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Receptor Tyrosine Kinase Specific for the Skeletal Muscle Lineage: Expression in Embryonic Muscle, at the Neuromuscular Junction, and after Injury

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Summary

While a number of growth factors have been described that are highly specific for particular cell lineages, neither a factor nor a receptor uniquely specific to the skeletal muscle lineage has previously been described. Here we identify a receptor tyrosine kinase (RTK) specific to skeletal muscle, which we term "MuSK" for muscle-specific kinase. MuSK is expressed at low levels in proliferating myoblasts and is induced upon differentiation and fusion. In the embryo, it is specifically expressed in early myotomes and developing muscle. MuSK is then dramatically downregulated in mature muscle, where it remains prominent only at the neuromuscular junction; MuSK is thus the only known RTK that localizes to the neuromuscular junction. Strikingly, MuSK expression is dramatically induced throughout the adult myofiber after denervation, block of electrical activity, or physical immobilization. In humans, MuSK maps to chromosome 9q31.3–32, which overlaps with the region reported to contain the Fukuyama muscular dystrophy mutation. Identification of MuSK introduces a novel receptor-factor system that seems sure to play an important and selective role in many aspects of skeletal muscle development and function.

Introduction

Growth factors produced by one cell and recognized by cell surface receptors on other cells provide for a major mode of intercellular communication. Specificity in this type of communication often depends upon the distribution of receptors for a given growth factor, which determines the cells that can respond to the factor. Some factors, such as insulin and its relatives, have broadly expressed receptors and thus simultaneously signal to, and elicit responses from, most cells in the body (Lee and Pilch, 1994). Other factors have receptors that are quite specific for particular cell types, allowing responses only by selected cellular targets. Examples of such factors include members of the nerve growth factor family, each of which acts on particular subpopulations of neurons and only at discrete stages of their development (Barbacid, 1993; Glass and Yancopoulos, 1993; Snider, 1994). There are several receptors that are exclusively expressed on endothelial cells and that allow for the lineage-specific actions of factors such as vascular endothelial cell growth factor (Fong et al., 1995; Sato et al., 1995). Similarly, many hemopoietic lineages can be specifically regulated by cytokines, such as erythropoietin and thrombopoietin, that act only on these lineages (Miyajima et al., 1992). While a number of generally acting growth factors have been shown to act on cells of the skeletal muscle cell lineage, neither a factor nor a receptor uniquely specific to this lineage has thus far been described. One could imagine that such a factor and its receptor, if they were to exist, might mediate critical aspects of skeletal muscle development, might somehow regulate muscle metabolism or function, might be involved in muscle maintenance, and could be therapeutically useful for muscle responses to stress and injury without affecting other cell systems.

Skeletal muscle is derived from mesodermal structures in the embryo that are termed somites (reviewed in Hauschka, 1994). By a process that is not completely understood, but that apparently involves myogenic determination transcription factors (MDFs) such as myf5, myogenin, and myoD, proliferating cells in the dorsal region of the somite become committed to the muscle lineage (reviewed in Li and Olson, 1992; Hauschka, 1994). Some of these myoblasts exit the cell cycle and migrate ventrally to form the myotomal region of the somite, from which axial muscles are derived. Other myoblasts migrate out from the lateral portion of each somite and instead form body wall and limb muscles. Myoblasts that exit the cell cycle begin to differentiate, start to express muscle-specific structural proteins, and eventually fuse to form multinucleated myotubes. Muscle development involves two waves of myotube formation: small numbers of primary myotubes seem initially to align fibers for individual muscles, and secondary myotubes are subsequently recruited to build upon this initial scaffold (Kelly and Zacks, 1969; Ontell and Kozeka, 1984). While the number of primary myotubes formed seems to be genetically determined, survival of these myotubes apparently depends upon functional innervation, and this survival in turn influences secondary myotube formation (Ashby et al., 1993a, 1993b).

Muscle innervation depends upon formation of a highly specialized synaptic connection, termed the neuromuscular junction (NMJ), between the incoming nerve and the muscle fiber (reviewed in McMahan, 1990; Burden, 1993; Hall and Sanes, 1993; Fallon and Hall, 1994). Before innervation, the entire surface of the multinucleated myofiber contains molecules, such as subunits of the acetylcholine receptor (AChR; α, β, δ, and γ subunits), that will later be highly localized to the NMJ. Localized aggregation at the endplate is apparently induced by secretion, from the motor nerve terminal, of a protein termed agrin (McMahan, 1990; Fallon and Hall, 1994). In addition to initiating agrin-mediated aggregation of preexisting AChR, innervation has dramatic effects on muscle gene expression (Burden, 1993). For example, onset of electrical activity suppresses expression of AChR α, β, δ, and γ mRNAs by all extrajunctional nuclei, while a neural factor released by the nerve terminal (perhaps corresponding to a member of the neuregulin family) promotes continued expression of α, β, and δ transcripts only by the subsynaptic nuclei and also seems to turn on expression of AChR ε mRNA in these nuclei (Burden, 1993; Falls et al., 1993; Jo et al., 1995). Developing myofibers are initially innervated by multiple axons that terminate at a single synaptic site. Subsequently, synapses are eliminated, leaving each myofiber innervated by a single axon and refractory to receiving additional innervation (Hall and Sanes, 1993). Denervation or other inhibitors of electrical activity, even in an adult muscle, will result in rapid and dramatic upregulation of α, β, δ, and γ mRNAs in extrajunctional nuclei in a fiber-wide pattern and will release the block on multiply innervating fibers (Burden, 1993; Hall and Sanes, 1993); thus, in many ways, denervation may resemble regression to a more embryonic state. Furthermore, mononucleated myogenic precursors that are intimately associated with myofibers but are quiescent in adult muscle, known as satellite cells, can be reactivated by denervation; satellite cells can also be activated in response to direct muscle trauma or in dystrophies (reviewed in Bischoff, 1994). Denervation or loss of muscle activity due to other pathological conditions has dire consequences for the muscle. A sharp drop in muscle protein synthesis and a rise in protein degradation lead to rapid muscle atrophy, reducing total muscle mass by 80% in the first 3 weeks following denervation (Engel and Stinnington, 1974). The effects of denervation are thought to be due to both interruption of electrical activity, which itself seems to have trophic effects upon muscle, as well as loss of muscle-sustaining factors (termed "myotrophic" factors) released by the nerve terminal (reviewed in Grinnel, 1994).

Growth factors have already been suggested to play important roles in many aspects of muscle development and function (reviewed in Olson, 1992; Hauschka, 1994). Fibroblast growth factors (FGFs) and transforming growth factor βs (TGFβs) may be important for mesoderm induction, which is required for subsequent muscle formation. The neural tube or notochord, and perhaps other embryonic structures, are thought to release factors that may induce muscle differentiation in the somite. Myoblast proliferation, in vitro, depends on serum that can be replaced to varying degrees by a variety of growth factors such as FGFs, TGFβs, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), the insulin-like growth factors (IGFs), and leukemia inhibitory factor (LIF); some of these factors also regulate differentiation and fusion, with TGFβs apparently blocking differentiation and IGFs promoting differentiation. It is unclear what role, if any, these factors play in regulating myoblast proliferation in vivo. It has been suggested that serum and growth factors maintain in vitro myoblast proliferation and prevent terminal differentiation by inactivating MDFs via phosphorylation cascades; interestingly, terminal differentiation correlates with down-regulation of many of these growth factor receptors. Factors released by the nerve terminal, such as the neuregulins, may be involved in regulating transcription from synaptic nuclei (Burden, 1993; Falls et al., 1993; Jo et al., 1995). Other factors found in peripheral nerve extracts, and IGFs and ciliary neurotrophic factor (CNTF) in particular (Grinnel, 1994; Helgren et al., 1994), may have myotrophic actions that reduce muscle wasting following denervation.

None of the aforementioned growth factors, however, acts solely on muscle lineage cells. Because other cell types utilize lineage-specific growth factors, and because identification of such a growth factor specific to skeletal muscle seems sure to have important consequences, we set out to obtain evidence for such a factor. Toward this end, we chose to determine whether we could identify a novel growth factor receptor specific to the skeletal muscle lineage. We focused on identifying a muscle-specific member of the receptor tyrosine kinase (RTK) superfamily (Schlessinger and Ullrich, 1992) because cDNAs encoding members of this superfamily can be molecularly cloned via approaches that exploit sequence similarities within the catalytic kinase domains of these receptors (e.g., Wilks, 1989; Lai and Lemke, 1991). We searched for RTKs expressed in denervated skeletal muscle, based on the notion that denervation may induce reexpression of embryonically important RTKs; denervation might also upregulate RTKs that might be involved in NMJ plasticity, in other responses dependent on muscle activity, or in muscle atrophy. This search resulted in the identification of a RTK we term "MuSK" for muscle-specific kinase. MuSK is expressed at low levels in proliferating myoblasts, is induced by differentiation and fusion, and is expressed in the developing myotomes of the embryo. MuSK remains highly expressed during muscle development and then becomes dramatically down-regulated in mature muscle, where it remains prominent only at the NMJ. Thus, MuSK appears to be involved in early muscle development and may also be important for function of the NMJ in the adult. Strikingly, MuSK expression is reinduced throughout the myofiber following denervation, block of electrical activity, and physical immobilization, suggesting additional roles for MuSK in the response to injury, atrophy, or changes in muscle activity. In addition, the *MuSK* gene maps to mouse chromosome 4 and its syntenic counterpart at human chromosome 9q31.3–32, which overlaps with the re-

gion reported to contain the Fukuyama muscular dystrophy mutation, raising the possibility that loss of MuSK function may account for this disorder.

The closest known relative of MuSK is a receptor cloned from the electric organ of the electric ray Torpedo californica, which was also found to be specifically expressed in the skeletal muscle of this organism (Jennings et al., 1993); because the electric organ is a specialized variant of skeletal muscle, MuSK and its orthologs appear to have crucial muscle lineage–restricted functions that are conserved across long evolutionary distances. Identification of MuSK and description of its restricted expression profile introduce a novel receptor–factor system that seems sure to play an important role in many aspects of skeletal muscle development and function.

Results

Molecular Cloning of MuSK from Denervated Skeletal Muscle

Based on the notion that denervation might result in reexpression of embryonically important muscle proteins, or induction of proteins involved in responses to injury or changes in muscle activity, we attempted to isolate cDNAs encoding RTKs expressed in denervated muscle. The polymerase chain reaction (PCR) was used in combination with oligonucleotides corresponding to two conserved regions (the DLAARN and DVWSYG motifs) found in most tyrosine kinase domains to amplify cDNA fragments from denervated muscle cDNA encoding portions of tyrosine kinases spanned by the conserved residues. A library of these cDNA fragments was constructed and screened to remove known kinases (see Experimental Procedures), leading to the identification of a novel candidate RTK. Probes specific for this candidate RTK were then hybridized to Northern blots containing RNA from several adult tissue sources, including normal adult skeletal muscle as well as denervated skeletal muscle. This Northern analysis revealed a very restricted expression pattern in adult tissues, with prominent expression only in denervated skeletal muscle (Figure 1A); very low levels of expression were also noted in innervated skeletal muscle, as well as in a limited number of other tissues including spleen and retina. The candidate RTK was thus designated MuSK for muscle-specific kinase and subjected to further detailed analysis as described below.

Structural Features of MuSK and Relationship to Torpedo RTK and the RORs

Longer cDNA clones encoding the entire coding region of MuSK were cloned from a denervated rat cDNA library and from human myoblast cDNA (see Experimental Procedures). The rat and human versions of MuSK exhibit all the canonical features of conventional RTKs—including a signal peptide, a large ectodomain, a transmembrane region, and a cytoplasmic domain containing the 12 subdomains that characterize a functional tyrosine kinase—and are highly homologous to each other (94% overall identity, with 91% identity in their ectodomains and transmembrane regions [45 differences in a total of 515 resi-

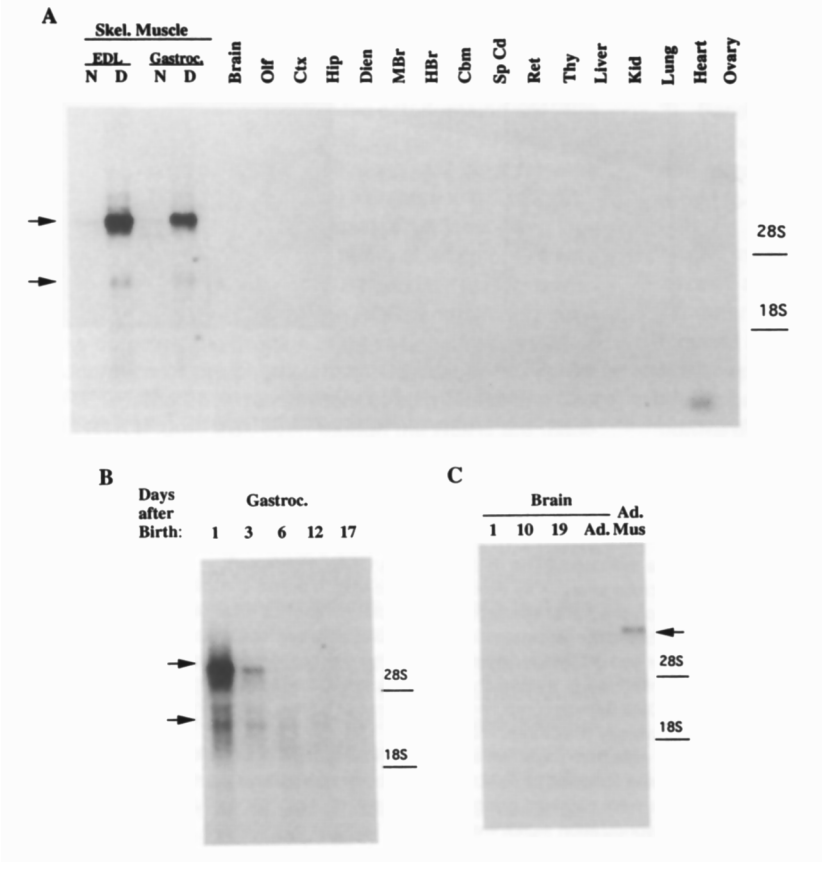

Figure 1. Northern Blot Analysis of *MuSK* Expression

Blots show detection of *MuSK* expression in adult rat tissue (A) and during early postnatal development of the rat gastrocnemius muscle (B) or brain (C). Arrows indicate the ~7.0 and ~3.5 kb *MuSK*-specific transcripts. N and D, naive and denervated extensor digitorus longus (EDL) and gastrocnemius skeletal muscle, respectively; Olf, olfactory bulb; Ctx, cortex; Hip, hippocampus; Dien, diecephalon; MBr, midbrain; HBr, hindbrain; Cbm, cerebellum; Sp Cd, spinal cord; Ret, retina; Thy, thymus; Kid, kidney.

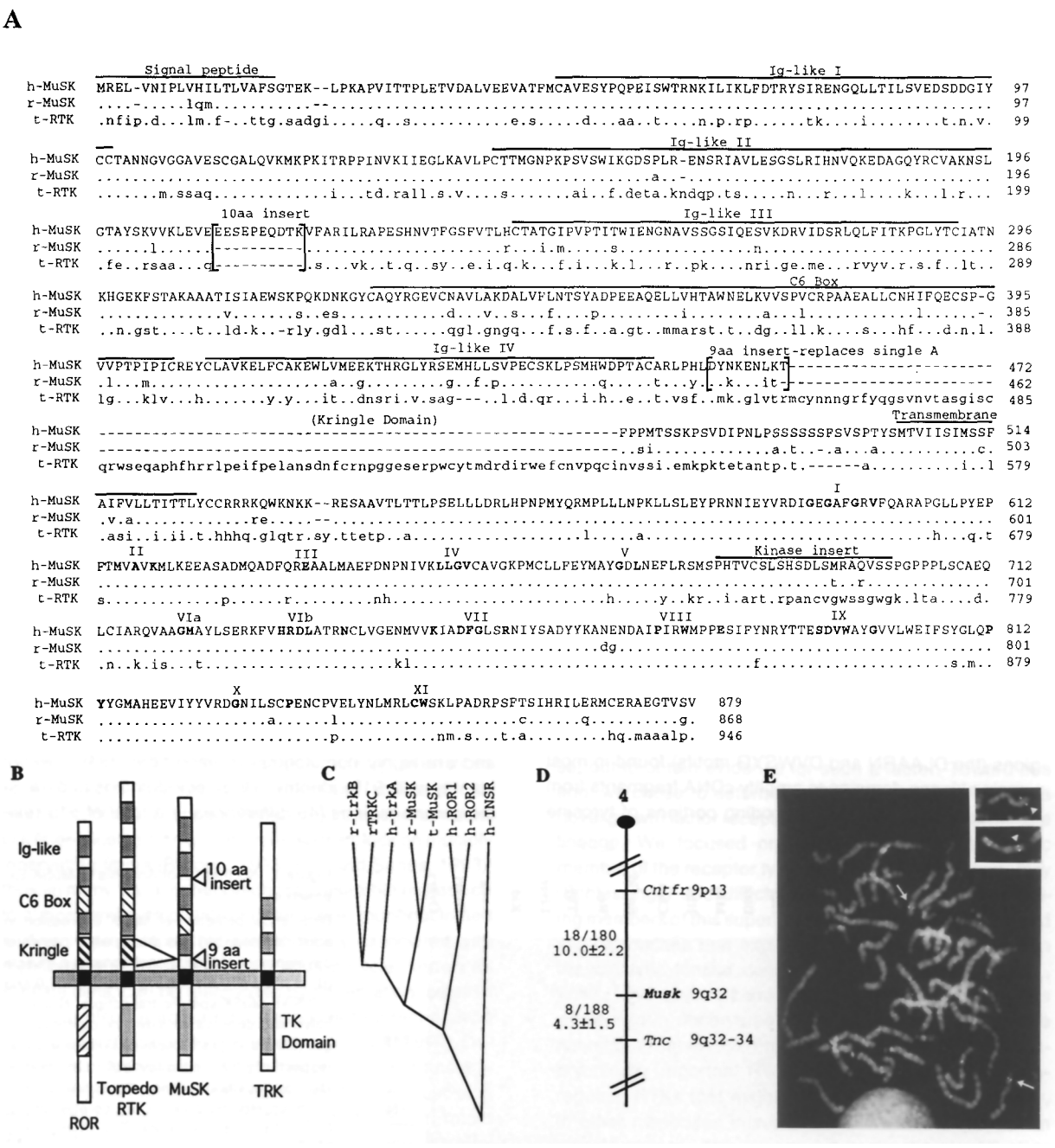

Figure 2. Sequence, Structural Comparisons, and Chromosomal Mapping of MuSK

(A) Comparison of human and rat MuSK amino acid sequence to that of Torpedo californica RTK (t-RTK). Only differences from the human MuSK sequence are indicated, with periods denoting identity to the human MuSK sequence, and hyphens denoting missing residues. The signal peptide, Ig-like domains, C6 box, Kringle domain, transmembrane domain, and conserved subdomains I–XI of the kinase region are indicated; conserved residues in the kinase subdomains are in boldface.

(B) Schematic representation of MuSK structure in comparison to that of Torpedo californica RTK and the Trks. Ig-like domains, the C6 Box, Kringle domains, kinase domains, and positions of MuSK insertions are indicated; the ROR kinase domain is differentially striped to indicate that it is more distant from the others.

(C) Cladogram comparing the evolutionary relationships between the indicated RTKs (h-INSR, human insulin receptor).

(D) Mapping of *Musk* to mouse chromosome 4 by interspecific backcross analysis as described in the text. A partial chromosome 4 linkage map showing the location of *Musk* in relation to linked genes is shown on the right. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the recombination frequencies between loci expressed in centimorgans (± SEM) are indicated to the left of the chromosome map. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution pattern. Positions of the depicted linked loci in human chromosomes are shown to the right. References for the map positions of most human loci can be obtained from the Genome Data Base, a computerized database of human linkage information maintained by The William H. Welsh Medical Library of The Johns Hopkins University (Baltimore, MD).

(E) In situ hybridization of a biotin-labeled *MuSK* probe to metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes. The chromosome 9 homologs are identified with arrows; specific labeling was observed at 9q32. The insert shows partial karyotypes of two chromosome 9 homologs illustrating specific labeling at 9q31.3–32 (arrow). Images were obtained using a Zeiss Axiophot microscope coupled to a cooled CCD camera. Separate images of DAPI-stained chromosomes and the hybridization signal were merged using image analysis software (NU200 and Image 1.52b).

dues] and 97% identity in their intracellular domains [11 differences in a total of 358 residues]; Figure 2A). Several distinct cDNAs were isolated encoding slightly different versions of human MuSK that differ by the presence of either 1 or 2 different insertions (a 10 amino acid insertion and a 9 amino acid insertion) in the ectodomain (Figures 2A and 2B). Determination of the structure of the *MuSK* gene reveals that these insertions result from alternative splicing that either includes or excludes small exons encoding the insertions (unpublished data); the splicing that results in inclusion of the small exon encoding the 9 amino acid insertion removes an alanine residue at the splicing junction (Figure 2A).

The ectodomains of rat and human MuSK can be subdivided into four immunoglobulin-like (Ig-like) domains, with the third and fourth Ig-like domains separated by a rather large spacer containing 6 conserved cysteines (Figures 2A and 2B, C6 box). Among all known RTKs, rat and human MuSK are most highly related to a RTK previously cloned from the electric organ of the electric ray Torpedo californica (Jennings et al., 1993). The Torpedo RTK contains all the major features of the rat and human MuSK proteins, but in addition includes a Kringle domain just after the fourth Ig-like domain (Figures 2A and 2B). Analysis of multiple MuSK transcripts as well as the *MuSK* gene indicates that Kringle-containing isoforms of MuSK do not exist (unpublished data). Torpedo RTK was found to be specifically expressed in the electric organ of the electric ray, which represents a specialized form of skeletal muscle, as well as in skeletal muscle of the electric ray (Jennings et al., 1993). The related tissue distributions of MuSK and Torpedo RTK, together with the high homology between these evolutionarily distant species (mammalian MuSK and Torpedo RTK display an overall amino acid identity of ~70%, including 61% identity between the ectodomains and 78% identity between the cytoplasmic domains), suggest that they may represent distant orthologs that have retained particular importance for the skeletal muscle lineage.

MuSK and Torpedo RTK display significant homology in their ectodomains to another family of receptor-like tyrosine kinases, comprised by ROR1 and ROR2 (Masiakowski and Carroll, 1992). While the RORs contain only three Ig-like domains, the position of these domains, as well as conservation of the characteristic C6 spacer box in the RORs, is remarkably reminiscent of both MuSK and Torpedo RTK (Figure 2B), even though the RORs are markedly less related to MuSK and Torpedo RTK within these regions (~30% amino acid identity) than MuSK and Torpedo RTK are to each other; interestingly, the RORs, like Torpedo RTK, contain Kringle domains. Thus, the RORs appear to be distant relatives of MuSK and Torpedo RTK, and the homology in their ectodomains suggests that they might recognize structurally related ligands.

Although the ectodomains of MuSK and Torpedo RTK are most related to the RORs, the kinase domains of MuSK and Torpedo RTK are most similar to the kinase domains of the Trks (Figures 2B and 2C), which serve as receptors for the neurotrophins and which mediate neuronal survival and differentiative responses (Barbacid, 1993; Glass and Yancopoulos, 1993; Snider, 1994).

MuSK Is Expressed in Early Embryonic Myotomes and Subsequently during Embryonic Muscle Development

To determine whether *MuSK* is expressed prominently in early stages of muscle differentiation, we first probed Northern blots containing RNA isolated from skeletal muscles of young rats. *MuSK* was prominently expressed in skeletal muscle of newborns and was rapidly downregulated shortly after birth (see Figure 1B); early developmental expression of *MuSK* was not noted in other tissues examined (e.g., brain; see Figure 1C). The finding that *MuSK* was specifically and prominently expressed in newborn muscle inspired an attempt to use in situ hybridization techniques to characterize further the temporal and spatial patterns of *MuSK* expression during embryonic development. A developmental study of rat embryos revealed that *MuSK* transcripts could not be detected during early somite formation prior to myotome differentiation (i.e., prior to embryonic day 11 [E11] in the rat, which corresponds to E9.5 in the mouse). However, *MuSK* expression was specifically noted in the developing myotomes of the rat at the time of their formation at E11 (Figure 3, top panels); expression was specific to the myotomes and not noted in the overlying dermatomes (Figure 3; and data not shown). Comparison of *MuSK* transcript distribution with that of one of the earliest markers of skeletal muscle progenitors, α-cardiac actin, revealed striking colocalization at E11, except that *MuSK* was not expressed in cardiac muscle as was α-cardiac actin (Figure 3). During further embryonic development, *MuSK* and α-cardiac actin continued to display striking colocalization outside of the heart, indicating that *MuSK* is not only expressed at the earliest stages of myotome development but also during myotube fusion and all later stages of embryonic muscle formation (Figure 3). Based on the Northern analysis discussed above, *MuSK* expression appears to stay high in developing skeletal muscle until shortly after birth, when its expression drops to low levels.

MuSK mRNA and Protein Induced by Denervation as well as Disuse in All Muscles Tested

To determine whether MuSK protein is as dramatically induced by denervation as is *MuSK* mRNA, we generated MuSK-specific antisera to immunoblot muscle extracts for MuSK protein. No MuSK protein could be detected from normal adult muscles, but high levels of a protein of ~100 kDa could be detected from denervated adult skeletal muscle (Figure 4); the same protein was detected by different antisera generated against different epitopes of MuSK, and detection could be specifically blocked using peptides used to generate the different antisera (data not shown). Marked MuSK protein induction occurred in three different types of denervated adult muscle: soleus, which is primarily composed of slow-twitch fibers; extensor digitalis longus (EDL), which is primarily composed of fast-twitch fibers; and gastrocnemius, which is a mixed fiber muscle (Figure 4A). MuSK protein induction was apparent by 2 days after denervation and reached maximum levels by 2 weeks after the nerve lesion (Figure 4B). In the absence of reinnervation, MuSK protein levels remained elevated

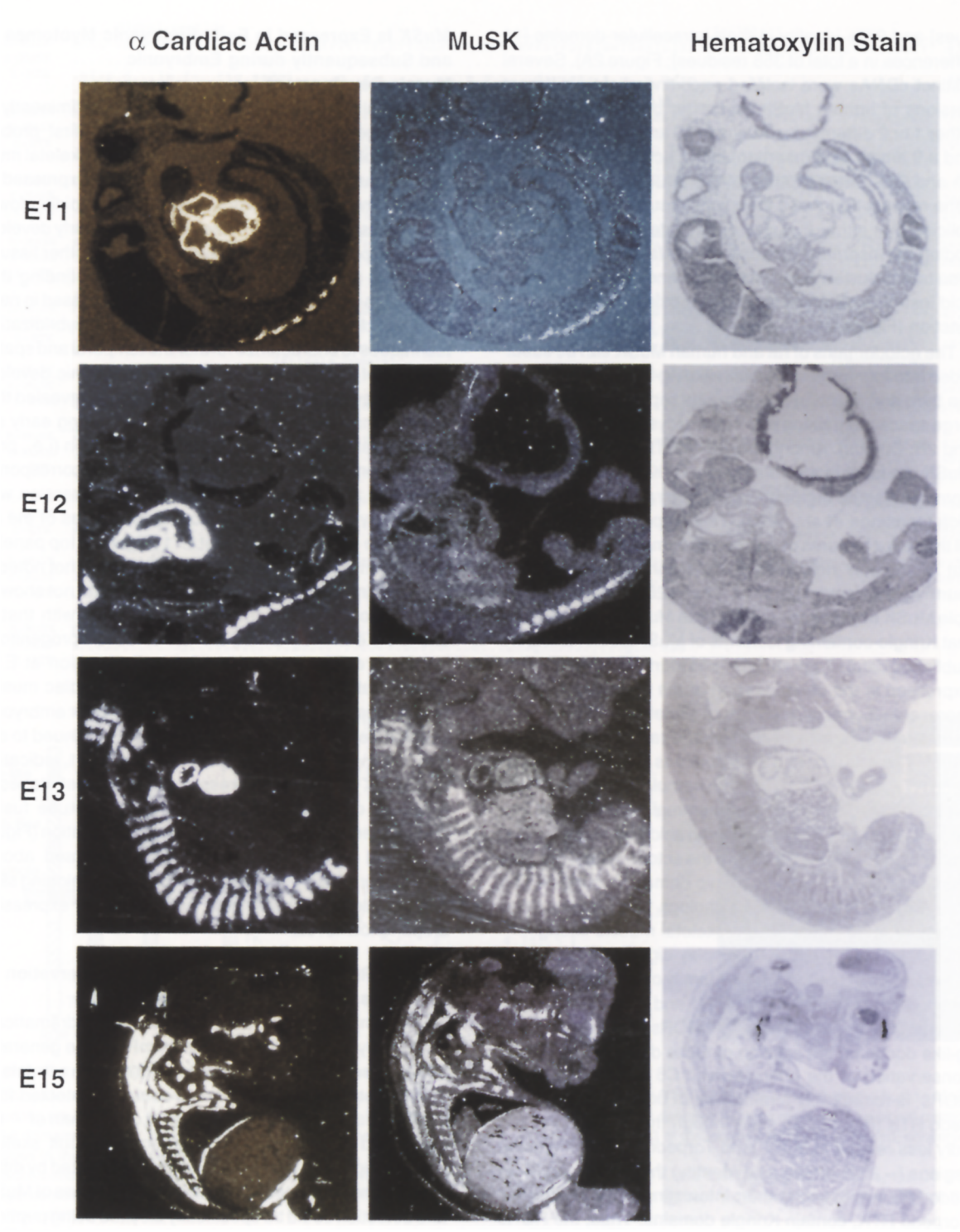

Figure 3. In Situ Localization of *MuSK* Transcripts in Rat Embryos

Low magnification views of sagittal sections of rat embryos displaying in situ hybridization of an antisense ^{35}S-labeled riboprobe for *MuSK*; in situ localization of α-cardiac actin transcripts in adjacent sections as well as hematoxylin-stained bright-field views are provided for comparison. Note the precise colocalization of the sites of expression of α-cardiac actin and *MuSK*, except for the complete absence of *MuSK* in the heart. Embryonic day (11–15) is indicated on the left.

for prolonged periods (up to 28 days), whereas, following a nerve crush injury that allowed for reinnervation, MuSK protein levels returned to baseline with reinnervation (Figure 4B).

Consistent with the notion that MuSK induction is caused by a decrease in muscle activity, physical immobilization (as a result of ankle joint fixation) resulted in a dramatic induction of both MuSK mRNA and protein levels

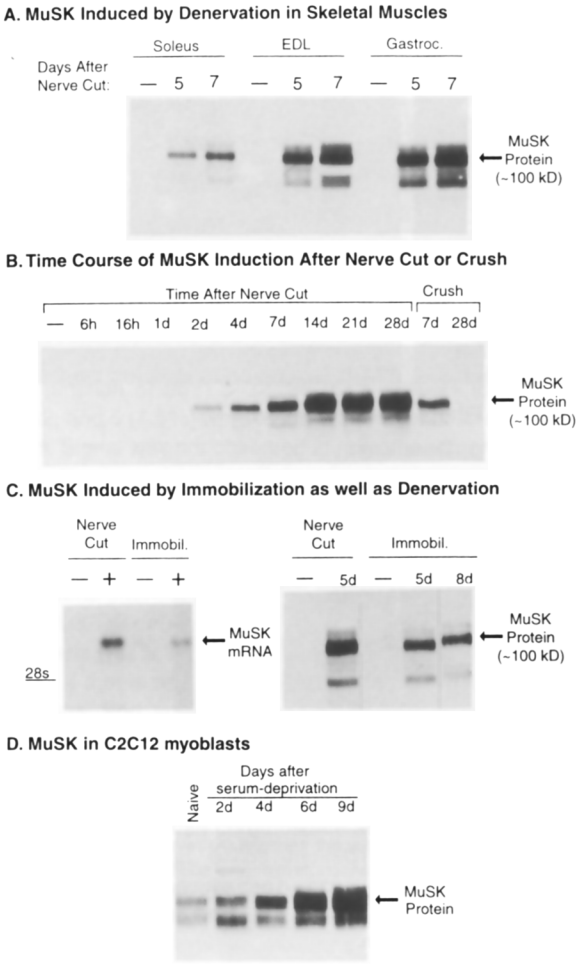

Figure 4. MuSK Induction in Denervation, Immobilization, and Myoblast Differentiation

(A) MuSK protein induction following denervation of different skeletal muscles; (B) time course of this induction in gastrocnemius following nerve cut or crush; (C) induction of MuSK mRNA and protein in gastrocnemius by immobilization as well as denervation; (D) induction of MuSK protein during C2C12 myoblast differentiation. Immunoblots or Northern analysis of MuSK protein or mRNA, as indicated; the number of days after the nerve lesion or immobilization is indicated.

in the gastrocnemius (Figure 4C). We also observed increased MuSK expression in muscle after block of electrical activity by application of tetrodotoxin to the sciatic nerve (data not shown).

MuSK Is Expressed in Myoblasts and Up-Regulated upon Fusion to Myotubes

To determine the expression profile of MuSK during the in vitro differentiation of myoblast cells, we examined levels of MuSK protein in the immortalized mouse myoblast cell line C2C12. MuSK was expressed at low levels in C2C12 myoblast cells prior to differentiation and fusion and gradually increased to high levels upon fusion induced by serum deprivation (Figure 4D).

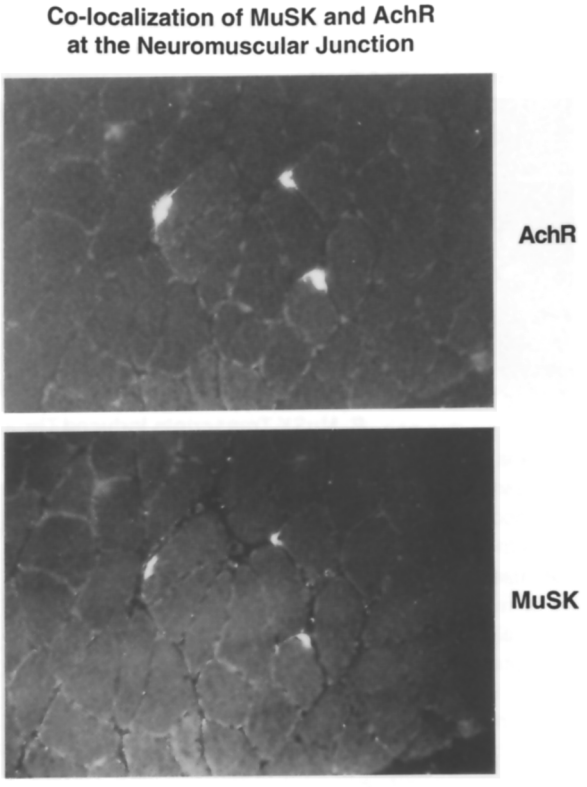

Figure 5. Colocalization of MuSK and AChR in Adult Skeletal Muscle

Frozen sections (10 μm thick) of the rat sternomastoid muscle are depicted. Antibodies against MuSK specifically stain neuromuscular synapses (bottom), which are simultaneously visualized using tetramethylrhodamine-labeled α-bungarotoxin, which detects AChRs (top).

MuSK Colocalizes with AChRs at the NMJ in Normal Adult Muscle

To investigate the subcellular localization of MuSK in adult skeletal muscle, immunofluorescence microscopy was used to compare MuSK distribution with that of the AChR, which is known to be confined to the postsynaptic membrane of the NMJ (Anderson and Cohen, 1974). Frozen sections of muscle were double labeled with tetramethylrhodamine-conjugated α-bungarotoxin, which specifically labels AChRs, or with antisera specific to MuSK detected with a fluorescein-conjugated secondary antibody. In normal adult muscle, MuSK-specific immunostaining was confined to neuromuscular synapses as judged by precise colocalization with AChRs (Figure 5, compare upper and lower panels); the MuSK immunostaining was blocked by the peptide used to raise the antisera. MuSK immunostaining was not evident in the nonsynaptic membrane of the myofiber or in the other cell types present in skeletal muscle.

Synaptic localization of AChRs in normal adult muscle appears to be due, in part, to the fact that the mRNAs encoding the AChR subunits are transcribed only in subsynaptic nuclei; prior to innervation, or following denervation, these mRNAs are transcribed by nuclei throughout the myofiber. To determine whether the NMJ localization of

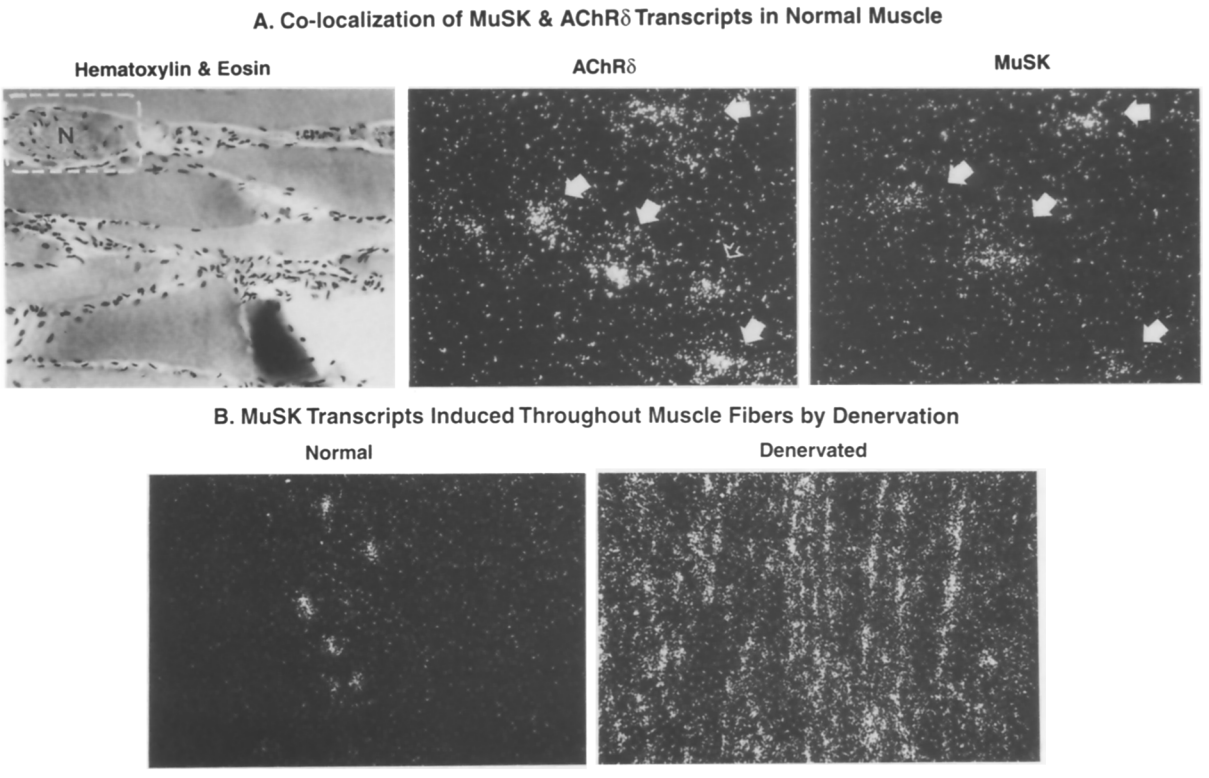

Figure 6. *MuSK* Transcripts Localize to Subsynaptic Nuclei in Normal Adult Diaphragm, while Transcripts Are Widely Expressed Following Denervation of EDL

In situ hybridizations performed using antisense ^{35}S-labeled riboprobes for *MuSK* and AChRδ on adjacent sections (A) reveal colocalization of *MuSK* and AChRδ transcripts, presumably by subsynaptic nuclei known to express specifically AChRδ transcripts in normal adult muscle. Bright-field view in (A) shows the position of the phrenic nerve, marking the central region of muscle containing abundant NMJs. In (B), comparison of localized *MuSK* expression (presumably by subsynaptic nuclei) in normal muscle (left) contrasts with widespread expression (apparently throughout myofiber, as noted by striped pattern overlying longitudinally sectioned myofibers) 4 days following denervation.

MuSK results from a similar mechanism, in situ hybridizations were performed on adjacent sections of normal adult diaphragm using probes specific for MuSK or an AChR subunit (the δ chain). Clusters of transcripts for both genes were detected only in the central region of the muscle, near the nerve, where NMJs are most abundant (Figure 6A); these clusters were specifically detected by antisense and not sense control probes. The finding that these clusters largely colocalized (Figure 6A) strongly suggests that in normal adult muscle the gene for MuSK, like the genes for the AChR subunits, is transcribed only by subsynaptic nuclei. Following denervation, *MuSK* transcripts become widely detectable throughout the myofibers (Figure 6B), indicating abolishment of the restriction of transcription to subsynaptic nuclei, as also occurs for AChR transcripts.

Altogether, the in situ hybridization data coupled with the immunolocalization studies provide compelling evidence that the subcellular distribution of MuSK closely parallels that of the AChR and is perhaps governed by similar mechanisms. That is, *MuSK* transcripts are widespread throughout developing myotubes and early muscle. In mature muscle, MuSK becomes highly localized to the NMJ, where it remains in normal adult muscle. Following denervation or other changes in muscle activity, MuSK becomes highly and widely expressed throughout the myofiber. Thus, these studies indicate that MuSK is the only known RTK that localizes to the NMJ, and its localization is dramatically regulated by innervation and muscle activity.

The *MuSK* Gene Maps to Mouse Chromosome 4 and Human Chromosome 9q31.3-32

To determine whether the *MuSK* gene might map near any known genetic mutations with phenotypes that might be expected to result from *MuSK* alteration, we determined the chromosomal location of the *MuSK* gene in mouse and man. In mice, the location was determined by interspecific backcross analysis using progeny derived from mating of [(C57BL/6J × Mus spretus) F1 × C57BL/6J] mice. This interspecific backcross mapping has been typed for over 1900 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). Genomic DNAs from C57BL/6J and M. spretus were digested with several restriction enzymes and analyzed by Southern blot hybridization for informative polymorphisms using a rat *MuSK* cDNA probe, revealing that a 14.0 kb M. spretus–specific EcoRI fragment polymorphism could be used to follow segregation of the *Musk* locus in backcross mice. Such an analysis revealed that

Musk is located on mouse chromose 4 in a region syntenic with human chromosome 9q (see Figure 2D).

Mapping the *MuSK* locus in humans confirmed the mouse mapping. To localize the human *MuSK* gene, we performed fluorescence in situ hybridization of a biotin-labeled genomic *MuSK* probe to normal human metaphase chromosomes. Hybridization of this probe resulted in specific labeling of only chromosome 9 (see Figure 2E). Specific labeling of 9q31–32 was observed in four (7 cells), three (5 cells), two (10 cell), or one (3 cells) chromatid(s) of the chromosome 9 homologs in 25 cells examined. Of 66 signals observed (66 of 100 21q chromatids from 25 metaphase cells were labeled), 25 (38%) were located at the junction of 9q31.3–32, 36 (54.5%) were located at 9q32, and 5 (7.5%) were located at the junction of 9q32–33.1. Signal was not observed at any other chromosomal site. We observed specific signal at 9q32 in a second hybridization experiment using this probe. These results indicate that the *MuSK* gene is localized to chromosome 9 bands 31.3–32, syntenic to the mouse map position.

Several interesting mutations map near the position of the *MuSK* gene in both mice and humans. For example, vacillans (*vc*) is a recessive mouse mutation on chromosome 4 that is associated with tremors, swaying of hindquarters, and substantially decreased muscle strength (Sirlin, 1956). Fukayama type congenital muscular dystrophy is a human recessive disorder that is the second most common type of childhood muscular dystrophy in Japan and maps to human chromosome 9q31–33 (Toda et al., 1993). Further study will be required to determine whether either of these diseases can be accounted for by mutations in the *MuSK* gene.

Discussion

We report the identification and characterization of rat and human versions of a RTK, termed MuSK, that appears to be quite specific for the skeletal muscle lineage. MuSK appears to be expressed at several critical periods during the life of skeletal, but not cardiac, muscle lineage cells. Initial embryonic expression of MuSK occurs in the early myotome. Expression stays high throughout skeletal muscle development in the embryo, during myotube fusion and subsequent differentiation, wherever skeletal muscle is found. Shortly after birth, MuSK expression plummets, and MuSK expression in normal adult skeletal muscle appears to be highly restricted to the NMJ, as is expression of AChRs. Following denervation or loss of muscle activity due to immobilization, MuSK expression is up-regulated throughout the myofiber and stays high unless reinnervation occurs.

The small number of RTKs that are known to be expressed in a lineage-specific manner have all been shown to serve critical roles for those lineages (Snider, 1994; Fong et al., 1995; Sato et al., 1995). The intriguing expression pattern of MuSK is highly suggestive of important functions for this receptor and its putative ligand in several aspects of skeletal muscle development and function. Expression of some MDFs, most notably myf5, which is apparently expressed in the somite prior to myotome formation, appears to precede that of MuSK (Li and Olson, 1992; Hauschka, 1994). On the other hand, MuSK expression may occur prior to the appearance of late-appearing MDFs such as MyoD (Li and Olson, 1992; Hauschka, 1994). Although a precise side by side analysis of MuSK expression compared with the MDFs must be performed, the current data would be consistent with the possibility that some of the MDFs are involved in initiating MuSK expression, while MuSK itself might regulate onset of later MDFs. Although most known growth factor receptors, such as those for FGFs, PDGF, and EGF, are rapidly down-regulated following cessation of the proliferative phase of myoblast development (Hauschka, 1994), MuSK expression remains high throughout early muscle development in vivo and increases as myoblasts differentiate during culture in vitro. Thus, in contrast to growth factors that primarily seem to maintain myoblast proliferation or the earliest phases of differentiation, the putative ligand for MuSK may continue to serve a critical role during later stages of differentiation.

In normal adult muscle, expression of MuSK is restricted to the NMJ. Thus, MuSK is the first RTK known to be expressed in this crucial subcellular location (Falls et al., 1993; Jo et al., 1995). Expression at the NMJ suggests an important signaling role that could be critical for the formation, organization, maintenance, or function of this highly specialized structure. Thus, MuSK could be involved in the early stages of agrin-mediated aggregation of AChRs; it has been noted that such aggregation involves tyrosine phosphorylation of AChRs (Wallace et al., 1991). As a growth factor receptor localized to the NMJ, MuSK also becomes a prime candidate for mediating the critical survival role played by incoming nerves on the primary myotube scaffold. MuSK may also be involved in promoting specific transcription of certain genes (including those for AChR subunits as well as MuSK itself) from subsynaptic nuclei, as has been suggested for the neuregulins, which appear to be released from nerve terminals and are thought to act on receptors at the NMJ (Falls et al., 1993; Jo et al., 1995). MuSK may well contribute to other responses mediated by the nerve terminal, perhaps due to regulated release by the terminal of the putative MuSK ligand. In addition to producing agrin, promoting myotube survival, and regulating expression at subsynaptic and extrajunctional nuclei, neural influences are clearly involved in the pruning of excess synapses formed early in development, in the continued editing of synapses throughout life, in the regulation of structural protein synthesis or degradation and resulting muscle growth or atrophy, in the determination of muscle fiber types, in the production of muscle-derived neurotrophic factors, and in an assortment of other processes. While some of these neural influences may depend in part upon nerve regulation of muscle activity, neural influences also appear to involve release of growth factors by the nerve terminal. For example, the rapid muscle atrophy resulting from denervation is thought to be due to both interruption of electrical activity, which itself seems to have trophic effects upon muscle, as well as loss of muscle-sustaining factors (termed "myotrophic" factors) released by the nerve terminal (Grinnel, 1994).

In fact, activity-mediated effects appear to be inextrica-

bly linked to changes in growth factors and their receptors. Muscle activity appears to regulate muscle-derived neurotrophic factors: denervation or muscle inactivation can markedly alter muscle production of such factors, and it has recently been shown that exercise and increased muscle activity lead to increased expression of neurotrophin-4 (Funakoshi et al., 1995). Denervation can also dramatically affect expression of growth factor receptors on skeletal muscle, which presumably alters responsivity of skeletal muscle to factors recognizing these receptors. Prior to the findings reported here for MuSK, the IGF and CNTF receptors represented the only known examples of growth factor receptors up-regulated by denervation (Davis et al., 1993; Grinnel, 1994; Helgren et al., 1994). Interestingly, exogenously provided IGF and CNTF both seem capable of blunting the muscle atrophy resulting from denervation (Grinnel, 1994; Helgren et al., 1994). Parallels with MuSK suggest that the ligand for this novel receptor may act as a very specifically acting growth factor for denervated skeletal muscle. MuSK is also the only known growth factor receptor whose expression is dramatically up-regulated not only by denervation but by physical immobilization of muscle as well, raising the possibility that the ligand for MuSK may be trophic in this instance as well; no other factors are known that may prevent disuse atrophy. Since muscle size and strength are normally regulated by the daily level of activity and work performed by the muscle, MuSK may also be involved in nonpathological regulation of muscle growth.

The finding that the ectodomain of MuSK resembles those of the RORs, while the kinase domain is instead similar to those of the Trk neurotrophic factor receptors, suggests that RTKs can evolve in a modular fashion. Thus, the ectodomain of MuSK may bind to a ligand unrelated to the neurotrophins but instead structurally similar to those recognized by the RORs, while the cytoplasmic domain of MuSK may mediate survival or differentiation responses more similar to those mediated by the Trks. The closest known relative of MuSK, in both its extracellular and cytoplasmic domains, is the receptor cloned from the electric organ of the electric ray Torpedo californica, which was also found to be expressed specifically in the skeletal muscle of this organism. Because the electric organ is a specialized variant of skeletal muscle, MuSK and its orthologs appear to have crucial skeletal muscle lineage-restricted functions that are conserved across long evolutionary distances. Further characterization of MuSK and identification of its ligand will help define a novel receptor–factor system that seems certain to play important roles in many aspects of skeletal muscle development and function.

Experimental Procedures

cDNA Library and Cloning

A cDNA library constructed using mRNA purified from denervated rat skeletal muscle (S. Davis, T. Aldrich, and G. D. Y., unpublished data) was used as a template for PCR using degenerate oligonucleotide primers corresponding to the amino acid sequences DLAARN (5′ primer) and DVWSYG (3′ primer), which are conserved sequences in tyrosine kinases (Hanks and Quinn, 1991). The respective sequences of the 5′ (sense) and 3′ (antisense) primers were 5′-TCTTGACTCGAGA(T,C)(T,C)T(N)GC(N)GC(N)(C,A)G(N)AA-3′ and 5′-GAATTCGAGCTCCC(A,G)(A,T)A(N)GCCCA(N)AC(A,G)TC-3′, where N indicates inclusion of all 4 bases at a given position and the tail sequences are underlined. The resulting PCR products were subcloned into the Bluescript (Stratagene, Inc., La Jolla, CA) plasmid, and a library of bacteria harboring these subclones was screened with probes specific to particular kinases to eliminate known kinases. The remaining clones were subjected to high throughput DNA sequencing, revealing a single novel RTK-like sequence we have termed MuSK. To obtain a full-length *MuSK* cDNA clone, a cDNA library was prepared using mRNA from denervated rat gastrocnemius muscle and the Uni-ZAP XR vector kit (Stratagene, Inc., La Jolla, CA). Library screening, plaque purification, and subcloning were performed as described (Valenzuela et al., 1993). To obtain the human *MuSK* sequence, rat probes were first used to isolate genomic phage clones spanning the entire human *MuSK* gene (D. M. V., E. R., and G. D. Y., unpublished data). DNA sequence spanning the initiation and termination codons was obtained and used to design exact oligonucleotide primers that allowed for PCR-based amplification (using the TaKaRa LA PCR kit optimized for long-range PCR, from Takara Shuzo Co., LTD, Shiga, Japan) of the entire human *MuSK* cDNA from mRNA prepared from human myoblasts (Clonetics, Inc., CA). DNA sequencing was performed using the ABI 373A DNA sequencer and *Taq* Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The human and rat *MuSK* cDNA sequences are listed under GenBank accession number U34985.

In Situ Hybridization

A DNA fragment, corresponding to amino acids 710–868 from the rat sequence depicted in Figure 2A, was subcloned into the Bluescript KS(+) plasmid (Stratagene, Inc., La Jolla, CA) for generating RNA probes specific for rat *MuSK*. The α-cardiac actin probe was kindly provided by M. P. Goldfarb, and the AChRδ probe by S. J. Burden. The ^{35}S-radiolabeled antisense or sense strand probes were transcribed off linearized plasmids and used for in situ hybridization as described previously (Valenzuela et al., 1993).

Immunoprecipitations, Immunoblotting, Immunostainings, and Labeled α-Bungarotoxin Stainings

Anti-peptide (to the peptides SLSHSDLSTRARVSSP and CSIHRILQRMCERAEGTVGV) polyclonal antisera were generated in rabbits by Research Genetics, Inc. (Huntsville, AL). Immunoprecipitations, immunoblotting, and immunostaining using the rat MuSK-specific antisera were performed as previously described (Jo et al., 1995; Stitt et al., 1995). Side by side comparison of immunostaining on frozen sections of rat sternomastoid muscle using tetramethylrhodamine-labeled α-bungarotoxin to detect AChRs or anti-MuSK-specific antisera detected using secondary fluorescein-conjugated antibodies was essentially performed as described previously (Jo et al., 1995).

Animal Surgeries

Nerve cuts and nerve crushes were performed as described previously (Helgren et al., 1994). For ankle joint fixation, rats were anesthetized with ketamine/xylazine, a 0.1 mg/ml solution of epinephrine was injected into the right heel, and a longitudinal incision was made over this region. A 23 gauge needle was inserted through the calcaneus and talus, into the shaft of the tibia. The needle was cut flush, and the wound was sutured. All animal use in this study was conducted in compliance with approved institutional animal care and use protocols and according to NIH guidelines (Guide for the Care and Use of Laboratory Animals, 1985, NIH publication no. 86-23).

Interspecific Backcross Mouse Chromosomal Mapping

Generation of mice used for the mapping, protocols, and strategy have been detailed previously (Copeland and Jenkins, 1991; Valenzuela et al., 1995). The *Musk* probe, a 540 bp EcoRV–NotI fragment of rat cDNA, was labeled with [α-^{32}P]dCTP using a nick translation labeling kit (Boehringer Mannheim); washing was done at a final stringency of 0.4× SSCP, 0.1% SDS at 65°C. A fragment of ~23 kb was detected in EcoRI-digested C57BL/6J DNA, and a fragment of 14.0 kb was detected in EcoRI-digested M. Spretus DNA. The presence or absence of the 14.0 kb EcoRI M. Spretus-specific fragment was followed in

backcross mice. A description of the probes and restriction fragment length polymorphisms for the loci linked to *Musk*, including CNTF receptor, tenasin c (*Tnc*, formerly *Hxb*) has been reported previously (Valenzuela et al., 1995).

Fluorescence In Situ Hybridization to Human Chromosomes

A 14 kb human *MuSK* genomic DNA fragment (D. M. V., E. R., and G. D. Y., unpublished data) was used as the probe. Fluorescence in situ hybridization was performed as described previously (Rowley et al., 1990).

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GenBank Accession Number

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