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Muscle-specific *trk*-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases

(*Torpedo californica*/electric organ/neuromuscular synapse)

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ABSTRACT **Little is known about the signaling pathways by which motoneurons induce synapses on muscle fibers, and no receptors for synapse-inducing signals have yet been identified. Because several other inductive events in development are mediated by receptor tyrosine kinases (RTKs), and because phosphotyrosine staining within muscle fibers is concentrated at synaptic sites, one possibility is that synapse-inducing signals are transduced by a RTK within the muscle fiber. We have used PCR to search for tyrosine kinases within the electric organ of the electric ray *Torpedo californica*, since this tissue is homologous to muscle but is much more densely innervated and is therefore a rich source of synaptic molecules. We have isolated a RTK that is specifically expressed in electric organ and skeletal muscle. The kinase domain of this receptor is related to the *trk* family of neurotrophin receptors, but unlike any previously described receptor, the extracellular region of this *Torpedo* RTK contains a kringle domain close to the transmembrane domain.**

A central problem in neurobiology is to understand how synapse formation is regulated. Synaptic development has been best studied at the vertebrate neuromuscular junction, where, following contact with the presynaptic motoneuron, the muscle fiber becomes highly specialized in the synaptic region (1). These specializations include the accumulation of acetylcholine receptors (AChRs) within the postsynaptic membrane, the localization of both intracellular and extracellular molecules to the synaptic site, and the local transcription of AChR subunit genes by the synaptic nuclei of the muscle fiber (2). Thus the formation of the postsynaptic apparatus represents a complex differentiation program that is induced by the motoneuron in a manner analogous to embryonic induction, except that the responding target is a region of a syncytial myofiber rather than an individual cell.

It is not known how many signals are required to induce these postsynaptic specializations, but one signal appears to be agrin (3), a basal lamina protein which is made by motoneurons and which causes clustering of AChRs in cultured myotubes (4). It is unclear, however, whether agrin is sufficient to induce all aspects of neuromuscular synapse formation, and several additional molecules have also been proposed as synaptic signals, including acetylcholine receptor inducing activity (ARIA) (5) and fibroblast growth factor (FGF)-related molecules (6). A key step in understanding the mechanism of synapse induction will be the identification of the receptor(s) for the inducing signals. Many types of cell–cell interaction are mediated by tyrosine phosphorylation, and in several cases inductive signals during embryonic development are known to be transduced by receptor tyrosine kinases (RTKs) (7–10). An attractive possibility, therefore, is that a tyrosine kinase within the myofiber may be involved in the induction of neuromuscular synapses. Consistent with this possibility, phosphotyrosine staining in myofibers is concentrated at neuromuscular synapses and is at least partly due to tyrosine phosphorylation of the AChR (11), while agrin (12), FGF (13), and ARIA (G. Corfas, D. Falls and G. Fischbach, personal communication) are all known to induce tyrosine phosphorylation in responding cells.

In this study we have used PCR to search for tyrosine kinases that may be involved in the transduction of synapse-inducing signals. As a source, we have used *Torpedo* electric organ, since this tissue is homologous to muscle but is much more densely innervated. Because of this dense innervation, the electric organ is a rich source of synaptic molecules, and many proteins that are concentrated at neuromuscular synapses, including the AChR subunits, acetylcholinesterase, agrin, and the 43- and 58-kDa subsynaptic proteins, were originally isolated from the electric organ (14). We describe here a receptor tyrosine kinase that is expressed specifically in electric organ and skeletal muscle.* This receptor is related to the *trk* family of neurotrophin receptors, but it differs from all previously described receptors in that it possesses a kringle domain in the extracellular region.

MATERIALS AND METHODS

Torpedo californica was obtained from Winkler Enterprises (San Pedro, CA). PCR was performed with cDNA from electric organ mRNA as a template. PCR primers were targeted to the sequences LHRDLAA and DVWS(F/Y)G, corresponding to domains VI and IX (16). Primer sequences were 5′-TTTGAATTCTBCAYMVGAYCTBGCHGC and 5′-TTTGGATCCRWANSWCCANACRTC (in which B = C, G, or T; Y = C or T; M = A or C; V = A, C, or G; H = A, C, or T; W = A or T; R = A or G; S = G or C; and N = any nucleoside).

PCR-derived clones were used to screen an electric organ cDNA library. Of 90,000 clones screened, 7 gave positive signals with one PCR probe. Inserts were analyzed by sequencing. Since none was full-length, a primer-extended library was constructed to obtain additional sequence. A composite sequence was assembled from the longest clones from each library. The entire coding region was sequenced on both strands.

Northern blots were probed with antisense RNA probes as described (15). Three different RTK probes were used, corresponding to nucleotides 1–1985 (which includes the entire extracellular region), 1724–3400 (which includes the entire intracellular region), and 1–565 (which encodes the N-terminal region); all three probes gave identical patterns of bands. The AChR α subunit probe was transcribed from a 272-bp fragment (nucleotides 441–713 according to ref. 17). The EF1α probe was transcribed from a full length *Xenopus* cDNA (18). Results were quantified by using a PhosphorIm-

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Abbreviations: RTK, receptor tyrosine kinase; AChR, acetylcholine receptor.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L11311).

ager (Molecular Dynamics, Sunnyvale, CA). Relative amounts of RTK and AChR transcripts in electric organ were determined by probing duplicate blots with similar amounts of each probe and correcting for probe length.

RESULTS

To identify tyrosine kinases that are expressed in the electric organ, we designed PCR primers targeted to two highly conserved domains present in all known tyrosine kinases (16). In contrast to the primers in earlier studies (19, 20), our primers were designed to be almost maximally degenerate, to amplify the widest possible range of tyrosine kinases present in the electric organ. PCR products of the appropriate size were subcloned and classified by sequencing. Seventy independent clones were examined, and these corresponded to 19 different sequences, including both intracellular and receptor-like tyrosine kinases. Three sequences were represented at much higher frequency than the others, suggesting they might be derived

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ATGAACTTTATCCCAGTCGACATTCCACTCTTGATGATCTTCCTTGTGACAACTGGGGGCTCAGCTGACGGAATCCTTCCCAAAGCTCCACAGATCACCAGTCCCTTGGAGACAGTGGAT  240
M  N  F  I  P  V  D  I  P  L  L  M  I  F  L  V  T  T  G  G  S  A  D  G  I  L  P  K  A  P  Q  I  T  S  P  L  E  T  V  D   40

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A  L  V  E  E  E  A  S  F  M  C  A  V  D  S  Y  P  A  A  E  I  T  W  T  R  N  N  I  P  I  R  P  F  D  T  R  Y  S  T  K   80

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M  K  P  K  I  I  R  P  P  T  D  V  R  A  L  L  G  S  K  V  V  L  P  C  S  T  M  G  N  P  K  P  A  I  S  W  F  K  D  E  160

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F  E  Y  S  R  S  A  A  L  E  V  Q  V  S  A  R  I  V  K  A  P  T  S  Q  N  V  S  Y  G  S  E  V  I  L  Q  C  K  A  T  G  240

TTTCCGATTCCCACCATCAAGTGGTTGGAGAATGGGAGAGCAGTCCCCAAGGGTTCGATACAGAATCGCATCAAGGGAGAGGTGATGGAATCTAGGCTGCGGGTCTATGTTACCAGACCT  960
F  P  I  P  T  I  K  W  L  E  N  G  R  A  V  P  K  G  S  I  Q  N  R  I  K  G  E  V  M  E  S  R  L  R  V  Y  V  T  R  P  280

TCACTGTTCACTTGCCTGACTACCAACAAGCACAATGAAGGAAGTACCACAGCAAAAGCCACTGCCACCCTGGATATCAAAGAATGGAGATTGTACAAAGGTGACTTGGGCTATTGCAGC 1080
S  L  F  T  C  L  T  T  N  K  H  N  E  G  S  T  T  A  K  A  T  A  T  L  D  I  K  E  W  R  L  Y  K  G  D  L  G  Y  C  S  320

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CACCATGACCCAGAAGCATGCACCAGAGTCTCTTTTCTTGACATGAAGAAGGGGCTCGTTACCAGAATGTGTTACAACAATAACGGGAGGTTTTACCAGGGATCGGTGAATGTCACTGCA 1560
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E  R  P  W  C  Y  T  M  D  R  D  I  R  W  E  F  C  N  V  P  Q  C  I  N  V  S  S  I  S  E  M  K  P  K  T  E  T  A  N  T  560

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P  S  T  S  A  T  Y  S  M  T  V  I  I  S  I  I  S  S  L  A  A  S  I  L  L  I  I  I  I  L  T  C  H  H  H  Q  K  G  L  Q  600

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Y  G  M  A  H  E  E  V  I  Y  Y  V  R  D  G  N  I  L  S  C  P  E  N  C  P  P  E  L  Y  N  L  M  R  L  C  W  S  N  M  P  920

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S  D  R  P  T  F  A  S  I  H  R  I  L  E  R  M  H  Q  R  M  A  A  A  L  P  V                                              946

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AAACTAGCCATGCCCAGTACATTGAAACACCCAATATTAAACCGTAGGTTCCAATTACCATCCCTCCCATTTTTTGTTCATGAACACGTTTCTCAATTATTACTGGATGTTCATTTAATT 3240

ATATATGATGACATTTTATGAGGGGCTTCAGTAAAGAAGACAGGGAGNACTGTCACCATGNGTAGAAGGCTCGTAACCAAAGGCCCCTAATTTAAAAAGAAAAGTCAGGGGATTTTTTTT 3360

TTTGTGGATTGTTCCAATCAACTTGAAAAAGAAGGAATTC                                                                                  3400
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FIG. 1. Nucleotide and deduced amino acid sequence of *Torpedo* RTK cDNA. The sequence was assembled from two overlapping cDNA clones. The sequence ends at a natural *Eco*RI site. The upstream in-frame stop codon is underlined. The deduced amino acid sequence includes a signal peptide (amino acid residues 1–22), four immunoglobulin (Ig)-like repeats (conserved cysteines at positions 51 and 101 in Ig I, 144 and 193 in Ig II, 236 and 285 in Ig III, 401 and 447 in Ig IV), a kringle domain (residues 464–542), a transmembrane domain (residues 571–591), and a tyrosine kinase domain (residues 658–933) with a short kinase insert of 20 amino acids (residues 749–768). There are four potential N-linked glycosylation sites (at positions 225, 340, 477, and 544).

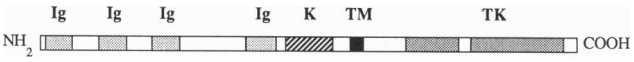

FIG. 2. Predicted structure of *Torpedo* RTK, showing immunoglobulin-like repeats (Ig), kringle domain (K), transmembrane domain (TM), and tyrosine kinase domain (TK).

from more abundant mRNAs. We therefore used these three clones to screen an electric organ cDNA library. One clone hybridized to 7 out of 90,000 plaques, while no hybridizing plaques were detected with the other two probes. Preliminary sequence analysis indicated that the 7 hybridizing clones all corresponded to the same or very similar transcripts. Since none of these clones was full length, we constructed a second cDNA library by using a specific oligonucleotide primer, and we assembled a full-length composite sequence (Fig. 1).

The cDNA sequence shown in Fig. 1 includes an open reading frame of 946 amino acid residues, counting from the first methionine. This reading frame begins with a consensus translation initiation sequence and is flanked by in-frame stop codons and therefore appears to be complete. The sequence encodes a RTK, the predicted structure of which is shown in Fig. 2. The protein sequence begins with a putative signal peptide and includes a single putative transmembrane domain. We have confirmed that the N terminus is extracellular by staining unpermeabilized cells that had been transfected with an epitope-tagged cDNA (data not shown). The intracellular (C-terminal) region includes a tyrosine kinase domain that is most closely related to the *trk* family of neurotrophin receptors (21), as shown in Fig. 3*A*. The tyrosine kinase domain shows 49%, 49%, and 47% identity to the corresponding domains of trk, trkB, and trkC, respectively, while these sequences show between 76% and 83% identity to each other. It is less closely related to Dtrk (32% identity). This receptor is therefore unlikely to be the *Torpedo* homologue of any of the known *trk* gene products but rather appears to be a somewhat divergent member of the *trk* family. The kinase domain is, however, more similar to the *trk* family than to any other known tyrosine kinase. The next-closest match was the *ret* protooncogene product (26), with 43% identity, but the *Torpedo* RTK contains many conserved residues that are diagnostic of the *trk* family (Fig. 3*A*) and which are absent from other tyrosine kinases, including ret. A further point of similarity with the *trk* family is the very short C-terminal sequence: most receptor tyrosine kinases have C-terminal sequences that extend for 50–300 amino acids beyond the conserved kinase domain (16), but the *trk* family all show C-terminal sequences of 15 or fewer, and that of the *Torpedo* receptor sequence is 13 amino acids.

The homology with the *trk* family is confined to the tyrosine kinase domain, and the extracellular region of the *Torpedo* receptor is not closely related to any of the *trk* family members, suggesting that it is probably not a receptor for a ligand related to nerve growth factor. The extracellular region contains four immunoglobulin-like repeats, similar to those found in a variety of receptors and adhesion molecules (27), as shown in Fig. 2. The spacing of these repeats would allow for another repeat between numbers 3 and 4, but although this region is cysteine rich and may therefore be highly folded, the conserved amino acids that surround the paired cysteines of immunoglobulin-like repeats are absent. The extracellular region also contains a single kringle domain (Fig. 3*B*), similar to those found in extracellular proteases (25). Fibronectin type II repeats, which share some homology with kringle domains (25), are present in several secreted and cell-surface molecules, including one transmembrane receptor, namely the IGF-II/mannose 6-phosphate receptor (28); the presence, however, of a *bona fide* kringle domain in a cell surface receptor is, to the best of our knowledge, unprecedented.

Northern blots revealed the presence of two transcripts in *Torpedo* electric organ, with estimated lengths of 3 and 5 kb. The sequence shown in Fig. 1 is likely to correspond to the

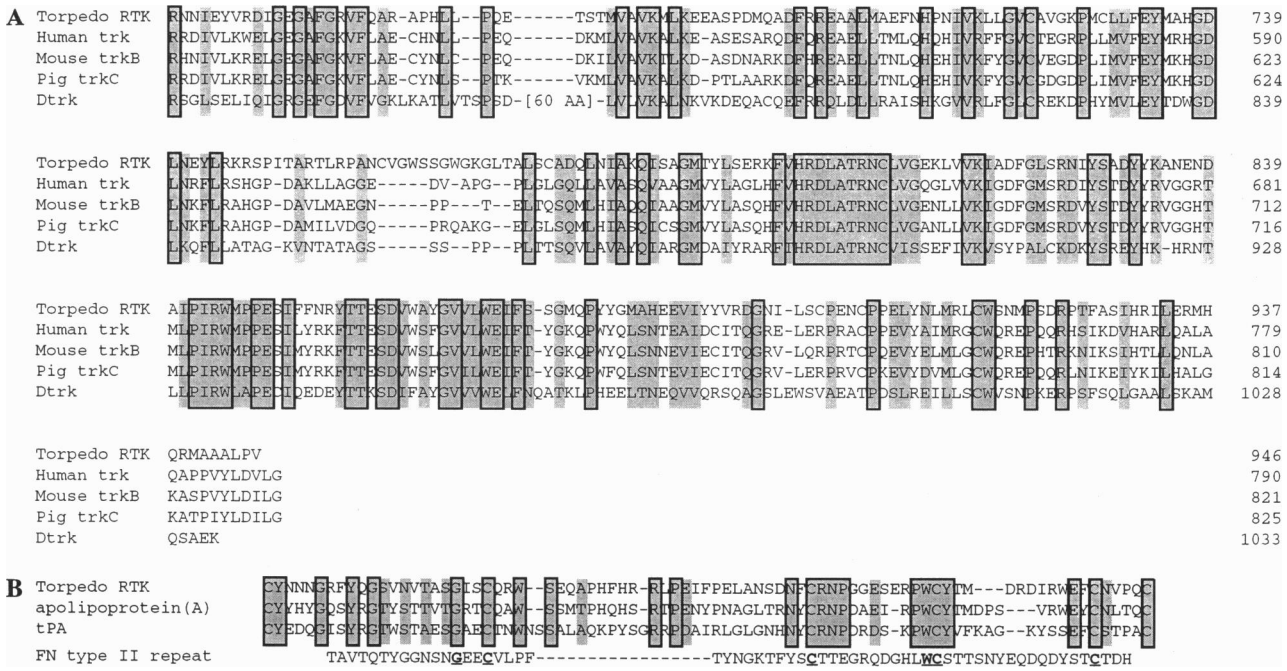

FIG. 3. (*A*) Alignment of *Torpedo* RTK kinase domain with other members of the *trk* family. Residues that are identical in all five sequences are boxed, and residues that are similar are shaded. Dashes indicate gaps inserted for optimal alignment. A 60-amino acid insertion in the Dtrk sequence (22) has been omitted for convenience. Residues in the *Torpedo* sequence that are diagnostic for the *trk* family include A662, F667, R702, F732, T805, and P847. (*B*) Alignment of RTK kringle domain with kringle 32 of human apolipoprotein(a) (23) and kringle 1 of human tissue-type plasminogen activator (tPA) (24). Identical residues are boxed, and similar residues are shaded. Dashes indicate gaps inserted for optimal alignment. The *Torpedo* sequence shows 48% identity with apolipoprotein(a) and 35% identity with tPA. The first type II repeat of bovine fibronectin (FN) is shown for comparison (alignment based on ref. 25). Residues that are conserved between FN type II repeats and kringle domains are underlined.

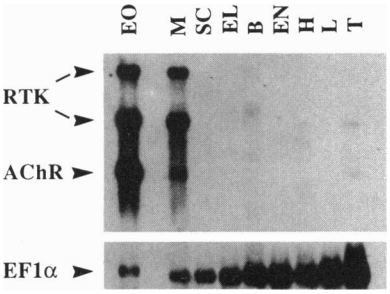

FIG. 4. *Torpedo* RTK is specifically expressed in electric organ and skeletal muscle. Adult *Torpedo* tissues were analyzed by Northern blotting for expression of RTK, AChR α subunit (AChR), and EF1α, a ubiquitous transcript of about 1.7 kb which serves as a control for RNA integrity. Each lane contains poly(A)-selected RNA corresponding to 80 μg of total RNA, from electric organ (EO), skeletal muscle (M), spinal cord (SC), electric lobe of brain (EL), remainder of brain (B), electric nerve (EN), heart (H), liver (L), or testis (T). The muscle sample contains a mixture of fast (white) and slow (red) muscle. Two major RTK transcripts are detected, with estimated sizes of 3 and 5 kb, based on comparisons with the AChR α subunit transcript (2.3 kb; ref. 17) and DNA standards. Both RTK transcripts are highly expressed in electric organ and muscle, and are also expressed at a lower level in testis. The faint bands visible in the brain and heart lanes hybridize to the RTK probe, but they have not been characterized. The blot was reprobed (without stripping) with the AChR probe; this probe is much shorter than the RTK probe, and so the intensities of the bands do not reflect the 60-fold difference in abundance between RTK and AChR transcripts. The blot was stripped and reprobed at low stringency with a *Xenopus* EF1α probe.

longer transcript, since it extends for 3.4 kb but does not include the poly(A) tail. Multiple transcripts have been observed for other receptor tyrosine kinase genes, and in some cases these are known to encode receptors with different signaling capabilities (29) or ligand specificities (30). We suspect, however, that the two transcripts observed here encode similar or identical protein sequences, because each transcript hybridizes to both N-terminal and C-terminal probes, and because we have not found different classes of coding sequences among our cDNA clones.

To examine the expression of the RTK in different tissues, we probed Northern blots of poly(A)-selected RNA with a probe corresponding to the entire coding region of the cDNA sequence. As shown in Fig. 4, the RTK is highly expressed in both electric organ and skeletal muscle and is expressed at much lower levels in other tissues. Although RNase protections revealed low levels of expression in all tissues examined except electric nerve (data not shown), even testis, the most highly expressing of these tissues, shows a level of expression approximately 1/10 that of skeletal muscle.

Since we were interested in the possibility that this receptor might be involved in synapse formation, we compared its expression with that of a known synaptic molecule, namely the AChR α subunit. In adult muscle, AChR mRNAs are largely confined to the synaptic region as a result of local transcription by synaptic nuclei (2). Because the electric organ is very densely innervated, it expresses high levels of AChRs: the AChR α subunit mRNA constitutes about 0.5% of total mRNA in the electric organ, and it is about 100-fold more abundant in electric organ than in skeletal muscle (Fig. 4). The RTK is expressed at about 1/50 the level of the AChR α subunit in electric organ (Fig. 4 and data not shown), but unlike the AChR, it is expressed at similar levels in electric organ and muscle, suggesting that its expression in muscle is unlikely to be restricted to synaptic nuclei. It remains to be determined whether the protein is concentrated at synaptic sites.

DISCUSSION

We have used PCR to search for tyrosine kinases in *Torpedo* electric organ, and we have identified a RTK that is highly expressed in both electric organ and skeletal muscle. This receptor shows several novel features: (*i*) It is largely muscle specific, unlike any other known RTK, (*ii*) it is related to the *trk* family in its kinase domain but not in its extracellular region, and (*iii*) the extracellular region contains a kringle domain, a structural motif that has not been found in any other type of receptor.

The tyrosine kinase domain of the *Torpedo* RTK is most closely related to the *trk* family of neurotrophin receptors, and the *Torpedo* sequence also resembles the *trk* family in having a very short C-terminal region beyond the kinase domain. The similarity between the kinase domains of the *Torpedo* RTK and the *trk* family suggests that both may act through the same downstream signaling pathways. The extracellular region of the *Torpedo* RTK, by contrast, is not closely related to the other members of the *trk* family: the four trk sequences described previously are all colinear in their extracellular regions (21), whereas the *Torpedo* RTK does not align with these sequences. Although the Torpedo RTK, like the other *trk* gene products, contains immunoglobulin repeats, these are a common feature of receptors and adhesion molecules, and the number and spacing of these repeats differ between the *Torpedo* sequence and the other trk sequences. We therefore suspect that the ligand for the *Torpedo* RTK is not likely to be a neurotrophin molecule related to nerve growth factor.

The extracellular region of the *Torpedo* receptor includes a single kringle domain close to the transmembrane domain. Kringle domains are highly folded structures (31) that are present in many of the extracellular serine proteases that are involved in blood clotting and fibrinolysis (25). Kringles are also present in hepatocyte growth factor (32) and apolipoprotein(a) (23), but both of these molecules appear to be derived from proteases, since they show extensive homology to proteases outside their kringle domains. To the best of our knowledge the presence of a kringle domain in a receptor, or indeed in any molecule otherwise unrelated to proteases, has not been reported previously. Kringle domains are involved in binding of tissue-type plasminogen activator (tPA) to fibrin and are required for fibrin-mediated stimulation of tPA protease activity (33), but it is not known how they regulate protease activity or whether kringle domains in other proteases play a similar role. The intron/exon structure of the kringle-containing proteases has suggested that they have evolved in modular fashion and that kringles can function as autonomous units (25, 34), and the presence of a kringle domain in a completely different type of protein, a receptor tyrosine kinase, would seem to confirm this view. The significance of the kringle domain in this receptor remains obscure. It has been suggested that extracellular proteolysis may play a role in neuromuscular synapse formation (35) and elimination of polyneuronal innervation (36), and it is therefore tempting to speculate that this kringle domain may interact with a protease-mediated signaling pathway, although we have no evidence for this at present. Although agrin has domains homologous to Kazal-type protease inhibitors (4), inhibitors of this class bind directly to the active sites of serine proteases (37) and are not known to interact with kringle domains.

Unlike the gene for any other known tyrosine kinase, this gene is highly expressed in both electric organ and skeletal muscle and is expressed at low levels or not at all in other tissues examined. We estimate that it constitutes about 0.01% of the electric organ mRNA pool, and it may well be the most abundant tyrosine kinase in the electric organ, since it is expressed much more abundantly than any of the other tyrosine kinase-like sequences that we isolated in a PCR screen designed to amplify all classes of tyrosine kinases. The function of this receptor is unknown, but the fact that it is specifically expressed in skeletal muscle suggests that it

performs a muscle-specific function. Since it is also highly expressed in electric organ, a noncontractile tissue that is specialized for synaptic transmission, and since phosphotyrosine staining in both muscle and electric organ is concentrated at synaptic sites (11), the most attractive possibility is that this receptor is involved in the formation or maintenance of neuromuscular synapses. Synapse formation in skeletal muscle fibers is induced by signals from the motoneuron, which induce the muscle fiber to undergo a complex differentiation program, including the concentration of AChRs at synaptic sites (1) and the local activation of AChR gene expression in synaptic nuclei (2). The signals for both processes are stably maintained within the synaptic basal lamina, since AChR clusters (4) and local transcription of AChR genes (2) are reinduced when muscle regenerates within its original basal lamina in the absence of innervation. Similar mechanisms are likely to operate in the electric organ, since AChRs are highly concentrated under presynaptic terminals (38), and AChR expression increases sharply at the time that neurons invade the electric organ (39). Electrocytes that are explanted prior to innervation do not undergo this increase, whereas if they are explanted after innervation, the increase continues, suggesting that a nerve-derived signal is required and that its effect persists after explantation (39) or denervation (38).

No receptor for a synapse-inducing signal has yet been isolated, although a putative agrin receptor has been detected by binding studies (40). This putative receptor is uniformly distributed on cultured myotubes but becomes concentrated at AChR clusters after agrin treatment. The high level of RNA encoding the RTK described here in muscle as well as electric organ suggests that the RNA is probably not confined to the synaptic region of the myofiber, but it remains possible that the protein is concentrated at synaptic sites. A receptor need not be localized, however, to transduce a local signal; the *Drosophila* gene torso, for example, encodes a RTK that mediates the local induction of terminal fates during embryogenesis (8), but the receptor is distributed throughout the syncytial blastoderm and is not confined to the terminal regions (41).

In conclusion, we have identified a RTK that is expressed specifically in skeletal muscle and electric organ. Future experiments are necessary to determine whether this receptor is involved in synapse formation or in other muscle-specific functions. In particular, it will be important to determine whether it is present at synaptic sites in skeletal muscle and/or electric organ and whether it is a receptor for known ligands such as agrin (4) or ARIA (5) that have been implicated in synapse formation. It will also be important to investigate the function of the kringle domain, since this structural motif is not present in any previously described receptor. Recent evidence, however, indicates that another RTK with a kringle domain is expressed in the developing *Drosophila* nervous system (C. Wilson, D. Goberdhan, and H. Steller, personal communication), indicating that this class of receptors has been conserved over a long period of evolution and raising the possibility that the *Torpedo* RTK described here may be a prototype for a more extended gene family.

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