MCN

Molecular and Cellular Neuroscience **17,** 1034–1047 (2001)
doi:10.1006/mcne.2001.0997, *available online at http://www.idealibrary.com on* IDEAL®

Expression of Eph Receptors in Skeletal Muscle and Their Localization at the Neuromuscular Junction

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The participation of ephrins and Eph receptors in guiding motor axons during muscle innervation has been well documented, but little is known about their expression and functional significance in muscle at later developmental stages. Our present study investigates the expression and localization of Eph receptors and ephrins in skeletal muscle. Prominent expression of EphA4, EphA7, and ephrin-A ligands was detected in muscle during embryonic development. More importantly, both EphA4 and EphA7, as well as ephrin-A2, were localized at the neuromuscular junction (NMJ) of adult muscle. Despite their relative abundance, they were not localized at the synapses during embryonic stages. The concentration of EphA4, EphA7, and ephrin-A2 at the NMJ was observed at postnatal stages and the synaptic localization became prominent at later developmental stages. In addition, expression of Eph receptors was increased by neuregulin and after nerve injury. Furthermore, we demonstrated that overexpression of EphA4 led to tyrosine phosphorylation of the actin-binding protein cortactin and that EphA4 was coimmunoprecipitated with cortactin in muscle. Taken together, our findings indicate that EphA4 is associated with the actin cytoskeleton. Since actin cytoskeleton is critical to the formation and stability of NMJ, the present findings raise the intriguing possibility that Eph receptors may have a novel role in NMJ formation and/or maintenance.

INTRODUCTION

Eph receptors represent the largest family of receptor tyrosine kinases (RTK). They are activated by ephrins, which are membrane-bound proteins being classified based on their modes of membrane anchorage: ephrins-A bind to membrane through the glycosylphosphatidylinositol linkage, while ephrins-B are transmembrane proteins. Accordingly, the receptors that interact preferentially with ephrins-A are classified as EphA and those with ephrins-B are known as EphB receptors (Gale *et al.,* 1996). Ephrins and Eph receptors play pivotal roles during nervous system development. They have been demonstrated to mediate topographic mapping in various parts of the central nervous system, as well as segmentation during hindbrain development and neural crest cell migration (reviewed by Flanagan and Vanderhaeghen, 1998). In addition, Eph receptors may also participate in the functioning of adult nervous system. Several Eph receptors and ephrin-B ligands are localized at the synapses of hippocampal neurons (Torres *et al.,* 1998; Buchert *et al.,* 1999). Moreover, ephrin-A5 has been demonstrated to promote long-term potentiation of hippocampal neurons (Gao *et al.,* 1998), and intrahippocampal infusion of ephrin-A5 immunoadhesin into mice could enhance their performance in learning (Gerlai *et al.,* 1999). These observations suggest that ephrins may play a role in mediating learning and memory, though little is known about the underlying mechanisms of how Eph receptors modulate synaptic functions in the brain.

Several lines of evidence indicate that ephrins are also involved in limb patterning and innervation. During the period of limb innervation, ephrin-A2 and ephrin-A5 are expressed in the limb bud (Ohta *et al.,* 1997; Iwamasa *et al.,* 1999), while EphA3 and EphA4 are expressed in different subtypes of motor neurons (Ohta *et al.,* 1996; Kilpatrick *et al.,* 1996). EphA4 and EphA7 are also expressed in the dorsal mesenchyme of the limb bud, and disruption of their expression affects pathfind-

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ing of specific motor neurons (Araujo *et al.*, 1998; Helmbacher *et al.*, 2000). In addition to being a guidance cue for axon navigation, ephrins are also expressed in muscle during early development and play a role in the topographic matching between motor neurons and muscle fibers (Feng *et al.*, 2000). However, all these studies focus on the roles of ephrins in axon guidance and topographic mapping of motor neurons, while the importance of ephrins in muscle beyond the period of limb innervation has not yet been addressed. Moreover, it remains largely unknown whether muscles express both Eph receptors and ephrins.

We have previously demonstrated the expression of ephrin-A3 and ephrin-A5 isoforms in muscle (Lai *et al.*, 1999). In particular, both ligands are expressed in muscle throughout development and their expression persists in adulthood. In the present study, we examine the expression of Eph receptors in skeletal muscle and explore the potential role in neuromuscular junction (NMJ) formation and maintenance. Prominent expression of two receptors, EphA4 and EphA7, is detected in embryonic muscle, and their expression persists in adult muscle. Importantly, both receptors, as well as the ligand ephrin-A2, are concentrated at the postsynaptic membrane on muscle fiber. Furthermore, their concentration at the NMJ is developmentally regulated, with the strongest synaptic localization occurring in adulthood. Our results raise the possibility of a new role for this family of RTK in the maintenance or functioning of NMJ. Moreover, NMJ may provide an alternative system to study the molecular mechanisms of how Eph receptor signaling affects synaptic structures and functions in the brain.

RESULTS AND DISCUSSION

Expression of EphA4, EphA7, and Ephrins-A in Skeletal Muscle

As a first step to examine the expression of different EphA receptors in skeletal muscle, RT-PCR, using specific primers to EphA3, A4, A5, A6, and A7, was performed. Two of the receptors, EphA4 and EphA7, were found to be prominently expressed in embryonic muscle (Fig. 1A). Detailed expression profiles of EphA4 and EphA7 in hindlimb muscle during development was examined by Northern blot analysis. A single transcript (~7 kb) for EphA4 and multiple transcripts (~6.8, 5.7, 4.0, and 3.2 kb) for EphA7 were detected in skeletal muscle throughout development. In addition, expression of both receptors was more prominent during early embryonic development and was down-regulated prior to birth. However, the transcripts of both receptors could be detected in adult muscle. Our previous study has revealed the expression of ephrin-A3 and ephrin-A5 in muscle during development by Northern blot analysis (Lai *et al.*, 1999). Both ligands were prominently expressed in muscle during early development, and their expression persisted in adult muscle. Likewise, the transcripts of the other three ephrin-A ligands were also expressed in muscle throughout development (Fig. 1B). Interestingly, the expression of ephrin-A2 and ephrin-A4 transcript decreased along development, while the expression of ephrin-A1 transcript remained relatively constant at various developmental stages. Moreover, RT-PCR indicated that ephrins-A1, A2, and A4, were expressed in adult muscle (Fig. 1C). Because there are multiple cell types, including non-muscle cells, present in skeletal muscle, it is possible that the expression of receptors and ligands may be found only in nonmuscle cells. To exclude this possibility, we investigate the expression of both Eph receptors and the ephrin-A ligands in the muscle cell line C2C12, which is derived from adult mouse leg muscle. Transcripts of expected sizes were detected in the differentiated C2C12 myotubes for both receptors and ligands (Fig. 1D), suggesting that they were indeed expressed in muscle fibres. Western blot analysis revealed a prominent band of about 120 kDa for both EphA4 and A7, indicating that the receptor proteins were expressed in muscle (Fig. 1E). Consistent with the Northern blot data, the protein expression of both receptors was down-regulated along development. Taken together, both EphA4 and EphA7, as well as all five ephrins-A, were expressed in skeletal muscle. More importantly, their expression remained in muscle after the period of limb innervation by motor axon. These observations therefore suggest that ephrins and Eph receptors may have other functions in muscle, in addition to acting as the guidance cues for pathfinding and target recognition of motor neurons.

Localization of EphA4, EphA7, and Ephrin-A2 at the NMJ of Adult Muscle

Previous study has reported the expression of Eph receptors in chick muscle by Western blot analysis, but the cellular distribution is unclear (Soans *et al.*, 1996). To determine the localization of Eph receptors and ephrins on muscle fibres, immunohistochemistry on adult muscle sections was performed using antibodies raised against the two receptors, EphA4 and EphA7, as well as ephrin-A2. To verify the absence of cross-reactivity of

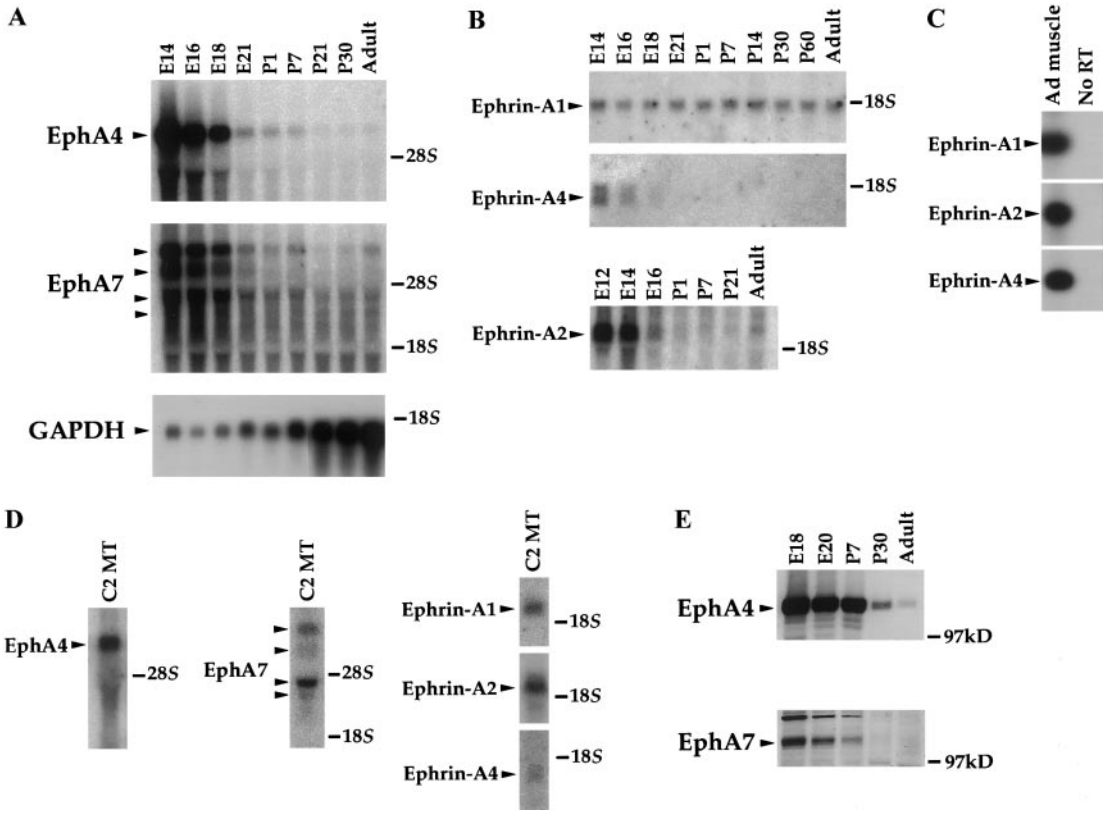

FIG. 1. Expression of EphA4, EphA7, and ephrins-A in skeletal muscle. (A) Northern blot analysis of EphA4 and EphA7 transcripts (indicated by arrowheads) in rat hindlimb muscle during development, including embryonic day 14 (E14), 16, 18, and 21, and postnatal day 1 (P1), 7, 21, and 30. The positions of ribosomal RNAs (28S and 18S) were indicated on the right. Hybridization with GAPDH probe was done as a loading control. (B) Northern analysis of ephrin expression in rat (ephrins-A1 and -A4) or mouse (ephrin-A2) hindlimb muscle during development. (C) Expression of ephrins-A1, A2, and A4 in adult (Ad) muscle examined by RT-PCR. The products of expected sizes (arrowheads) were amplified from adult muscle cDNA. In the negative control (No RT), no Reverse Transcriptase was added. (D) Expression of the Eph receptors and ephrins in the muscle cell line C2C12 myotubes (C2 MT). (E) Regulation of EphA4 and EphA7 protein expression during development of hindlimb muscle. The ~120-kDa band (indicated by arrowheads) represented the EphA4 and EphA7 proteins recognized by the specific antibodies (see Experimental Methods).

the EphA4 and EphA7 antibodies, expression constructs encoding full-length EphA4 and EphA7 were individually transfected into Cos-7 cells. The EphA4 antibody (sc-921) recognized a band of ~120 kDa in Cos cells transfected with EphA4 but not EphA7. In contrast, the EphA7 antibody (sc-917) recognized the 120-kDa band in Cos cells transfected with EphA7 but not EphA4 (Fig. 2A). Similarly, the specificity of the ephrin-A2 antibody had been tested by immunoblotting lysate of Cos cells transfected with either ephrin-A2 or ephrin-A3. Only lysate of Cos cells transfected with ephrin-A2 gave rise to the band of ~40 kDa (data not shown). The specificities of the antibodies in immunostaining were also examined. Cos cells transfected with ephrin-A2 were stained intensely by the ephrin-A2 antibody. On the other hand, Cos cells that overexpressed ephrin-A3 were not stained by the ephrin-A2 antibody (Fig. 2B, left panels). Similarly, the EphA4 antibody (sc-921) could only stain Cos cells transfected with EphA4 but not EphA7 (Fig. 2B, middle panels), while the EphA7 antibody (sc-917) specifically stained the EphA7-transfected cells (Fig. 2B, right panels).

Immunohistochemical study using antibody against EphA4 (sc-921) revealed that EphA4 was stained considerably stronger at the NMJ than the extra-junctional regions of the muscle fibres (Fig. 3A, i and ii); the NMJ staining of EphA4 was confirmed by another antibody (Sek_1), which recognized a nonoverlapping epitope of EphA4 (Fig. 3A, iii and iv). Similarly, antibody against EphA7 (sc-917) stained strongly at the junctional regions of adult muscle sections (Fig. 3B, i and ii); the synaptic staining of EphA7 could also be demonstrated

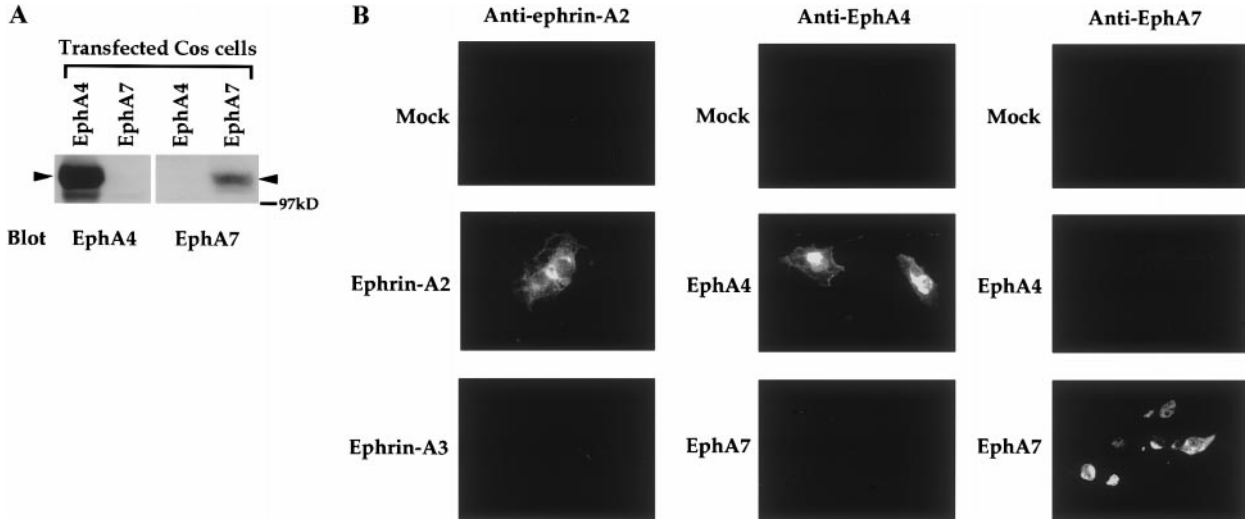

FIG. 2. Specificities of the antibodies against EphA4, EphA7, and ephrin-A2. (A) Full-length expression constructs of EphA4 and EphA7 were transfected into Cos7 cells, and the cell lysates were immunoblotted by the antibody against EphA4 or EphA7. EphA4 and EphA7 (indicated by arrowheads) were recognized by their corresponding antibodies and little cross-reactivity was observed. (B) Cos7 cells transfected with ephrin-A2, ephrin-A3, or the expression vector pMT21 (Mock) were stained by the anti-ephrin-A2 antibody (left panels). Only the cells transfected with ephrin-A2 were stained by the antibody. Similarly, the antibody against EphA4 only stained the EphA4- but not EphA7- or mock-transfected Cos cells (middle panels), while the EphA7 antibody specifically stained the EphA7-transfected cells (right panels).

by another EphA7 antibody (sc-1015) that recognized a distinct epitope of EphA7 (Fig. 3B, iii and iv). The specificity of staining was confirmed by the elimination of signal when the antibodies were preincubated with 10-fold excess of their respective blocking peptides (Fig. 3A, v and vi and Fig. 3B, v and vii). Thus, our findings indicate that both EphA4 and EphA7 are localized at the NMJ on adult muscle fibres. Since activation of Eph receptors require their interactions with ephrins, we further examine the localization of ephrin in adult muscle. Double immunostaining using anti-ephrin-A2 antibody revealed that ephrin-A2 was also localized at the adult NMJ (Fig. 3C, i and ii), and the synaptic staining of ephrin-A2 was eliminated when the antibody was preincubated with the blocking peptide (Fig. 3C, iii and iv).

There are three cell types which constitute the adult NMJ: the presynaptic terminal of motor axon, the postsynaptic muscle fiber, and the terminal Schwann cell. The concentration of Eph receptors at the NMJ therefore does not necessarily imply the localization occurs on the postsynaptic membrane. To rule out the possibility that the expression of Eph receptors is restricted to the terminal Schwann cells, double immunostaining of EphA7 and AChR was compared with that of S-100, a marker for terminal Schwann cells (Jessen and Mirsky, 1991). While there was some overlap between S-100 and AChR staining, considerable portion of the S-100 staining extended beyond that of AChR (Fig. 3D, upper panel). On the other hand, the staining of EphA7 overlapped with that of AChR to a much greater extent (Fig. 3D, lower panel). Thus, it is highly unlikely that the localization of Eph receptor at the NMJ is exclusively contributed by the terminal Schwann cells.

It is possible that the receptors localized at NMJ are present on the presynaptic terminal of motor neurons rather than the postsynaptic apparatus on muscle fiber. To confirm the concentration of EphA4, EphA7, and ephrin-A2 on the postsynaptic specializations, immunostaining was performed on denervated muscle. Upon denervation, the axon terminal normally degenerates by day 4 after nerve cut. However, staining by antibody against EphA4, EphA7, or ephrin-A2 remained at the NMJ twenty days after denervation (Figs. 4A–4C). In contrast, antibody against the presynaptic protein synapsin I stained intensely at the adult NMJ but the staining disappeared at day 4 after denervation (Fig. 4D). Although both the ligands and receptors remained at the NMJ after nerve injury, the intensity of staining at the synapses reduced considerably. This expression profile is similar to that observed for the localization of some other postsynaptic proteins, such as the muscle specific kinase (MuSK) and neuregulin (NRG), in denervated muscle (Bowen *et al.*, 1998; Rimer *et al.*, 1998).

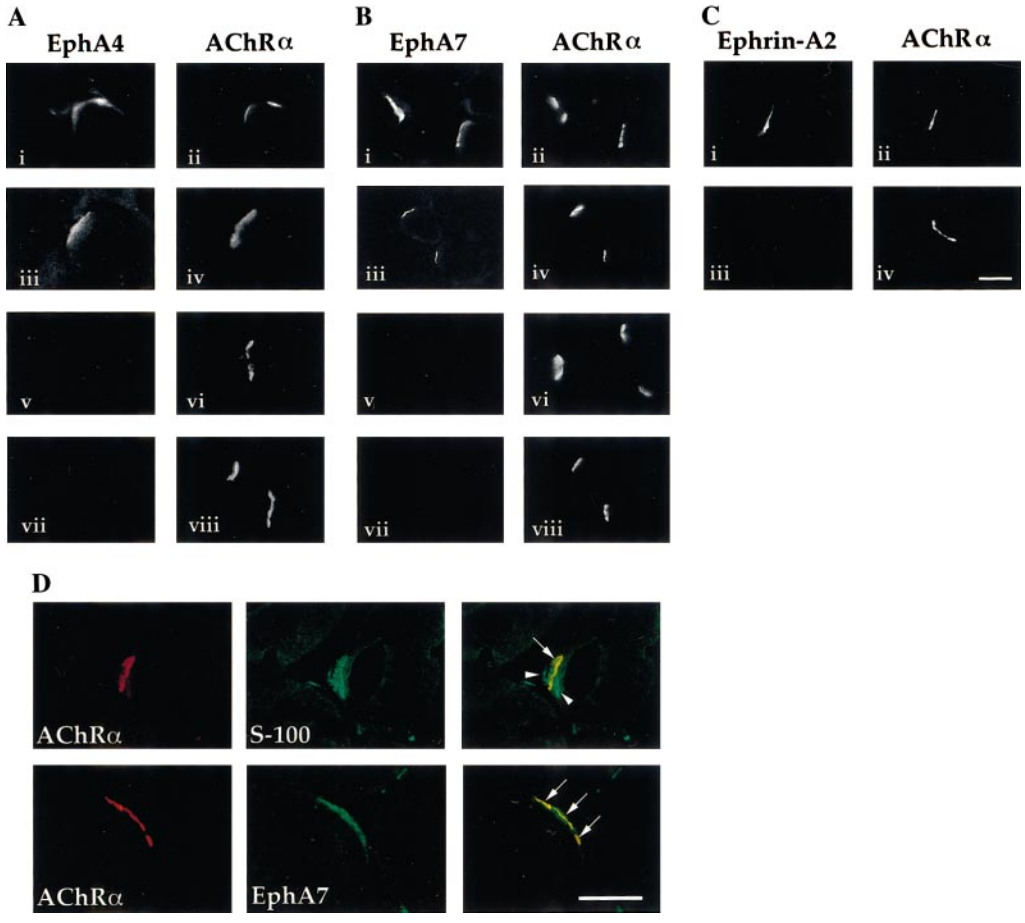

FIG. 3. Localization of EphA4, EphA7, and ephrin-A2 at the NMJ of adult rat muscle. (A) Double immunostaining indicated the co-localization of AChRα (ii and iv, detected by rhodamine-conjugated α-bungarotoxin) and EphA4 (i and iii). The NMJ staining of EphA4 was confirmed by two different antibodies, sc-921 (i) and Sek_1 (iii) (see Experimental Methods). (B) Immunostaining using two different EphA7 antibodies, sc-917 (i) and sc-1015 (iii), revealed that EphA7 was colocalized with AChRα (ii and iv). (C) Colocalization of ephrin-A2 (i) and AChRα (ii). The staining at NMJ was absent when the antibodies were pre-incubated with the corresponding blocking peptides (A v; B v, vii, and C iii) or in the absence of EphA4 antibody (A vii), while the AChRα staining served as positive control (A vi, viii, B vi, viii, and C iv). (D) Double immunostaining of S-100 and AChRα (upper panels) or EphA7 and AChRα (lower panels) of adult rat muscle sections (6 μm). The staining of S-100 and EphA7 (green) was compared with that of AChRα (red), and the overlapping region was indicated by yellow colour. While there was some overlapping staining of the Schwann cell marker S-100 and AChRα (arrow), much of the S-100 staining extended beyond that of AChRα (arrowheads). Twenty-nine NMJs were observed and twenty of them showed similar degree of nonoverlapping. In contrast, of the 27 NMJs analyzed, 22 of them showed significant overlapping staining between EphA7 and AChRα (arrows). Scale bars: 40 μm.

To further confirm the concentration of Eph receptors on the postsynaptic side of NMJ, the localization of EphA7 was compared with that of SV2, which is concentrated in the presynaptic terminals of motor neurons. Much of the EphA7 staining did not overlap with the SV2 staining, indicating that the localization of EphA7 was distinct from that of SV2 (Fig. 4E). Taken together, our data strongly suggests that there is localization of EphA4, EphA7, and ephrin-A2 at the postsynaptic apparatus.

Localization of EphA4, EphA7, and Ephrin-A2 at NMJ Is Developmentally Regulated

To examine whether the two receptors and ephrin-A2 were localized at NMJ during early development, muscle sections from rats at different developmental stages were stained by antibodies against EphA4, EphA7, and ephrin-A2. While strong immunoreactivity against either receptor as well as ephrin-A2 was detected in muscle fibers at embryonic day 20, extensive staining was observed on both the plasma membrane and cyto-

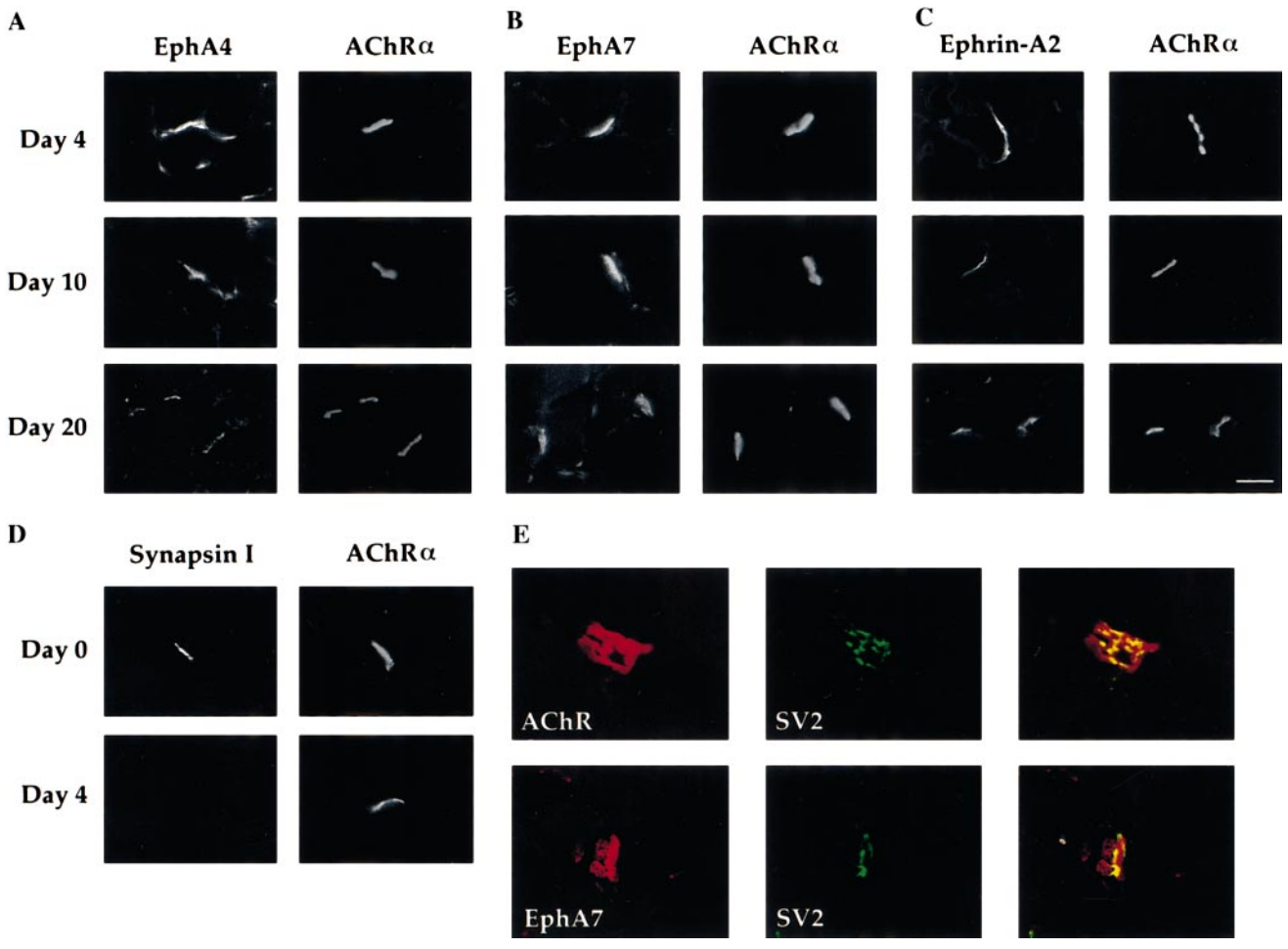

FIG. 4. Localization of Eph receptors and ephrin at NMJ after denervation. (A) Double immunostaining was performed on muscle sections from gastrocnemius of rats at day 4, day 10, or day 20 after sciatic nerve cut. Using the antibody against EphA4 and rhodamine-conjugated α-bungarotoxin, it was found that EphA4 remained at the NMJ for 20 days after denervation. (B) EphA7 remained at the NMJ at day 4, day 10, and day 20 after denervation, as revealed by the antibody against EphA7. (C) Ephrin-A2 staining remained at NMJ at day 4, day 10, and day 20 after denervation. Note that the synaptic staining of both receptors and ephrin became weaker after denervation. (D) Immunostaining using antibody against the presynaptic protein synapsin I revealed strong synaptic staining before denervation (Day 0), but the staining disappeared at day 4 after sciatic nerve cut, indicating the degeneration of motor axon terminus. (E) Double immunostaining was performed on longitudinal sections of adult muscle to compare the localization of EphA7 or AChR with the presynaptic protein SV2. The staining of EphA7 or AChRα (red) was compared with that of SV2 (green), and the overlapping region was indicated by yellow color. The localization of both EphA7 and AChR was distinct from that of SV2. Scale bar: 40 μm.

plasm with little concentration at the NMJ (Figs. 5A–5C). Similarly, no apparent NMJ localization was observed at postnatal day 2. EphA4 and EphA7 were found to be concentrated at certain synaptic sites at postnatal day 7, but significant extrasynaptic staining on muscle fibers was also observed. By postnatal day 14 and day 21, the synaptic staining increased considerably, while at adult stage, the extrasynaptic staining was greatly reduced and both receptors were mostly localized at the NMJ. Interestingly, the developmental regulation of ephrin-A2 localization at the NMJ was similar to that of the two Eph receptors, i.e., synaptic localization was found at postnatal day 7 and increased at later developmental stages. The temporal changes in synaptic and extrasynaptic staining of the different proteins were quantified (Figs. 5D and 5E, see Experimental Methods). Taken together, while the expression of ephrin-A2, EphA4, and EphA7 in muscle decreased during development, their concentration at the postsynaptic apparatus increased at later developmental stages.

During postnatal development of the NMJ, junctional remodelling occurs in which junctional folds form by

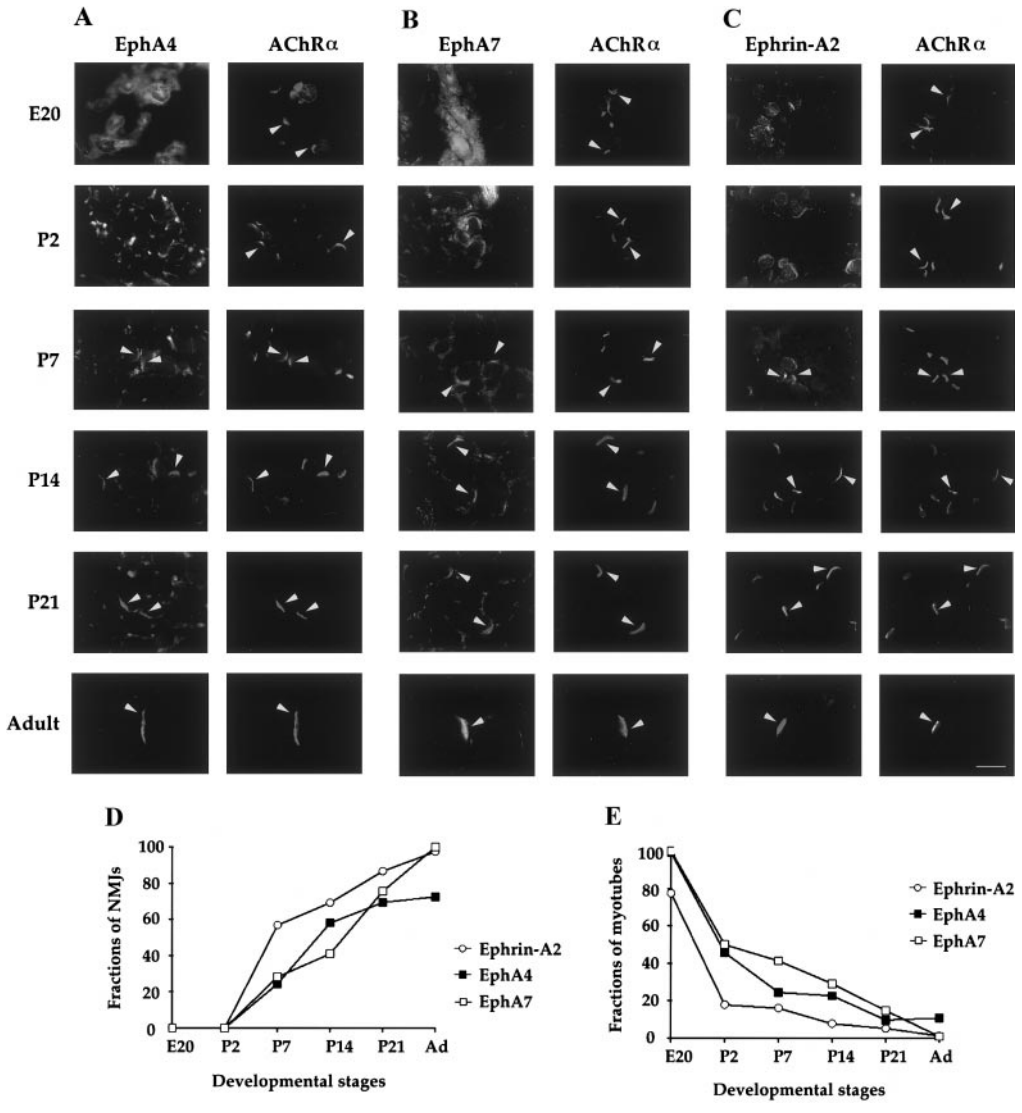

FIG. 5. Developmental regulation of the synaptic localization of EphA4, EphA7, and ephrin-A2 in skeletal muscle. Double immunostaining using the antibody against EphA4 (A), EphA7 (B), or ephrin-A2 (C) and rhodamine-conjugated α-bungarotoxin was performed on muscle sections from rats of different developmental stages. Arrowheads indicated the positions of NMJ. No apparent synaptic localization was observed at embryonic day 20 for both receptors and ephrin-A2. In contrast, considerable concentration at the NMJ was found at postnatal day 7, and the synaptic localization increased through postnatal day 21 to adult. Scale bar: 40 μm. (D) Quantification of the synaptic staining of ephrin-A2 and Eph receptors along development. The fractions of NMJs that were clearly labeled stronger than the extrajunctional regions by the different antibodies were counted at various developmental stages. Synaptic staining of ephrin and Eph receptors were found from postnatal day 7 and increased at later stages. (E) Quantification of the extrasynaptic staining of ephrin-A2 and Eph receptors along development. The extrasynaptic staining of ephrin-A2 and Eph receptors decreased along development, as revealed by the decline in fractions of myotubes that were free of α-bungarotoxin staining but were labelled strongly by the Eph receptors or ephrin-A2 antibody.

postnatal day 7 and the extent of folding increases until postnatal day 21 (Bewick *et al.*, 1996). Coincidentally, ephrin and Eph receptors started to be concentrated at some NMJs at postnatal day 7 and their junctional localization increased at day 14 and 21. Thus, it is possible that the developmentally increased concentration of ephrin-A2 and Eph receptors at the NMJ is resulted from the postsynaptic fold formation. Indeed, it was suggested that the temporal changes in the junctional localization of cytoskeletal proteins such as dystrophin and β-spectrin were related to the junctional fold formation (Bewick *et al.*, 1996). However, while the

junctional localization of ephrin and Eph receptors increased at postnatal stages, the extrajunctional labeling of the proteins decreased significantly along development (Fig. 5E), while in the cases of dystrophin and β-spectrin, extrajunctional labeling could be clearly seen at adult. Therefore, while fold formation remains a plausible mechanism to account for the increased concentration of ephrin and Eph receptors at the NMJ, there may be other mechanisms that contribute to their synaptic localization.

Regulation of EphA4 and EphA7 Expression in Muscle

The localization of synaptic proteins can be accomplished by selective transcription in sub-synaptic nuclei as well as electrical activity-induced suppression of extrasynaptic nuclei transcription. To determine whether the expression of EphA4 and EphA7 transcripts was regulated by nerve activity, Northern blot analysis was performed with RNAs prepared from muscles of denervated rats. The transcripts of EphA4 and EphA7 increased by about 2 to 3 folds just one day after denervation, and rapidly returned to basal level at day 2, while the level of AChRα transcript remained high at day 20 (Fig. 6A). The transient increase in EphA4 and EphA7 transcripts expression was not observed in the muscles of sham-operated rats, indicating that the regulation in expression was not resulted from inflammation due to surgical exposure of the sciatic nerve. The up-regulation of EphA4 and EphA7 proteins after denervation was also observed by Western blot analysis (Fig. 6B). Interestingly, while the transcripts of EphA4 and EphA7 returned to basal level by day 4 after nerve injury (Fig. 6A), the level of EphA4 and EphA7 proteins remained high after day 4 (Fig. 6B), indicating that the regulation of EphA4 and EphA7 expression by nerve activity occurred at both mRNA and protein levels.

NRG is suggested to be the nerve-derived factor which enhances the local gene expression of synaptic proteins (Martinou *et al.*, 1991). The regulation of EphA4 expression by NRG was examined by Western blot analysis. The level of EphA4 proteins in differentiated C2C12 myotubes increased by about 2.5-fold upon treatment with NRG for 24 and 48 hours (Fig. 6C, left panel). In addition, the effect of NRG on EphA4 expression was specific, since the expression of EphA7 proteins remained similar after NRG treatment. Northern blot analysis also revealed an increase in EphA4 transcript expression by NRG (Fig. 6C, right panel). In contrast, the expression of EphA7 or GAPDH transcript was not increased by NRG treatment. Taken together, our study suggests that, in addition to junctional fold formation, the concentration of EphA4 at NMJ may involve the repression of extrasynaptic gene expression by nerve activity, as well as localized synaptic synthesis enhanced by NRG. In this regard, it is noteworthy that synaptic localization of NRG increases substantially after birth (Jo *et al.*, 1995). Since our data reveals that concentration of EphA4 at NMJ was only observed by late postnatal stages, the increased EphA4 expression by NRG may contribute to the synaptic localization of EphA4 at the NMJ. Furthermore, the synaptic localization of Eph receptors in the hippocampus is mediated by association with PDZ proteins (Torres *et al.*, 1998; Buchert *et al.*, 1999). It remains to be seen whether Eph receptors also anchor to the postsynaptic specializations on muscle fibers via association with PDZ proteins.

Association between EphA4 and Cortactin

The synaptic localization of ephrin-A2 as well as EphA4 and EphA7 during late postnatal stages suggests that they may affect the synaptic structure and / or functioning of the NMJ. Activation of Eph receptors may modulate synaptic cytoskeleton and in turn affect the stability of the postsynaptic apparatus. Indeed, the collapse of neuronal growth cone induced by Eph receptors is mediated by rearrangement of actin cytoskeleton (Meima *et al.*, 1997). To examine the link between Eph receptor signalling and actin cytoskeleton in muscle, the association of EphA4 with cortactin was examined. Cortactin is an actin binding protein that has been demonstrated to link the signalling pathways of specific RTKs to the actin cytoskeleton (Maa *et al.*, 1992; Zhan *et al.*, 1993), and has been postulated to be a potential mediator of Eph receptor action (Brückner and Klein, 1998). EphA4 was coimmunoprecipitated with cortactin in muscle at various developmental stages (Fig. 7A, left panel), suggesting that the receptor is associated with cortactin *in vivo*. Relatively low amount of EphA4 was coimmunoprecipitated with cortactin in adult, which would be explained by the low expression of EphA4 in adult muscle (Fig. 1E). The association of cortactin with EphA4 is specific, since MuSK or EphA7, which are both prominent in embryonic muscle, were not coimmunoprecipitated with cortactin in embryonic day 20 muscle (Fig. 7A, right panel). In addition, exogenous EphA4 expressed in the muscle cell line C2C12 was also coimmunoprecipitated with cortactin (Fig. 7B), indicating that the association between the two molecules occurs in muscle cell context. Consistent with the results of immunoprecipitation experiment on embryonic day 20 muscle, exogenous EphA7 expressed in trans-

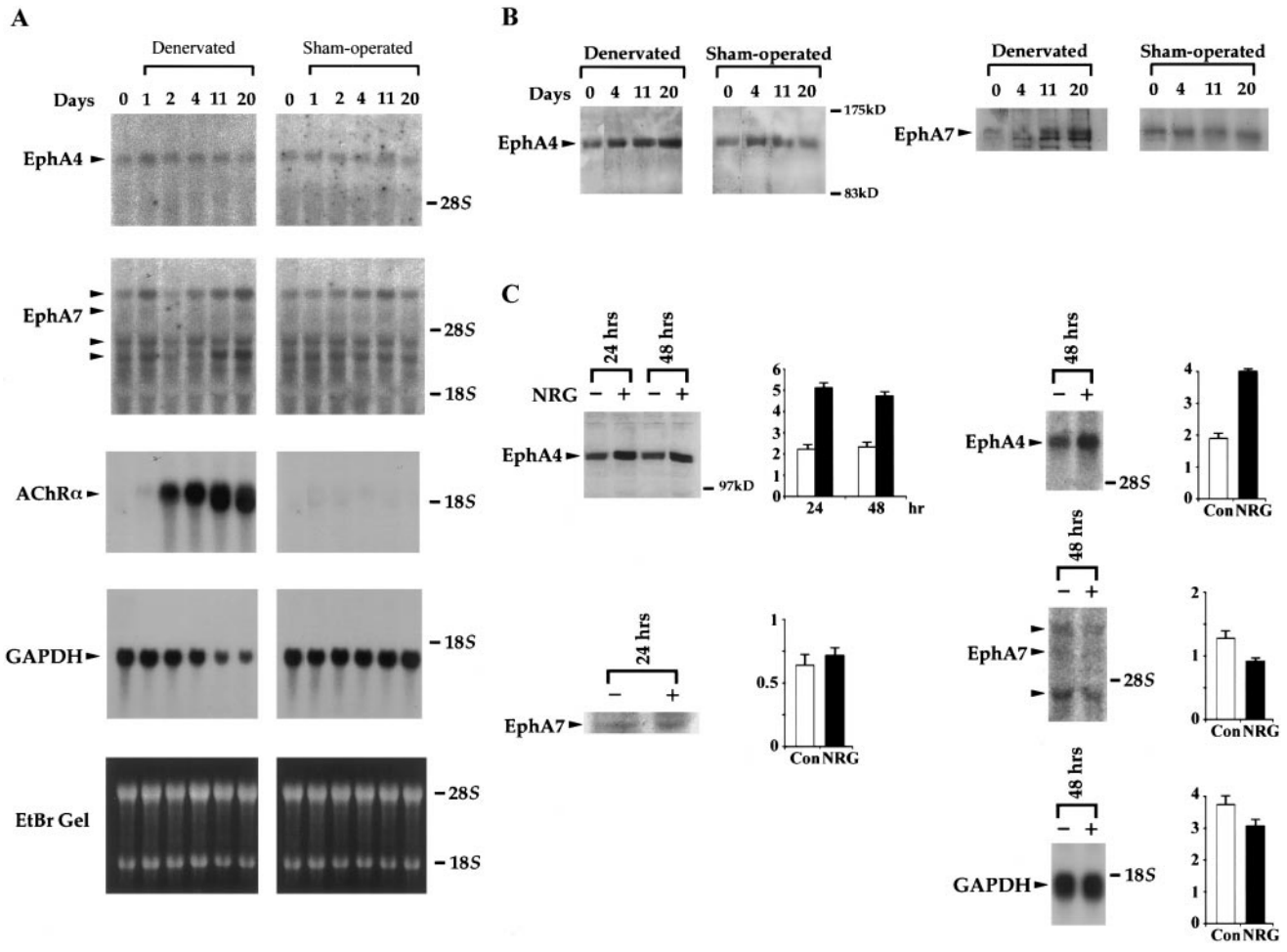

FIG. 6. Regulation of Eph receptors expression in muscle after nerve injury and NRG treatment. (A) Expression of EphA4, EphA7, AChR, and GAPDH transcripts (arrowheads) in gastrocnemius at different times after denervation or sham operation. Ethidium bromide-stained gel and hybridization with GAPDH represented as the RNA loading control. (B) Regulation of EphA4 and EphA7 protein expression after denervation or sham operation. Membrane proteins of gastrocnemius from rats at different time points after operations were immunoblotted by the antibody against EphA4 or EphA7. (C) Increased expression of EphA4 protein and transcript upon NRG treatment. C2C12 myotubes were cultured in the absence (−) or presence (+) of NRG (3 nM) for 24 or 48 h, and cell lysates were immunoblotted with the antibody against EphA4 or EphA7 (left panel). The expression of EphA4 but not EphA7 protein was increased by NRG treatment. The appearance of EphA4 in the Western blot as a doublet might be explained by the two isoforms that differed in 50 amino acids in the cytoplasmic domain (Gilardi-Hebenstreit *et al.*, 1992). Total RNA of C2C12 myotube treated with (+) or without (−) NRG for 48 h was analyzed in Northern blot and probed with EphA4 or EphA7 (right panel). Treatment of C2C12 myotubes by NRG specifically increased the expression of EphA4 but not EphA7 transcripts. Hybridization with GAPDH indicated equal loading of RNA. The results were expressed as arbitrary units and quantified (for EphA7, the intensity for the largest transcript of ~ 6.8 kb was measured; mean ± SD, $n = 3$).

fected C2C12 was not coimmunoprecipitated with cortactin (Fig. 7B).

Previous studies have demonstrated the phosphorylation of cortactin on tyrosine residues upon activation of RTKs such as fibroblast growth factor receptor and epidermal growth factor receptor, and tyrosine phosphorylation of cortactin would regulate its interaction with actin (Maa *et al.*, 1992; Zhan *et al.*, 1993; Huang *et al.*, 1997). In our study, the tyrosine phosphorylation of cortactin was examined in Cos cells transfected with EphA4. Upon overexpression in Cos cells, EphA4 was autophosphorylated (Fig. 7C, left panel), which may be due to receptor oligomerization and subsequent activation as suggested previously (van der Geer *et al.*, 1994). Tyrosine phosphorylation of cortactin was increased in Cos cells overexpressing EphA4 (Fig. 7C, right panel).

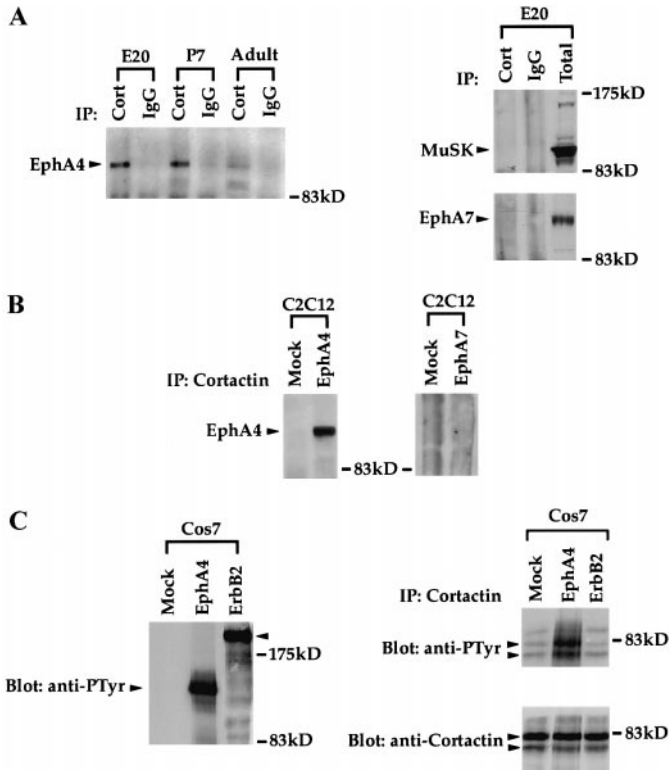

FIG. 7. Association of EphA4 with cortactin and tyrosine phosphorylation of cortactin by activated EphA4. (A) Membrane proteins of E20, P7, or adult rat muscle were immunoprecipitated by mouse IgG (IgG) or anti-cortactin antibody (Cort), and the products were immunoblotted by EphA4 antibody (left panel). EphA4 (arrowhead) was immunoprecipitated by anti-cortactin antibody but not mouse IgG. In addition, MuSK or EphA7 was not coimmunoprecipitated with cortactin in E20 rat muscle (right panel), indicating the specificity in the association between cortactin and different membrane proteins. (B) The association between EphA4 and cortactin in muscle cells was also examined by immunoprecipitating cortactin from total proteins of C2C12 transfected with vector (Mock) or EphA4 and then probed with EphA4 antibody. EphA4 was coimmunoprecipitated with cortactin in the muscle cell line. In contrast, EphA7 was not coimmunoprecipitated with cortactin in transfected C2C12. (C) Total proteins of Cos7 cells transfected with the expression vector pMT21 (Mock) or full-length constructs of EphA4 or ErbB2 were immunoprecipitated by antibodies against EphA4 or ErbB2 (left panel) or anti-cortactin antibody (right panel), and the products were immunoblotted by anti-phosphotyrosine antibody. Tyrosine phosphorylation of cortactin was induced in Cos cells that over-expressed EphA4 but not ErbB2 (right panel). Equal loading was indicated by reblotting the membrane with anti-cortactin antibody. The two bands at about 80 kDa represented two isoforms of cortactin, as reported previously (Ohoka and Takai, 1998).

Moreover, the tyrosine phosphorylation of cortactin by EphA4 is specific, since transfection of Cos cells with another RTK, ErbB2, did not result in the increased phosphorylation of cortactin, despite the fact that ErbB2 was tyrosine-phosphorylated (Fig. 7C). Similarly, overexpression of EphA7 in Cos cells did not lead to increased phosphorylation of cortactin (data not shown).

Cortactin has been demonstrated to associate with synaptic proteins in the brain (Boeckers *et al.*, 1999; Naisbitt *et al.*, 1999), while in muscle, cortactin was found to be located at the NMJ at the early stage of AChR clustering process (Peng *et al.*, 1997). Our finding has provided the first demonstration on the physical and functional association between EphA4 and cortactin. Recent study has demonstrated the ability of Eph receptors to regulate excitatory synapse formation (Dalva *et al.*, 2000), and it remains to be seen whether Eph receptors play a similar role in NMJ formation. An actin-based cytoskeleton is assembled at the postsynaptic apparatus of NMJ (Froehner, 1991), and actin polymerization and cortactin localization has been demonstrated to accompany with agrin-induced AChR clusters formation (Dai *et al.*, 2000). The relatively high expression of EphA4 in embryonic muscle and its interaction with cortactin therefore raises the possibility that it participates in NMJ formation during early development. Alternatively, Eph receptors may function to regulate the stability of AChR clusters during maturation of the NMJ. Actin has been demonstrated to affect the stability of AChR clusters in cultured muscle cells (Connolly, 1984), and the interaction between ephrin and Eph receptors at the NMJ may modulate the actin cytoskeleton and in turn affect the synaptic stability and function. In this context, it is noteworthy that ephrin and Eph receptors are localized at the NMJ at late postnatal stages, during which the stability of AChR is higher than that of newborn rats (Slater, 1982).

Several RTKs, such as MuSK, ErbB, and TrkB, have been implicated to play critical roles in mediating the formation and maintenance of NMJ (Glass and Yancopoulos, 1997; Martinou *et al.*, 1991; Jo *et al.*, 1995; Gonzalez *et al.*, 1999). Our present study represents the first demonstration that Eph receptors and ephrin are localized at the postsynaptic specializations on muscle fibers. Furthermore, their synaptic localization is developmentally regulated *in vivo.* The present findings potentially open up new avenue of research on the functions of this family of RTK in the formation and/or maintenance of NMJ. In addition, our understanding on the roles of Eph receptors at neuronal synapses of the CNS only begins recently, and it is interesting to study the similarities and differences between neuronal synapses and NMJ in terms of the expression and function of Eph receptors. For example, our study shows that little localization of EphA4 and EphA7 is observed at NMJ at embryonic stages, while EphB2 and ephrins-B

are synaptically localized in embryonic hippocampal neurons in culture (Torres *et al.*, 1998). It will be interesting to examine when the receptors start to localize at central synapses *in vivo.* Furthermore, little is known about the regulation of Eph receptors expression in neuron. Our study raises the question of whether the expression of Eph receptors in neuron is also regulated by electrical activity and NRG. Finally, cortactin is associated with the NMDA receptor/PSD-95 complex in hippocampal neurons (Naisbitt *et al.*, 1999). It will be important to further investigate whether the association between EphA4 and cortactin also occurs in neurons. Interestingly, it was recently demonstrated that EphB2 but not EphA4 directly interacted with NMDA receptors in neurons (Dalva *et al.*, 2000). Our findings suggest that EphA4 can possibly be linked to NMDA receptors indirectly through cortactin. Since ephrin-A5 can induce LTP of hippocampal neurons (Gao *et al.*, 1998), study of the physical and functional association between Eph receptors and the NMDA receptor complexes may shed new light on the potential mechanisms underlying the induction of LTP by ephrin. NMJ is widely regarded as a good model system to study the development of neuronal synapses in the brain, owing to the relative accessibility of the NMJ as opposed to the technical difficulties in the study of neuron–neuron synapses (Sanes and Lichtman, 1999; Lee and Sheng, 2000). The present findings raise the possibility of using NMJ as an alternative model system to understand the mechanisms underlying the localization and functions of Eph receptors at neuronal synapses.

EXPERIMENTAL METHODS

Antibodies and Plasmids

Rabbit polyclonal antibodies against EphA4 (sc-921), EphA7 (sc-917 and sc-1015), ephrin-A2 (sc-912), and ErbB2 (sc-284) were purchased from Santa Cruz Biotechnology, Inc. The antibodies sc-921, sc-1015, and sc-912 were raised against peptides mapping near the carboxyl terminus of EphA4, EphA7, and ephrin-A2, respectively, while the antibody sc-917 recognizes an epitope located near the amino terminus of EphA7. Another rabbit polyclonal antibody against the intracellular domain of EphA4 (Sek_i; Irving *et al.*, 1996) was kindly provided by David G. Wilkinson (National Institute for Medical Research, London, England). The MuSK antibody was custom-made by Research Genetics that was raised against the amino acids TLPSELLLDRLHPNPMYQ. Antibodies against cortactin and phosphotyrosine were purchased from Upstate Biotech., while anti-synapsin I and anti-S-100 antibodies were purchased from Molecular Probes Inc. and Dako, respectively. The anti-SV2 supernatant was obtained from the Developmental Studies Hybridoma Bank (IA). The full-length EphA4 cDNA was amplified from embryonic day 18 mouse brain RNA by RT-PCR using Vent Polymerase with the primers AGGAGCAGCGTTGGCACC and TCTATTCGGTACTGGCTC; full-length ephrin-A2 cDNA was amplified from mouse muscle cDNA using the primers ATGGCGCCCGCGCAGCG and CACTAGGAGCCCAGAAGG. Both were subcloned into the expression vector pMT21. Expression construct encoding full-length EphA7 was kindly provided by Thomas Ciossek (Max-Planck Institute, Martinsried, Germany).

Tissue Culture

The muscle cell line C2C12 was cultured in DMEM supplemented with 20% FBS. The cells were differentiated into myotubes by culturing in DMEM supplemented with 2% Horse Serum for four days before RNA extraction. The production of recombinant NRG-β was described previously (Fu *et al.*, 1999). Cultured C2C12 myotubes were treated with NRG (3 nM) for various durations and total RNA and proteins were extracted. Transient transfection of C2C12 and Cos-7 was performed using lipofectamine (Gibco BRL).

Surgical Procedure, RNA Extraction, Northern Blot, and RT-PCR Analysis

The denervation surgery and sham operation on adult rat was performed as previously described (Ip *et al.*, 1996). Total RNA were prepared from muscle of rats at different developmental stages, as well as from gastrocnemius of operated rats, and analyzed by formaldehyde RNA gel as described (Ip *et al.*, 1996). cDNA fragments originated from the extracellular regions of EphA4 and EphA7, partial rat GAPDH sequence corresponding to nucleotides 238 to 1036, and full-length AChRα cDNA, were used to hybridize the RNA blots. Results were confirmed in three independent experiments, and representative data was shown. To examine the expression of ephrins in adult muscle, RT-PCR was performed as described previously (Lai *et al.*, 1999), using primers specific for ephrins-A1, A2, and A4, and Southern blot analysis with probes specific for the respective ephrins was performed.

Protein Extraction, Immunoprecipitation, and Western Blot Analysis

Frozen tissues were homogenized in buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF) that contained 1 mM DTT, 1 mM PMSF, 5 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 2 μg/ml antipain, and 1 mM sodium orthovanadate. To extract membrane proteins, the samples were centrifuged at 14,000 rpm for 90 min, and the pellet was solubilized with the homogenization buffer that contained all the inhibitors and 1% NP-40. Protein samples were analysed in 6% SDS–PAGE and probed with the antibodies sc-921 (1:2000), sc-917 (1:1000), and Sek_I (1:5000). For coimmunoprecipitation, membrane proteins were extracted using the homogenate buffer that contained 0.1% NP-40, incubated with anti-cortactin antibody at 4°C overnight, and then protein G–Sepharose for 1 h. The samples were then immunoblotted by the antibodies against EphA4 (Sek_I), EphA7 (sc-917), or MuSK. To assay for tyrosine phosphorylation of cortactin, Cos7 cells were transfected with EphA4, EphA7, ErbB2, or the pMT21 expression vector by lipofectamine (Gibco). Three days after transfection, the cells were serum-starved in DMEM (without penicillin/streptomycin) for 16 h. They were then lysed by RIPA at 4°C for 1 h, and then immunoprecipitated by antibodies against cortactin, EphA4 (sc-921), EphA7 (sc-917), or ErbB2 overnight. The samples were immunoblotted by anti-phosphotyrosine antibody. The membrane was then stripped and reprobed with anti-cortactin antibody.

Immunohistochemistry

Hindlimb muscle from rats of different developmental stages and gastrocnemius muscle of denervated rats were put into isopentane and frozen by liquid nitrogen. Muscle sections (10 μm) were cut and mounted onto gelatin/poly-l-lysine-coated slides. They were fixed by 2% paraformaldehyde/5% sucrose for 15 min at room temperature. After blocking with 10% FBS, the sections were permeabilized using 0.1% Triton X-100, and incubated with antibodies against EphA7 (sc-917 or sc-1015 at the dilution of 1:100), EphA4 (sc-921 or Sek_I, diluted 1:100 and 1:150, respectively), ephrin-A2 (1:200), S-100 (1:200), synapsin I (1:500), or SV2 (1:100) at 4°C for 1 to 2 days. After incubation with secondary antibody (FITC-anti-mouse or rabbit-IgG, 1:1000), the AChR clusters were labeled by rhodamine-conjugated α-bungarotoxin (Molecular Probes Inc.) for 30 min at 37°C. Sections were mounted with mowiol and analysed under Leica fluorescence or Bio-Rad confocal microscopes. To test the specificity of staining, antibody was preincubated at 4°C overnight with 10-fold excess of the peptide against which the antibody was raised.

To quantify the double immunostaining of S-100/AChR and EphA7/AChR (Fig. 3D), 29 and 27 NMJs were, respectively, analyzed under confocal microscope, and the proportions of NMJs that showed similar extent of overlapped staining as shown in Fig. 3D were counted. To compare the distribution of AChR, EphA7, and SV2 at the NMJ, double immunostaining on longitudinal adult muscle sections was performed. Fifteen to 20 en face views of NMJs were analyzed for each set of double staining, and representative views were shown (Fig. 4E). To qunatify the synaptic staining of Eph receptors and ephrin-A2 at different developmental stages (Fig. 5D), the fractions of NMJs that were clearly labeled stronger than the extrajunctional regions were counted. Twenty to 60 NMJs were scored for each developmental stage examined. On the other hand, myotubes that were free of α-bungarotoxin staining but were labeled strongly by the Eph receptors or ephrin-A2 antibody were scored in order to quantify the extrasynaptic staining of Eph receptors and ephrin-A2 (Fig. 5E). Around 120–500 myotubes were scored for each developmental stage examined.

ACKNOWLEDGMENTS

We are grateful to David G. Wilkinson and Thomas Ciossek for the EphA4 antibody and expression construct of EphA7, respectively. We also thank Ka-Chun Lok and Ada W. Y. Fu for technical support. This study was supported by the Research Grants Council of Hong Kong (RGC 568/95M, RGC 6107/98M) and Hong Kong Jockey Club. N. Y. Ip was the recipient of Croucher Foundation Senior Research Fellowship.

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Received November 27, 2000
Revised March 29, 2001
Accepted April 13, 2001