24 h, and total RNA was extracted by the guanidinium thiocyanate method as described previously (36). Total RNA was analyzed by formaldehyde RNA gel, and Northern hybridization against cDNA probes corresponding to full-length AChE and partial sequence of fibronectin or tropomyosin (37) was performed as described previously (36).

Analysis of AChE Expression in EphA4 Null Mice.—Wild-type, heterozygous, or homozygous null mice were genotyped as described (38). Hindlimb muscle from EphA4^{-/-} mice and their wild-type littermates at postnatal day 14 were homogenized, and the cytosolic and membrane fractions were prepared as described above. The difference in AChE expression was consistently observed in two different pairs of wild-type and EphA4 null mice, and the representative result was shown. In addition, to eliminate the possible variation arising from different muscles, AChE expression was also compared between the gastrocnemius muscle from two different pairs of wild-type and EphA4 null mice originated from two distinct litters, and similar down-regulation of AChE protein level in EphA4 null mice was observed (data not shown).

RESULTS

Activation of the Jak/Stat Pathway by EphA4.—To screen for transcription factors that represent downstream targets of Eph receptor signaling, we examined potential signaling molecules activated by EphA4 overexpressed in COS7 cells. The cell lysate was then immunoblotted with antibodies that specifically recognized phosphorylated forms of various transcription factors. It was found that two members of the Stat family, Stat1

FIG. 2. Activated EphA4 induced tyrosine phosphorylation of Stat1 and Stat3 via Jak2. A, COS7 cells were transfected with EphA4 (A4) or kinase-dead (KD) mutant of EphA4. Two days after transfection, the cells were treated with Me₂SO or the Jak2 inhibitor AG490 (100 μM) for 6 h before cell lysate was collected and immunoblotted with antibodies against phospho-Stat1, phospho-Stat3 (left panel), phospho-Jak2, and phospho-Tyk2 (right panel). The presence of AG490 significantly reduced the induction of Jak/Stat tyrosine phosphorylation by EphA4. Immunoblotting the membranes with antibodies specific for Stat1α, Stat3, and Jak2 indicated similar loading. V, vector. B, COS7 cells were transfected with the kinase-dead (KD) mutant of EphA4 or EphA4 (A4) and treated with Me₂SO or different concentrations of AG490 (50–100 μM) for 6 h. The lysate was immunoblotted by antibodies against phospho-Stat3 and phospho-FAK (left panel). AG490 inhibited the tyrosine phosphorylation of Stat3 and FAK induced by overexpressed EphA4. EphA4 or EphA4KD was immunoprecipitated from transfected cells by anti-EphA4 antibody and immunoblotted by anti-phospho-tyrosine antibody (right panel). The autophosphorylation of EphA4 was not affected by AG490 even at higher concentration (100 μM). Immunoblotting the membrane with anti-EphA4 indicated that similar amount of EphA4 was immunoprecipitated in each sample.



and Stat3, were tyrosine-phosphorylated upon overexpression of EphA4 (Fig. 1A). The phosphorylation occurred on Tyr⁷⁰¹ and Tyr⁷⁰⁵ of Stat1 and Stat3, respectively, which were essential for their dimerization. The induction of Stat1 and Stat3 phosphorylation required the kinase activity of EphA4, because overexpression of a kinase-dead mutant of EphA4 (EphA4KD), in which the Lys⁶³⁰ was changed to Arg, failed to induce the tyrosine phosphorylation (Fig. 1A). To verify that the transcriptional activity of the Stat proteins was also induced by EphA4 activation, COS7 cells were co-transfected with EphA4 and a luciferase reporter construct that was linked to the Stat1-responsive enhancer element (GAS-Luc). Overexpression of EphA4 significantly induced the transcriptional activity of Stat1 (Fig. 1B).

Stat proteins are important components of the signaling pathways activated by many cytokines. The receptor complexes of cytokines lack an intrinsic kinase activity and require the Janus kinase (Jak), a family of non-receptor tyrosine kinase, which consists of Jak1, Jak2, Jak3, and Tyk2, to phosphorylate the Stat proteins (39). To examine whether EphA4 could activate the Jaks, the lysate of transfected COS7 cells was immunoblotted with antibody that specifically recognized Tyr^{1007/1008} of mouse Jak2 or Tyr^{1054/1055} of human Tyk2, which are essential for their kinase activity. Overexpression of EphA4 but not EphA4KD induced the Jak2 and Tyk2 phosphorylation (Fig. 1C). Taken together, these results demonstrate that both the Jaks and the Stat proteins were tyrosine-phosphorylated upon activation of EphA4. Furthermore, the resulting tyrosine phosphorylation of Stat1 correlated with increased transcriptional activity.

Stat1 and Stat3 might be tyrosine-phosphorylated directly by the autophosphorylated EphA4, or as in the case of cytokine receptors, their phosphorylation was induced by the Jaks. To distinguish between these two possibilities, the transfected cells were pre-treated with the Jak2 inhibitor AG490, which

specifically inhibited Jak2 but not other tyrosine kinases (40). The presence of AG490 significantly reduced the tyrosine phosphorylation of Stat1, Jak2, and Tyk2, and completely abolished the EphA4-induced tyrosine phosphorylation of Stat3 (Fig. 2A). AG490 also significantly decreased the EphA4-induced phosphorylation of the tyrosine kinase FAK (Fig. 2B). In contrast, AG490 did not affect the kinase activity of EphA4, as revealed by the similar level of EphA4 autophosphorylation at various concentrations of AG490 (Fig. 2B). These results therefore suggested that activation of EphA4 would recruit Jak2 to phosphorylate Stat1 and Stat3.

Jak2 Was Constitutively Associated with EphA4—To study further the interactions between EphA4, Jak2, and Stat proteins, the cell lysate of COS7 cells transfected with EphA4 was subjected to immunoprecipitation by antibody against Jak2, and the product was immunoblotted by anti-EphA4 antibody. EphA4 was co-immunoprecipitated with Jak2 by the anti-Jak2 antibody (Fig. 3A, upper panel). The association between EphA4 and Jak2 was confirmed in the reciprocal immunoprecipitation (Fig. 3A, middle panel). Interestingly, although EphA4KD did not induce tyrosine phosphorylation of Jak/Stat proteins (Fig. 1, A and C), co-immunoprecipitation experiments showed that it could still interact with Jak2 (Fig. 3A). Thus it was likely that the association between Jak2 and EphA4 was constitutive and did not require tyrosine phosphorylation of the Eph receptor. The specificity of the co-immunoprecipitation was indicated by the absence of Stat1 in the EphA4 complex (Fig. 3A, lower panel). The interaction between EphA4 and Jak2 was further verified by GST pull-down assay. The N-terminal (contained the JH domains 3–7) or C-terminal half (contained the JH domains 1–3) of Jak2 was individually linked to GST. Much stronger interaction was observed for EphA4 and GST-JH3–7 when compared with GST-JH1–3 (Fig. 3B), indicating that EphA4 mainly interacted with the N-terminal half of Jak2.

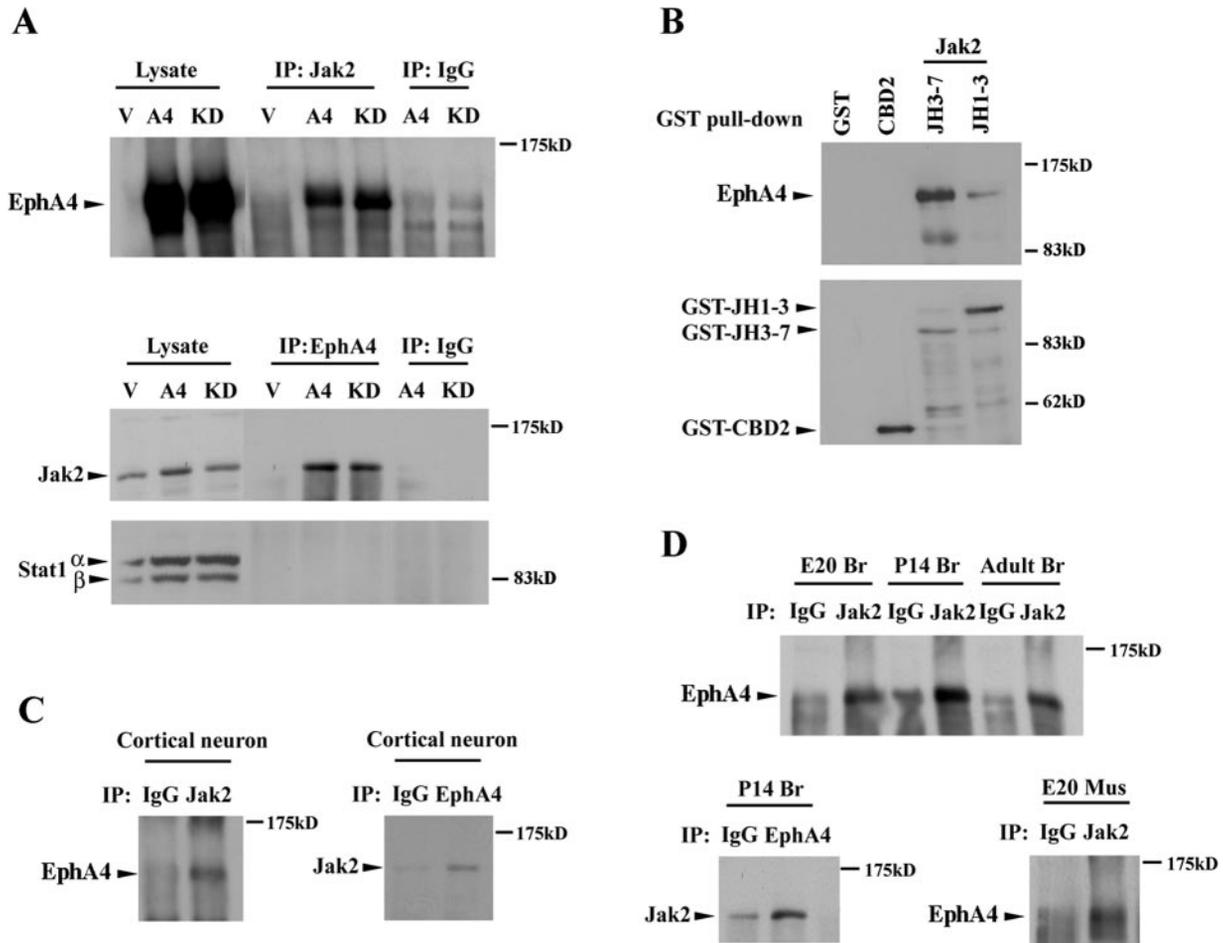


FIG. 3. EphA4 interacted with Jak2 *in vitro* and *in vivo*. *A*, EphA4 (A4), kinase-dead (KD) mutant of EphA4, or vector (V) was transfected into COS7 cells, and the cell lysate was immunoprecipitated (IP) by anti-Jak2 antibody and immunoblotted by antibody against EphA4 (*upper panel*). In the negative control, rabbit IgG was used in the immunoprecipitation. Both the wild-type EphA4 and the kinase-dead mutant was co-immunoprecipitated with Jak2. The association between EphA4 and Jak2 was verified in reciprocal immunoprecipitation, in which Jak2 was co-immunoprecipitated by anti-EphA4 antibody (*middle panel*). The association between Jak2 and EphA4 was specific, since Stat1 was not immunoprecipitated (IP) by anti-EphA4 antibody (*lower panel*). Similar experiments showed that Stat3 was not co-immunoprecipitated with EphA4 (data not shown). *B*, the N-terminal (JH domains 3–7) or C-terminal half (JH domains 1–3) of Jak2 was linked to GST, and the fusion proteins were purified and used to pull down EphA4 from lysate of transfected COS7 cells. EphA4 was mainly pulled down by the N-terminal half of Jak2 that contained the JH3 to JH7 domains (*upper panel*). The specificity of the pull-down assay was indicated by the absence of EphA4 in the pull-down reaction using GST alone or GST-CBD2 (cytokine-binding domain 2) of LIF receptor. Similar amounts of different GST fusion proteins were used in the pull-down assay, as confirmed by re-blotting the membrane with anti-GST antibody (*lower panel*). *C*, Jak2 was associated with EphA4 in neurons. Cortical neurons, which expressed endogenous EphA4 and Jak2, were cultured for 15 days, and the cell lysate was subjected to immunoprecipitation by anti-Jak2 antibody. EphA4 was detected in the immunoprecipitated reaction by anti-Jak2 antibody but not rabbit IgG (*left panel*). Likewise, Jak2 was reciprocally immunoprecipitated by anti-EphA4 antibody but not rabbit IgG (*right panel*). *D*, the interaction between EphA4 and Jak2 occurred *in vivo*. Membrane proteins of rat brain (*Br*) at different developmental stages (embryonic day 20, postnatal day 14, and adult) were subjected to immunoprecipitation by anti-Jak2 antibody, and the product was immunoblotted by EphA4 antibody. Significantly stronger signal of EphA4 was detected in the immunoprecipitation reaction by anti-Jak2 antibody compared with the IgG negative control (*upper panel*). The association between EphA4 and Jak2 in postnatal day 14 rat brain was verified by the reciprocal immunoprecipitation (*lower panel, left*). EphA4 was also co-immunoprecipitated by anti-Jak2 antibody from rat hindlimb muscle (*Mus*) at embryonic day 20 (*lower panel, right*).

To examine whether the association between Jak2 and EphA4 was observed in cells that endogenously expressed the two proteins, the immunoprecipitation was performed using the cell lysate of cultured cortical neurons. Similar to the results in transfected COS7 cells, Jak2 and EphA4 was co-immunoprecipitated, and the association was observed in the reciprocal immunoprecipitation (Fig. 3C). Finally, to examine whether the interaction between Jak2 and EphA4 occurred *in vivo*, the co-immunoprecipitation was performed using membrane protein fractions of rat brain and muscle. The association between Jak2 and EphA4 was observed in both brain and muscle (Fig. 3D).

Induction of Stat3 Tyrosine Phosphorylation by Ephrin-A1 in Cultured Myotubes—To confirm the activation of Jak/Stat pathway by EphA receptor signaling, the muscle cell line C2C12, which endogenously expressed EphA4 and EphA7 (33),

was differentiated into myotubes and treated with ephrin-A1-Fc fusion protein. Ephrin-A1 increased the tyrosine phosphorylation of Stat3, but not Stat1, in differentiated C2C12 myotubes (Fig. 4A and data not shown). Moreover, the enhancement of Stat3 phosphorylation was abolished by AG490, indicating the requirement of Jak2 in the ephrin-A1-induced Stat3 phosphorylation (Fig. 4B). Ephrin-A1 also down-regulated the phosphorylation of ERK in C2C12. The presence of AG490, however, did not affect the ephrin-A1-regulated ERK phosphorylation, thereby indicating the specific involvement of Jak2 in the ephrin-mediated Stat3 phosphorylation in muscle (Fig. 4B).

A previous study from our laboratory (33) has demonstrated the prominent concentration of EphA4 at the postsynaptic apparatus of the adult NMJ. Although the expression of Jak/Stat proteins in muscle has been reported (41, 42), little was known

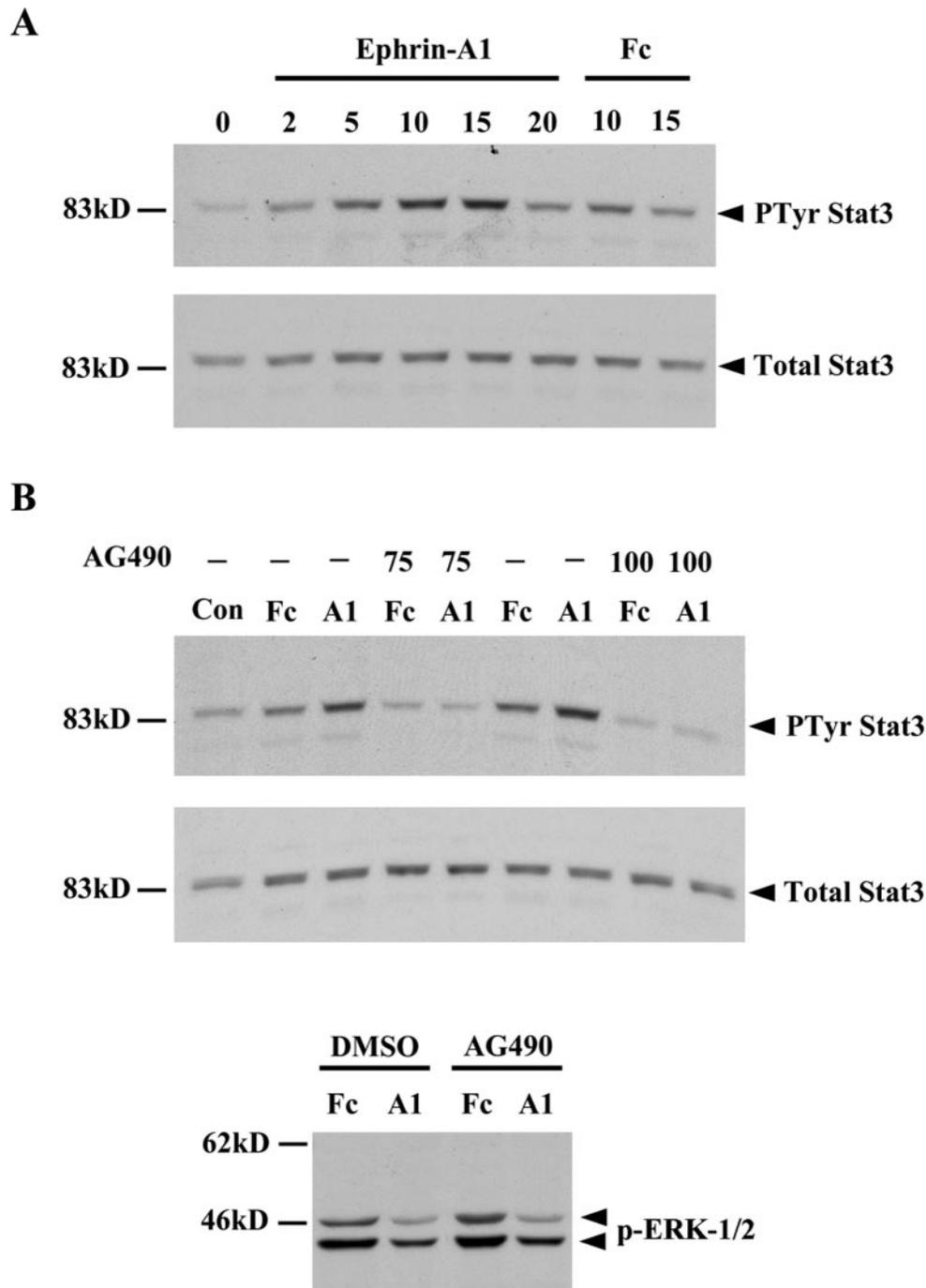


FIG. 4. Induction of Stat3 tyrosine phosphorylation by ephrin-A1 in cultured myotubes. *A*, the muscle cell line C2C12, which expressed endogenous EphA4, was differentiated into myotubes and treated with ephrin-A1 (2 μ g/ml) for 2–20 min. Equivalent amount of Fc fragment of IgG (Fc) was used as the negative control. The cell lysate was immunoblotted by anti-phospho-Stat3 antibody (*upper panel*). Induction of Stat3 tyrosine phosphorylation was observed upon treatment with ephrin-A1 compared with the Fc negative control. The membrane was stripped and re-probed with Stat3 to indicate similar loading (*lower panel*). *B*, the induction of Stat3 tyrosine phosphorylation by ephrin-A1 was dependent on Jak2. Differentiated C2C12 myotubes were pre-treated with AG490 (75 or 100 μ M) or Me₂SO (*DMSO*) for 6 h before treating with ephrin-A1 or Fc in the presence of AG490 or Me₂SO. In the negative control (*Con*), neither Fc nor ephrin-A1 was added. The cell lysate was immunoblotted by anti-phospho-Stat3 antibody (*upper panel*). The ephrin-A1-induced tyrosine phosphorylation of Stat3 was observed in the presence of Me₂SO but not the Jak2 inhibitor AG490. Similar loading was indicated by blotting the membrane with Stat3. The specificity of the action of AG490 was confirmed by immunoblotting the lysate with anti-phospho-ERK antibody (*lower panel*). The threonine/tyrosine phosphorylation of ERK-1/2 was reduced after treatment with ephrin-A1 for 10 min in the presence of Me₂SO, and AG490 did not affect the ephrin-A1-induced down-regulation of ERK-1/2 phosphorylation.

about their localization in muscle fibers. The localization of Jak/Stat proteins in muscle sections was therefore examined by immunohistochemistry. Both Stat1 and Stat3, as well as Jak2, were largely confined to the NMJ of adult muscle (Fig. 5). The specificity of staining was verified by the absence of fluorescence when the antibodies were pre-incubated with the corresponding blocking peptides. Taken together, the induction of

Stat3 phosphorylation by ephrin-A1 in C2C12 myotubes and the concentration of EphA4 and Jak2/Stat3 at the NMJ have raised the possibility that the activation of EphA4 in muscle might trigger the Jak/Stat pathway and regulate gene transcription at the NMJ.

Regulation of AChE Expression in Muscle by Jak/Stat Proteins—To elucidate the possible roles of Jak/Stat proteins at

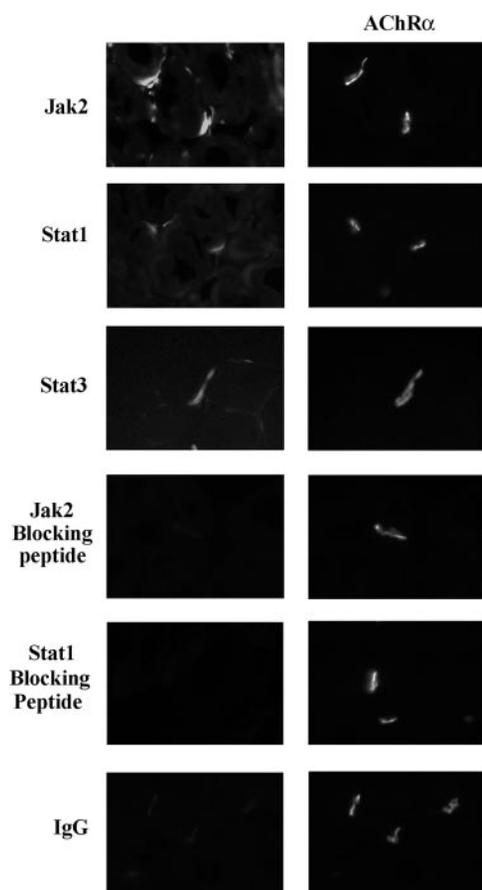


FIG. 5. Localization of Jak/Stat proteins at the neuromuscular junction. Double immunofluorescence staining of adult rat gastrocnemius muscle sections indicated the co-localization of Jak2, Stat1, and Stat3 (left panel) with acetylcholine receptor (*AChR α*), which was detected by rhodamine-conjugated α -bungarotoxin (right panel). The specificity of staining was confirmed by the absence of fluorescence when the antibodies against Jak2 and Stat1 were pre-incubated with the corresponding peptides against which the antibodies were raised or in the negative control in which the primary antibody was substituted by rabbit IgG.

the NMJ, differentiated C2C12 myotubes were incubated with either Me_2SO or AG490 for 2 days. Total RNA was then extracted and genes that were differentially expressed were identified by microarray hybridization. Out of the 6256 genes that were expressed in C2C12 myotubes and identified by the microarray chips, 186 genes were found to be down-regulated by 2-fold or more in the presence of AG490 (data not shown). One of the genes was AChE, the enzyme that catalyzes the hydrolysis of the neurotransmitter acetylcholine at the NMJ (43). Northern blot analysis confirmed the decrease in AChE transcript level by AG490 (Fig. 6A). The expression of tropomyosin remained constant in the presence of AG490, indicating the specificity of the inhibitor.

Regulation of AChE Protein Expression by Ephrin-A1/EphA4 in Muscle—The identification of AChE as the potential target gene of the Jak/Stat pathway in cultured myotubes led us to examine whether activation of the ephrin/Eph signaling could regulate the expression of AChE in muscle. Although ephrin-A1 could increase the tyrosine phosphorylation of Stat3 in C2C12 myotubes (Fig. 4, A and B), incubation with ephrin-A1 for 12, 24, or 48 h did not change the expression level of AChE transcript (Fig. 6B, upper panel and data not shown). Interestingly, when we investigated the effect of ephrin-A1 on the expression of the cell adhesion molecule fibronectin in muscle, a reduction in the fibronectin transcript level by ephrin-A1 was consistently observed, which was not abolished by AG490 (Fig.

6B, lower panel). Taken together, these findings indicated that ephrin-A1 could down-regulate the expression of fibronectin mRNA in C2C12 myotubes via a distinct signaling pathway that did not involve Jak2.

Although stimulation of C2C12 myotubes with ephrin-A1 did not change the expression of AChE transcript, the expression of AChE protein was regulated upon ephrin treatment. An increase in AChE protein level was consistently observed when C2C12 myotubes were treated with ephrin-A1 for 24 h (Fig. 6C). More importantly, the induction of AChE protein by ephrin-A1 was abolished by AG490 (Fig. 6C), indicating the involvement of Jak2 in the regulation of AChE protein expression in cultured myotubes. To verify whether the ephrin-regulated AChE expression in muscle occurred *in vivo*, the expression of AChE protein in hindlimb muscle of EphA4 null mice was analyzed. Mice that lacked EphA4 exhibited a dramatic reduction of AChE level in the hindlimb muscle when compared with the wild-type mice (Fig. 6D). Four different pairs of wild-type and EphA4 null mice were analyzed, and the difference in AChE protein level was consistently observed. These results therefore indicate that ephrin/Eph signaling in muscle can regulate the expression of AChE protein in muscle. Moreover, since AChE is localized at the NMJ and is responsible for hydrolyzing the neurotransmitter acetylcholine at the NMJ, the present data strongly suggest a functional role of ephrin/Eph receptor signaling in the development and functioning of the NMJ.

DISCUSSION

Our understanding of the molecular mechanisms of Eph receptors in axon guidance, cell adhesion, and cell migration has advanced rapidly in recent years (6, 13). In contrast, very little was known about the underlying mechanisms of how Eph receptors were involved in synaptic development and plasticity at the neuronal synapse or the NMJ, which could be long lasting and might involve the regulation of gene expression. The present study has identified a novel signaling pathway of EphA4, which involves the activation of the Jak/Stat proteins. We found that autophosphorylation of EphA4 led to the activation of Jak2, which in turn phosphorylated Stat1 and Stat3 and enhanced the transcriptional activity. Our study also provides the first evidence that the Jak/Stat proteins play a potential role at the synapse by regulating the expression of AChE at the NMJ. Furthermore, the present study demonstrates a possible function of ephrins/Eph receptors at the NMJ in terms of regulating the expression of AChE, an enzyme critical for neurotransmission at the NMJ. In particular, the ephrin-induced AChE expression requires the activity of Jak2. Finally, we provide evidence that ephrin-A1 can down-regulate the expression of fibronectin mRNA in muscle via a Jak2-independent mechanism. Together, these findings provide an important insight into the signal transduction of Eph receptors that leads to the regulation of gene transcription and protein expression.

Jak, a family of non-receptor tyrosine kinases that includes Jak1, Jak2, Jak3, and Tyk2, and their downstream targets Stat transcription factors were first identified as important components of the signaling pathways activated by the neuropoietic cytokines. Later studies, however, have reported the phosphorylation and activation of Stat transcription factors by various RTK, such as the receptors of epidermal growth factor (EGF), neuregulin, and platelet-derived growth factor (44–47). Although the Jaks are also phosphorylated by these RTK, they may not be responsible for phosphorylating the Stat proteins. Indeed, both EGF receptor and platelet-derived growth factor receptor can phosphorylate Stat proteins by a Jak-independent pathway (45, 46), whereas the neuregulin receptor ErbB-in-

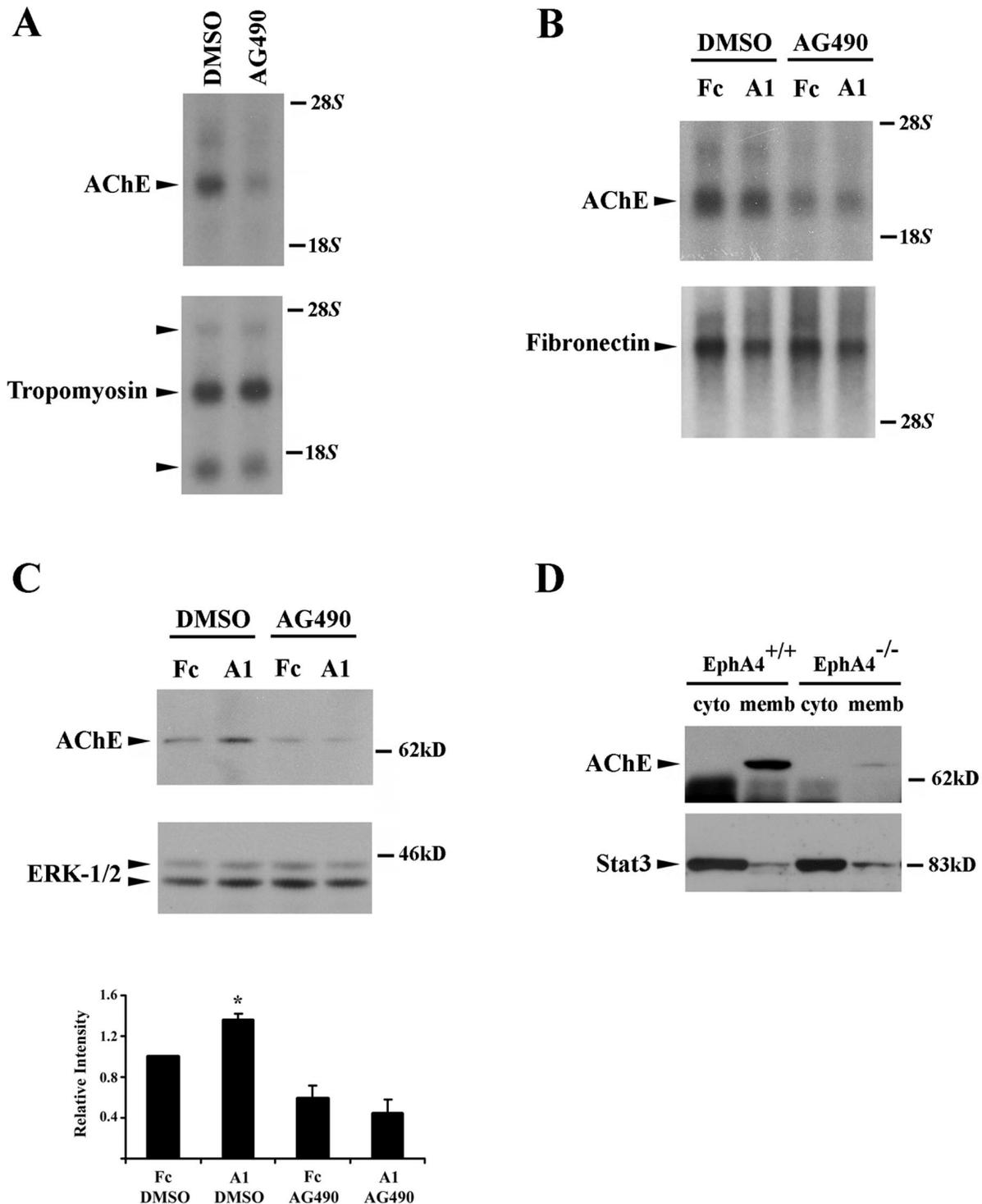


FIG. 6. Regulation of acetylcholinesterase expression by ephrin/Eph signaling in muscle was dependent on Jak2. *A*, differentiated C2C12 myotubes were treated with Me₂SO (DMSO) or AG490 (75 μ M) for 24 h, and total RNA was analyzed in Northern blot and hybridized with acetylcholinesterase (AChE, upper panel). The level of AChE transcript was significantly reduced by AG490. Similar results were obtained when lower concentration (25 μ M) of AG490 was added (data not shown). The membrane was re-probed with tropomyosin to indicate similar loading of RNA (lower panel). The positions of ribosomal RNAs (28 S and 18 S) were indicated. *B*, differentiated C2C12 myotubes were treated with ephrin-A1 or Fc (2 μ g/ml) for 24 h in the presence of Me₂SO or AG490 (75 μ M). Northern blot analysis with AChE (upper panel) and fibronectin (lower panel) showed that ephrin-A1 decreased the expression of fibronectin transcript, and the reduction was not affected by the presence of AG490. *C*, differentiated C2C12 myotubes were pre-treated with Me₂SO or AG490 (75 μ M) for 3 h and then incubated with ephrin-A1 or Fc (2 μ g/ml) for 24 h in the presence of Me₂SO or AG490. The cell lysate was immunoblotted by antibody against AChE (upper panel). The expression of AChE protein was increased by ephrin-A1, and the induction was abolished by AG490. The membrane was stripped and re-probed with antibody against ERK-1/2 to indicate similar loading (lower panel). The intensity of the AChE bands was quantified after normalization with that of the ERK-2 bands (mean \pm S.E., $n = 4$). The difference in band intensity between Fc and ephrin-A1 in the presence of Me₂SO was statistically significant (*, $p < 0.001$). *D*, expression of AChE protein in muscle of wild-type and EphA4 knockout mice. Cytosolic and membrane fractions of hindlimb muscles from wild-type (+/+) and EphA4 null mice (-/-) at postnatal day 14 were immunoblotted by antibody against AChE (upper panel). The level of AChE protein was significantly reduced in EphA4^{-/-} mice, and the difference was consistently observed in four different pairs of wild-type and homozygous null mice. The membrane was stripped and re-probed with antibody against Stat3 (lower panel) to indicate similar loading of proteins between the +/+ and -/- mice.

duced phosphorylation of Stat proteins is mediated by Src (47). The present study, however, suggests that Jak2 is the important tyrosine kinase that phosphorylates Stat1 and Stat3 after the activation of EphA4. Unlike the EGF receptor, which interacts directly with Stat1, no interaction between EphA4 and Stat1 (Fig. 3A) or Stat3 (data not shown) was observed in the co-immunoprecipitation. On the other hand, the interaction between EphA4 and Jak2 was demonstrated in reciprocal co-immunoprecipitation and GST pull-down assays. Moreover, the EphA4-induced Stat1 and Stat3 tyrosine phosphorylation was significantly inhibited by the Jak2 inhibitor AG490, which did not inhibit other tyrosine kinases such as Src (40). Interestingly, the presence of AG490 also significantly decreased the EphA4-induced phosphorylation of FAK. It is noteworthy that FAK has been reported to be a downstream target of Jak2 (48, 49), suggesting that activated EphA4 phosphorylates FAK via Jak2. The results also indicate that activation of Jak2 by EphA4 does not only lead to tyrosine phosphorylation of the Stat proteins but also other substrates of Jak2, such as FAK.

The identification of Jak2 as the interacting protein of EphA4 adds this kinase to the list of non-receptor tyrosine kinases, including FAK, Src, and Abl, that serve as candidate downstream targets of Eph receptor signaling. It is noteworthy, however, that the association between Jak2 and EphA4 appears to be constitutive and does not require tyrosine phosphorylation of the receptor, since both the wild-type EphA4 and the kinase-dead mutant were co-immunoprecipitated with Jak2 to a similar extent. The interaction of Jak2 with EphA4 is therefore different from that of the Src kinase, which interacts with the phosphotyrosine residues of the activated Eph receptors via the SH2 domain (26–28). The interaction between Abl and EphB2 is more complex and involves both a phosphorylation-dependent mechanism that requires the SH2 domain of Abl, as well as a phosphorylation-independent mechanism that requires the C terminus of Abl (30). Thus, different non-receptor tyrosine kinases utilize distinct mechanisms to interact with Eph receptors.

The constitutive association between EphA4 and Jak2 is reminiscent of the interaction between Jak2 and EGF receptors, which is pre-associated with Jak2 before stimulation by EGF (47). Jak consists of seven Jak homology (JH) domains (34). The GST pull-down experiment suggested that the N-terminal half of Jak2 that contained JH3 to JH7 was mainly responsible for interacting with EphA4. Detailed mutational analysis will be required to elucidate which domain(s) of Jak2 interacts with EphA4.

Our previous findings have revealed the unexpected expression of EphA4 in muscle and its localization at the NMJ (33). We are therefore interested to explore the functional consequences after activation of the EphA4-Jak-Stat pathway in muscle. Previous studies (41, 50, 51) have reported the expression of Jak/Stat proteins in muscle, and they have been implicated to mediate the proliferation of myoblasts in response to LIF or growth hormone. However, little was known about their functional significance in differentiated myotubes. Immunohistochemistry revealed the concentration of Jak2, Stat1, and Stat3 at the adult NMJ, raising the intriguing possibility that they may be involved in the development and/or functioning of the NMJ in muscle fiber. Interestingly, Jak2 and Stat3 have been reported recently (52) to be distributed in the postsynaptic density of the brain, although evidence that they are involved in the development or functioning of neuronal synapses is still lacking. The observation that AChE expression was significantly reduced by the Jak2 inhibitor AG490, as well as the synaptic localization of Jak/Stat proteins at neuronal synapses or NMJ, therefore strongly suggests a novel role of these mol-

ecules in the development and functioning of the synapses.

Ephrin-A1, which induced tyrosine phosphorylation of Stat3 but not Stat1 in C2C12 myotubes, did not regulate the expression of AChE mRNA. However, the expression of AChE protein was increased by ephrin-A1 in C2C12 myotubes, thus raising the intriguing possibility that ephrin-A1 may enhance the translation and/or stability of AChE protein in muscle. Moreover, the ephrin-A1-induced AChE protein level was abolished by AG490, indicating the involvement of Jak2. The signaling pathway that links Jak2 to protein synthesis and/or stability remains to be determined.

The regulation of AChE protein expression by the EphA receptor was further verified by the study of EphA4 knockout mice. The level of AChE protein was significantly reduced in the muscles of EphA4 null mice, thus providing further evidence for the regulation of AChE expression in muscle by the ephrin/Eph receptor signaling. It is noteworthy, however, that the expression of AChE in muscle is regulated by innervation of the motor neuron (53). EphA4 is not only expressed in muscle but is also present in the motor neuron, and the positioning of the motor neuron within the spinal cord is affected in EphA4-deficient mice (54). Thus, it is possible that the observed reduction of AChE expression in EphA4 null mice can result either directly from the absence of EphA4 signaling at the postsynaptic muscle side or indirectly from defective muscle innervation, or both. The last possibility is supported by the observation that the difference in AChE expression between wild-type and EphA4 null mice was consistently greater than the extent of AChE induction by ephrin-A1 in C2C12 myotubes (compare Fig. 6, C and D).

Although ephrin-A1 did not induce the expression of AChE transcript, it did regulate the expression of fibronectin mRNA in C2C12 myotubes. Many studies have reported that the activation of Eph receptor signaling can reduce cell adhesion (13). However, it is generally believed that the change in cell adhesion is short term and involves modulation of the actin cytoskeleton. The observation that long term treatment with ephrin-A1 led to reduced expression of fibronectin suggests a novel mechanism underlying the regulation of cell adhesion by Eph receptor signaling. The detailed signaling pathways and the transcription factors involved in the regulation remain to be determined.

Taken together, the present study has identified the Jak/Stat proteins as novel targets of EphA4 signaling. Moreover, it provides important insights into the potential significance of Eph receptors at the NMJ. Because of the critical roles of AChE in neurotransmission between motor neuron and muscle fiber (43), our study strongly suggests a crucial role of EphA4 in the development and functioning of the NMJ. In addition, it is plausible that ephrin-A1 is involved in the development or maturation of the NMJ by regulating the expression of fibronectin, one of the components of the basal lamina at the NMJ (55). It will be interesting to explore whether the expression of AChE and fibronectin is similarly regulated at the neuronal synapses in the brain.

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