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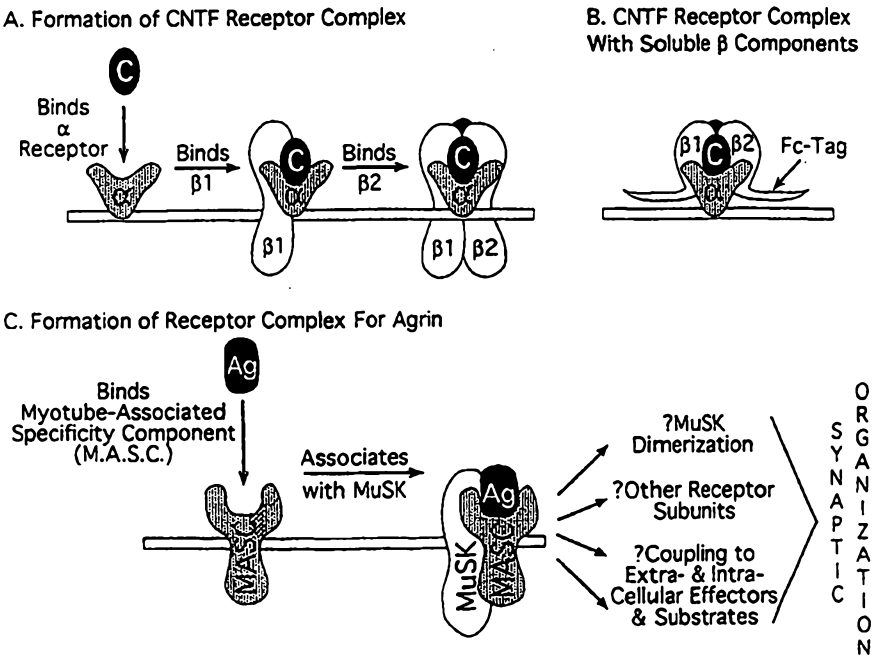
(54) Title: NOVEL TYROSINE KINASE RECEPTORS AND LIGANDS



(57) Abstract

The present invention provides for a gene, designated as musk, that encodes a novel tyrosine kinase receptor expressed in high levels in denervated muscle. The invention also provides for an isolated polypeptide which activates MuSK receptor. The invention further provides for a polypeptide which is functionally equivalent to the MuSK activating polypeptide. The invention also provides assay systems that may be used to detect and/or measure ligands that bind the musk gene product. The present invention also provides for diagnostic and therapeutic methods based on molecules that activate MuSK.

NOVEL TYROSINE KINASE RECEPTORS AND LIGANDS

INTRODUCTION

The present invention provides for novel molecules capable of activating a tyrosine kinase receptor, and methods of making and use thereof.

BACKGROUND OF THE INVENTION

The ability of polypeptide ligands to bind cells and thereby elicit a phenotypic response such as cell growth, survival or differentiation in such cells is often mediated through receptor tyrosine kinases. The extracellular portion of each receptor tyrosine kinase (RTK) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand-recognizing characteristic. Binding of a ligand to the extracellular domain results in signal transduction via an intracellular tyrosine kinase catalytic domain which transmits a biological signal to intracellular target proteins. The particular array of sequence motifs of this cytoplasmic, catalytic domain determines its access to potential kinase substrates (Mohammadi, et al., 1990, Mol. Cell. Biol., 11: 5068-5078; Fantl, et al., 1992, Cell, 69:413).

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The tissue distribution of a particular tyrosine kinase receptor within higher organisms provides relevant data as to the biological function of the receptor. For example, the localization of a Trk family receptor, TrkB, in tissue provided some insight into the potential biological role of this receptor, as well as the ligands that bind this receptor (referred to herein as cognates). Thus, for example, in adult mice, trkB was found to be preferentially expressed in brain tissue, although significant levels of trkB mRNAs were also observed in lung, muscle, and ovaries. Further, trkB transcripts were detected in mid and late gestation embryos. In situ hybridization analysis of 14 and 18 day old mouse embryos indicated that trkB transcripts were localized in the central and peripheral nervous systems, including brain, spinal cord, spinal and cranial ganglia, paravertebral trunk of the sympathetic nervous system and various innervation pathways, suggesting that the trkB gene product may be a receptor involved in neurogenesis and early neural development as well as play a role in the adult nervous system.

The cellular environment in which an RTK is expressed may influence the biological response exhibited upon binding of a ligand to the receptor. Thus, for example, when a neuronal cell expressing a Trk receptor is exposed to a neurotrophin which binds that receptor, neuronal survival and differentiation results. When the same receptor is expressed by a fibroblast, exposure to the neurotrophin results in proliferation of the fibroblast (Glass, et al., 1991, Cell 66:405-413). Thus, it appears that the extracellular domain provides the determining factor as to the ligand specificity, and once signal transduction is initiated the cellular environment will determine the phenotypic outcome of that signal transduction.

A number of RTK families have been identified based on sequence homologies of their intracellular domains. For example, two members of the TIE (tyrosine kinase with immunoglobulin and EGF homology domains) family, known as TIE-1 and TIE-2, have 79% sequence homology in their

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intracellular region (Maisonpierre, et al., 1993, Oncogene 8:1631-1637). Although these receptors share similar motifs in their extracellular domain, only 32% of the sequences are identical.

A receptor having a kinase domain that is related to the Trk family has been identified in the electric ray Torpedo californica and may play a role in motor neuron induced synapses on muscle fibers. Jennings, et al. Proc. Natl. Acad. Sci. USA 90: 2895-2899 (1993). This kinase was isolated from the electric organ, a tissue which is a specialized form of skeletal muscle. The tyrosine kinase domain of this protein is related to the Trk family, while the extracellular domain is somewhat divergent from the Trks. The protein was found to be expressed at high levels in Torpedo skeletal muscle, and at much lower levels in adult Torpedo brain, spinal cord, heart, liver and testis.

Often such novel RTKs are identified and isolated by searching for additional members of known families of tyrosine kinase receptors using, for example, PCR-based screens involving known regions of homology among Trk family members. (See, for example, Maisonpierre, et al., 1993, Oncogene 8: 1631-1637). Isolation of such so called "orphan" tyrosine kinase receptors, for which no ligand is known, and subsequent determination of the tissues in which such receptors are expressed, provides insight into the regulation of the growth, proliferation and regeneration of cells in target tissues. The identification and isolation of novel RTKs may be used as a means of identifying new ligands or activating molecules that may then be used to regulate the survival, growth, differentiation and/or regeneration of cells expressing the receptors. Further, because RTKs appear to mediate a number of important functions during development, the identification and isolation of such receptors, ligands and activating molecules enhances our understanding of developmental processes and may improve our ability to diagnose or treat abnormal conditions.

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For example, the above described methods may be used to study an event that occurs during development of the neuromuscular junction (NMJ) - the localization of acetylcholine receptors at the synapse. It has long been known that important signals are exchanged across the NMJ (Nitkin et al., 1987, J.Cell.Biol. 105: 2471-2478; Hall, Z.W. and Sanes, J.R., 1993, Cell/Neuron (Suppl.) 72/10: 99-121; Bowe, M.A. and Fallon, J.R., 1995, Ann. Rev. Neurosci. 18: 443-462; Sanes, J.R., 1995, Devel. Biol. 6: 163-173; Burden, S.J., et al., 1995, Devel. Biol. 6: 59-65). These signals include the chemical transmitter, acetylcholine, which is released from vesicles in the nerve terminal, recognized by acetylcholine receptors (AChRs) on the muscle, and ultimately results in electrical activation and contraction of the muscle.

Muscle also provides neurotrophic factors that support survival of motor neurons (DeChiara, T. et al., 1995, Cell 83: 313-322), and the nerve may in turn provide myotrophic factors that maintain muscle mass (Helgren, M.E., et al., 1994, Cell 76: 493-504). Reciprocal signaling interactions are also critical both for the formation and maintenance of the neuromuscular junction itself. Such signals regulate recognition of nerve-to-muscle contact, arrest the growth of the incoming nerve ending, and induce formation of a highly specialized nerve terminal marked by a polarized arrangement of synaptic vesicles and active zones. Simultaneously, precisely juxtaposed with respect to the nerve terminal, a complex molecular apparatus forms on the muscle membrane. This specialized postsynaptic structure, termed the motor endplate, comprises a tiny patch on the muscle membrane which is characterized by a dense clustering of particular proteins; some of these may receive nerve-derived signals, as AChRs are known to do, while others may be involved in creating the molecular scaffold for this post-synaptic specialization.

Signals produced by the nerve induce postsynaptic clusters by at least two mechanisms. First, these signals can induce redistribution of pre-existing

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molecules that are initially expressed throughout the myofiber, and second, they can induce localized transcription of specific genes only by subsynaptic nuclei underlying the NMJ. Between the nerve terminal and the motor endplate is a narrow synaptic cleft containing a complex basal lamina. This basal lamina is distinguished from the adjacent extracellular matrix by the accumulation of a number of proteins, such as acetylcholinesterase and s-laminin. The synaptic basal lamina also serves as a reservoir for signaling molecules exchanged between nerve and muscle.

While the reciprocal interactions between nerve and muscle have been intensively explored for decades, many questions still remain concerning the precise nature of the signals involved in formation of the NMJ. The realization that empty sheaths of the synaptic basal lamina could induce formation of both nerve terminal specializations and motor endplates suggested that key signaling molecules might be embedded in the extracellular matrix (Sanes, J.R. et al., 1978, J.Cell. Biol. 78: 176-198; Burden, S.J., et al., 1979, J.Cell. Biol. 82: 412-425; McMahan, U.J. and Slater, C.R., 1984, J.Cell. Biol. 98: 1453-1473; Kuffler, D.P., 1986, J.Comp. Neurol. 250: 228-235). Indeed, recent findings indicate that a protein discovered for its AChR-inducing activity and thus termed ARIA (Jessell, T.M., et al., 1979, PNAS (USA) 76: 5397-5401; Falls, D.L., et al., 1990, Cold Spring Harbor Symp. Quant. Biol. 55: 397-406; Falls, D.L., et al., 1993, Cell 72: 801-815) which can increase the expression of several of the AChR subunit genes (Harris, D.A., 1989, et al., Nature 337: 173-176; Martinou, J.-C., et al., 1991, PNAS (USA) 88: 7669-7673; Jo, S.A., et al., 1995, Nature 373: 158-161; Chu, G.C., et al., 1995, Neuron 14: 329-339), is localized to the synaptic basal lamina (Jo, S.A., et al., 1995, Nature 373: 158-161; Goodearl, A.D., et al., 1995, J.Cell. Biol. 130: 1423-1434). Molecular cloning has revealed that ARIA corresponds to a factor alternatively referred to as neuregulin, NDF, heregulin or glia growth factor, and binds to the erbB family of RTKs (Carraway, K.L. and Burden, S.J., 1995, Curr. Opin. Neurobiol. 5: 606-612). Interestingly, neuregulin production has been demonstrated in

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motor neurons and neuregulin receptors, erbB3 and erbB4, have recently been localized to the motor endplate, supporting the idea that nerve-derived neuregulin provides an important signal to muscle that regulates transcription from subsynaptic nuclei (Altiok, N., et al., 1995, EMBO J. 14: 4258-4266; Moscoso, L.M., et al., 1995, Dev. Biol. 172: 158-169; Zhu, X., et al., 1995, EMBO J. 14: 5842-5848).

Another protein, known as agrin, was isolated from the synaptic basal lamina based on its ability to cause redistribution of pre-existing AChRs into clusters on the surface of cultured myotubes (Godfrey, E.W., et al., 1984, J.Cell. Biol. 99: 615-627; Rupp, F., et al., 1991, Neuron 6: 811-823; Tsim, K.W., et al., 1992, Neuron 8: 677-689). In contrast to neuregulin, agrin does not appear to regulate AChR expression. However, agrin causes the clustering of a number of synaptic components, along with AChRs, in cultured myotubes (Wallace, B.G., 1989, J.Neurosci. 9: 1294-1302).

A variety of data are consistent with the notion that agrin also acts in vivo to induce and maintain the postsynaptic membrane specialization. Most important among these are the findings that the most active forms of agrin are exclusively made by neurons and are deposited in the synaptic basal lamina (Ruegg, M.A., et al., 1992, Neuron 8: 691-699; Ferns, M., et al., 1993, Neuron 11: 491-502; Hoch, W., et al., 1993, Neuron 11: 479-490), and that antibodies to agrin block nerve-induced clustering of AChRs on cultured myotubes (Reist, N.E., et al., 1992, Neuron 8: 865-868).

The precise mechanism of action of agrin remains a mystery (Sealock, R. and Froehner, S.C., 1994, Cell 77: 617-619). Agrin is known to induce tyrosine phosphorylation of AChRs, and inhibitors of tyrosine phosphorylation block agrin-mediated clustering (Wallace, B.G., et al., 1991, Neuron 6: 869-878; Wallace, B.G., 1994, J.Cell. Biol. 125: 661-668; Qu, Z. and Huganir, R.L., 1994, J.Neurosci. 14: 6834-6841; Wallace, B.G., 1995, J.Cell. Biol. 128: 1121-1129).

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Intriguing recent findings have revealed that agrin can directly bind to α-dystroglycan, an extrinsic peripheral membrane protein that is attached to the cell surface by covalent linkage to β-dystroglycan, which in turn couples to the intracellular cytoskeletal scaffold via an associated protein complex (Bowe, M.A., et al., 1994, Neuron 12: 1173-1180; Campanelli, J.T., et al., 1994, Cell 77: 673-674 ; Gee, S.H., et al., 1994, Cell 77: 675-686; Sugiyama, J., et al., 1994, Neuron 13: 103-115; Sealock, R. and Froehner, S.C., 1994, Cell 77: 617-619).

Extrasynaptically, the dystroglycan complex binds laminin on its extracellular face, and couples to the actin scaffold via a spectrin-like molecule known as dystrophin. At the synapse however, agrin (via its own laminin-like domains) may be able to substitute for laminin, whereas utrophin (a dystrophin related protein) replaces dystrophin as the link to actin (reviewed in (Bowe, M.A. and Fallon, J.R., 1995, Ann. Rev. Neurosci. 18: 443-462)). The dystroglycan complex co-clusters with AChRs in response to agrin in vitro, and components of this complex are concentrated at the endplate in vivo (reviewed in (Bowe, M.A. and Fallon, J.R., 1995, Ann. Rev. Neurosci. 18: 443-462)).

Recent evidence suggests that a 43 kD cytoplasmic protein, known as rapsyn, anchors AChRs to a sub-synaptic cytoskeleton complex, probably via interactions with the dystroglycan complex (Cartaud, J. and Changeux, J.P., 1993, Eur. J. Neurosci. 5: 191-202; Apel, E.D., et al., 1995, Neuron 15: 115-126). Gene disruption studies reveal that rapsyn is absolutely necessary for clustering of AChRs, as well as of the dystroglycan complex. However, other aspects of NMJ formation, involving presynaptic differentiation and synapse-specific transcription, are seen in mice lacking rapsyn (Gautam, M., et al., 1995, Nature 377: 232-236).

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Despite the findings that agrin can bind directly to α-dystroglycan, and that AChRs and the dystroglycan complex are linked and co-cluster in response to agrin, the role of dystroglycan as an agrin receptor remains unclear (Sealock, R. and Froehner, S.C., 1994, Cell 77: 617-619; Ferns, M., et al., 1996, J. Cell Biol. 132: 937-944). It has recently been reported that a 21kD fragment of chick agrin is sufficient to induce AChR aggregation (Gesemann, M., et al., 1995, J. Cell. Biol. 128: 625-636). Dystroglycan could be directly involved in activating signalling pathways that appear to be required for clustering, such as those involving tyrosine phosphorylation, by an unknown mechanism (for example, via association with a cytoplasmic tyrosine kinase).

Alternatively, dystroglycan could be involved in couplings of agrin not only to AChRs but to a novel signalling receptor. It also remains possible that dystroglycan does not play an active or required role in initiating clustering, and is merely among an assortment of post-synaptic molecules that undergo clustering. Recent evidence indicates that the agrin fragment that is active in inducing AChR aggregation does not bind to α-dystroglycan and a structural role in aggregation, rather than a signal transfer role, has been proposed for the binding of agrin to α-dystroglycan (Gesernann, M., et al., 1996, Neuron 16: 755-767).

SUMMARY OF THE INVENTION

The tyrosine kinase, termed "MuSK" for "muscle specific kinase," is expressed in normal and denervated muscle, as well as other tissues including heart, spleen, ovary or retina (See Valenzuela, D., et al., 1995, Neuron 15: 573-584). The tyrosine kinase has alternatively been referred to as "Dmk" for "denervated muscle kinase." (See PCT International Application No. PCT/US94/08039 published 1 February 1996 as WO 96/02643 entitled Denervated Muscle Kinase (DMK), A

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Receptor Of the Tyrosine Kinase Superfamily). Thus, the terms MuSK and Dmk may be used interchangeably. The protein appears to be related to the Trk family of tyrosine kinases.

According to the present invention novel polypeptides have been identified which activate the MuSK receptor.

The term "MuSK activating molecule" as used herein refers to a molecule which is capable of inducing phosphorylation of the MuSK receptor in the context of a differentiated muscle cell. One such activating molecule is agrin as described in the Examples set forth herein.

In one embodiment, the polypeptide of the invention is the portion of human agrin which activates the MuSK receptor and is encoded by a nucleotide sequence contained in the vector designated as pBluescript human Agrin-1 (pBL-hAgrin1) that was deposited with the American Type Culture Collection on December 12, 1995 under ATCC Accession No. 97378. The present invention also relates to isolated polypeptides which are functionally equivalent to this polypeptide.

The invention therefore provides for an isolated nucleic acid molecule having a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence contained in the vector designated as pBL-hAgrin 1 (ATCC Accession No. 97378) or a fragment thereof which encodes the portion of human agrin which activates the MuSK receptor;

(b) a nucleotide sequence that differs from the nucleotide sequence of (a) by the presence of an insert corresponding to the insert at position Z in Figure 15 and/or by the absence of an insert corresponding to the insert at position Y in Figure 15 and/or by the presence of a nucleic acid sequence encoding a further insert having the amino acid sequence VLSASHPLTVSGASTPR immediately following the insert at position Y in Figure 15 which encodes a polypeptide which activates the MuSK receptor;

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(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes a polypeptide which activates the MuSK receptor.

The invention further provides an isolated nucleic acid molecule encoding the portion of human agrin which activates the MuSK receptor, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 15 or a fragment thereof encoding at least amino acids 300 to 492;

(b) a nucleotide sequence that differs from the nucleotide sequence of (a) by the absence of an insert corresponding to the insert at position Y in Figure 15 and/or by the absence of an insert corresponding to the insert at position Z in Figure 15 and/or by the presence of a nucleic acid encoding a further insert having the amino acid sequence VLSASHPLTVSGASTPR immediately following the insert at position Y in Figure 15;

(c) a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of (a) or (b) and which encodes a polypeptide of substantially the same number of amino acids and which activates the MuSK receptor; and

(d) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from any of the nucleotide sequences of (a), (b) or (c) and which encodes a polypeptide which activates the MuSK receptor.

The nucleic acid molecules of the invention may contain or omit a nucleotide sequence insert so that the encoded polypeptide contains or omits the eight amino acids ELANEIPV at the position corresponding to amino acid position 1780 as shown in Figure 14 (insert Z in Figure 15). The nucleic acid may alternatively

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contain or omit a nucleic acid insert so that the encoded polypeptide contains or omits the four amino acids KSRK at the position corresponding to amino acid position 1643 as shown in Figure 14 (insert Y in Figure 15). A further 17 amino acid insert VLSASHPLTVSGASTPR may immediately follow the insert Y.

Stringent conditions as used herein are those which (1) employ low ionic strength and high temperature for washing, for example, 0.15 M NaCl/ 0.015 M sodium citrate /0.1% NaDodS0_4 at 50°C, or (2) use during hybridization of a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCI, 75 mM sodium citrate at 42°C.

The invention also provides for a method of promoting the growth, survival or differentiation of a MuSK receptor expressing cell comprising administering to the MuSK receptor expressing cell an effective amount of agrin or a portion thereof effective to active the MuSK receptor. The method may be practised in vitro or in vivo. In one embodiment of this method, the agrin is human agrin. In another embodiment of this method, the MuSK receptor expressing cell is a cell which is normally found in the heart, spleen, ovary, retina or skeletal muscle. In another embodiment, the MuSK receptor expressing cell is a cell which has been genetically engineered to express the MuSK receptor.

The present invention further provides polypeptides encoded by the nucleic acids of the invention. Such polypeptides may optionally be modified by covalent attachment of a polyethylene glycol molecule.

The invention further provides vectors, including expression vectors, which comprise a nucleic acid molecule according to the invention, host-vector systems comprising such vectors in a suitable host cell and methods of producing polypeptides of the invention which comprise growing host cells of such a system under conditions permitting production of the polypeptide and recovering the polypeptide so

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produced.

The present invention also includes a method of treating a patient suffering from a disease or disorder affecting muscle comprising administering to the patient a polypeptide or nucleic acid of the invention or a derivative thereof. The invention also relates to the use of a polypeptide or nucleic acid of the invention in the manufacture of a medicament for the treatment of a disease or disorder affecting muscle.

The invention yet further relates to pharmaceutical compositions comprising a polypeptide or nucleic acid of the invention and a pharmaceutically acceptable carrier.

The present invention also includes a method of producing a monoclonal or polyclonal anti-agrin antibody or fragment thereof wherein a polypeptide of the invention or a fragment or derivative thereof is employed as an immunogen.

The invention further relates to the vector pBL-hAgrin 1 (deposited as ATCC 97378).

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BRIEF DESCRIPTION OF THE FIGURE

FIGURE 1 - Nucleotide and deduced amino acid (single letter code) sequences of rat MuSK. The nucleotide sequence encoding mature MuSK begins around nucleotide 192.

FIGURE 2 - Northern blot showing distribution of MuSK in the rat during early development. Lane 1: Total embryo E9; Lane 2: Total embryo E11; Lane 3: Placenta E11; Lane 4: Embryo head E12; Lane 5: Embryo body E12; Lane 6: Embryo spinal cord E12; Lane 7: Placenta E12; Lane 8: Embryo head E13; Lane 9: Embryo body E13; Lane 10: Embryo brain E17; Lane ll: Embryo brain P1; Lane 12: Embryo brain P10; Lane 13: Embryo brain P19; Lane 14: Adult brain; Lane 15: Adult muscle; Lane 16: Adult denervated muscle; where day of sperm positivity is designated as day E1, and day of birth is designated as day P1.

FIGURE 3 - Northern blot showing distribution of MuSK in adult rat tissues. Lane 1: Brain; Lane 2: Olfactory bulb; Lane 3: Cortex; Lane 4: Hippocampus; Lane 5: Thalamus/hypothalamus; Lane 6: Midbrain; Lane 7: Hindbrain; Lane 8: Cerebellum; Lane 9: Spinal Cord; Lane 10: Thymus; Lane ll: Spleen; Lane 12: Liver; Lane 13: Kidney; Lane 14: Lung; Lane 15: Sciatic Nerve; Lane 16: Retina; Lane 17; Heart; Lane 18: Ovary; Lane 19: Muscle; Lane 20: Denervated muscle.

FIGURE 4 - Nucleotide and deduced amino acid (single letter code) sequences of human MuSK receptor.

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FIGURE 5 - Schematic representation of genomic DNA encompassing the three kinase domain exons of the mouse MuSK gene, of the targeting vector constructed, and of a mutant locus following successful targeting. The three exons of the MuSK kinase domain are indicated as black boxes, containing the indicated kinase subdomains (SD). The PGK-neo and MC1-tk cassettes are indicated as open boxes. The novel EcoRI (R) and NcoI (N) fragments generated following successful targeting are labeled. The 5' EcoRI/HpaI probe used to detect the endogenous and mutant EcoRI fragments was derived from genomic DNA not included in the targeting construct. B, BamHI; Hp, HpaI; S, SpeI (sites included within parentheses are destroyed in the cloning process).

FIGURE 6 - MuSK Knockout Mice - Southern blot of tail DNA from wild-type, heterozygous and homozygous F2 progeny showing the endogenous and mutant EcoRI fragments detected by the 5' RI/HpaI probe, as well as the endogenous NcoI fragments detected by the kinase region probe, which are absent in the homozygous mutant.

FIGURES 7A - 7D - Post-mortem histological analysis of lung demonstrating that the alveoli air sacs in the MuSK$^{-/-}$ newborn are not expanded (Figure 7A) as they are in the lung of the control littermate (Figure 7B), indicating that mutant pups do not take a single breath. Post-mortem histological analysis of hindlimb musculature reveals that MuSK$^{-/-}$ mice (Figure 7C) possess grossly normal muscle architecture similar to that of control mice (Figure 7D).

FIGURES 8A - 8C - Agrin induces AChR clustering in myotubes from control but not MuSK$^{-/-}$ mice. Myotubes derived from control and MuSK$^{-/-}$ mice were treated overnight with varying concentrations of agrin$_{4,8}$, stained with rhodamine-conjugated α-bungarotoxin (α-BGT) to label surface AChRs, and then either photographed at 64X magnification under rhodamine optics (Figure 8A, challenge with 100 nM agrin depicted) or subjected to AChR

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cluster quantitation (Figure 8B, each point represents the mean ± SEM of forty myotube segments). Total AChRs on the myotubes before agrin treatment was determined by binding with ^{125}I-α-BGT (Figure 8C, each bar represents the mean ± SEM CPM bound per μg of total cell protein (control: N=6; MuSK -/-: N= 5).

FIGURES 9A - 9D - c-agrin$_{4,8}$ and c-agrin$_{0,8}$ specifically induce rapid tyrosine phosphorylation of MuSK receptors. C2C12 and primary rat myoblasts were differentiated into myotubes and stimulated with conditioned media from COS cells transfected with a plasmid control (Mock) or plasmids encoding the various forms of soluble agrin, with conditioned media containing neuregulin, or with purified bFGF or insulin, as labelled. Stimulations were for ten minutes using 10 nM concentrations of the various factors, except as indicated in Figures 9C and 9D. Following factor challenges, the cells were lysed and subjected to immunoprecipitations (I.P.) for either the MuSK or ErbB3 receptors as indicated, then immunoblotted for phosphotyrosine levels. Only agrins containing the eight amino acid insert at the Z position, but not other factors, could induce MuSK phosphorylation (Figure 9A). Agrin could not induce phosphorylation of another muscle receptor, ErbB3 (Figure 9B). MuSK phosphorylation occurred at low agrin concentrations (Figure 9C) and very rapidly in response to agrin (Figure 9D).

FIGURES 10A & 10B - Agrin can not detectably bind to the isolated ectodomain of MuSK. Agrin was assayed for its binding to immobilized MuSK-Fc or to an immoblized agrin-specific monoclonal antibody (mAb), each coupled to a BIAcore sensorchip surface (Figure 10A); bindings to the MuSK-Fc surface were also done in the presence 2 mM Ca^{++} or heparin (0.01 mg/ml), as indicated, while bindings to the antibody surface were also competed with excess soluble monoclonal antibody or MuSK-Fc (each at 25

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μg/ml), as indicated. Reciprocally, binding of soluble MuSK-Fc or monoclonal antibody to immobilized agrin was assayed by first binding conditioned media transfected with a plasmid control (Mock) or a plasmid encoding c-agrin4,8 ($cAg_{4,8}$) to nitrocellulose, followed by detection using either the soluble MuSK-Fc or the agrin-specific monoclonal antibody, as indicated (Figure 10B); TrkB-Fc detection of nitrocellulose-immobilized BDNF served as an additional control.

FIGURE 11 - Agrin can only induce MuSK phosphorylation in the context of a differentiated myotube: evidence for a myotube-specific accessory component. Agrin-inducible phosphorylation of an introduced chick MuSK receptor was evaluated in a clone of C2C12 myoblasts stably transfected with a chick MuSK expression vector. The introduced chick MuSK is expressed regardless of whether this C2C12 clone is undifferentiated ("Undif") or differentiated into myotubes ("Dif") (middle panel), in contrast to the endogenous mouse MuSK, which is only expressed in differentiated cells (bottom panel). However, the chick MuSK can only be inducibly phosphorylated in response to agrin when it is assayed in differentiated myotubes (top panel). The chick MuSK displays the same specificity for activation by the various agrin isoforms (each at 10 nM for ten minutes) as does the endogenous mouse MuSK (compare transfected chick MuSK and endogenous mouse MuSK in upper panel).

FIGURES 12A-12C. Relevant models for the agrin/MuSK receptor complex. Figure 12A - Schematic representation depicting the step-wise assembly of the multi-component receptor complex for ciliary neurotrophic factor (CNTF); b1, gp130; b2, LIFRb. Figure 12B - Schematic depiction of the use of soluble b receptor components (Fc-tagged) to build a CNTF receptor complex attached to the cell surface via only one of its components, the non-signaling a component; surface binding of the soluble b components can be detected using antibodies recognizing the Fc tag. Figure 12C - Schematic representation of one of several possible models of the MuSK receptor complex for agrin,

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depicting requirement for a myotube-associated specificity component (M.A.S.C.) and possible interactions to additional components that may be required for signaling or coupling to various effectors or substrates; these couplings may be mediated extracellularly (for example via agrin binding to the dystroglycan complex) or intracellularly (for example via interactions of SH2 domain-containing proteins to phosphorylated tyrosines on MuSK).

FIGURES 13A - 13C. Evidence for an agrin/MuSK receptor complex utilizing a myotube-specific accessory component. Figure 13A - Formation of agrin/MuSK complexes on the surface of myotubes: undifferentiated (Undiff.) or myotube-differentiated (Diff) C2C12 cells were assayed for their ability to bind either MuSK-Fc or a control receptor-Fc fusion (TrkB-Fc), in the absence or presence of various agrin isoforms (provided in conditioned media from transient COS transfections); specific binding of MuSK-Fc to the myotube surface, which is enhanced by exogenously provided agrin, is suggested to involve complexes analogous to those depicted in Figure 12B. Figure 13B - Direct binding of agrin to MuSK is demonstrated by cross-linking analysis. Radiolabelled agrin (a recombinant c-terminal fragment (or portion) of human agrin, termed $hAgrin_{4,8}$) at 1 nM was chemically cross-linked to the surface of myotubes. Following cross-linking, lysates were immunoprecipitated with a MuSK-specific antibody (lane 1). The cross-linking was also done in the presence of excess (150 nM) unlabelled agrin (lane 2), while the immunoprecipitation was also done in the presence of excess peptide (corresponding to that used to generate the antibody) to block the MuSK precipitation; positions of the agrin/MuSK complex, as well as of various forms of unbound monomeric and dimeric agrin (see text), are indicated. Figure 13C - Inhibition of agrin-induced AChR clustering by MuSK-Fc: agrin-induced AChR-clustering (using 10 nM $c\text{-}agrin_{4,8}$) was performed on C2C12 myotube cultures in the presence of varying concentrations of soluble MuSK-Fc or a control receptor-Fc fusion (Ret-Fc); the soluble MuSK-Fc specifically inhibits, presumably by forming inactive

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complexes on the cell surface with agrin and the myotube-specific accessory component.

FIGURE 14 - Amino acid (single letter code) sequence of rat agrin indicating Y and Z sites of amino acid inserts found in splice variants.

FIGURE 15 - Nucleotide and amino acid (single letter code) sequences of human agrin expression construct including the signal peptide and flg tag (FLAG tag). The start of the coding region for the active C-terminal fragment (portion) of human agrin 4-8 is indicated. Also indicated are the position Y and position Z insert sites at which the 4 and 8 amino acid inserts are located. Throughout this application, references to human agrin 4,8; c-agrin 4,8; or human c-agrin 4,8 indicate the active C-terminal fragment (portion) of human agrin 4-8 as set forth in the Figure.

FIGURE 16 - Results of phosphorylation assay showing that the active C-terminal 50kD portion of human agrin 4,8 and the truncated delta 9 portion of human agrin can each induce phosphorylation of the MuSK receptor.

FIGURE 17 - Results of pharmacokinetic study comparing serum half-lives of active C-terminal 50kD portion of human agrin 4,8 (c-agrin 4,8) with active C-terminal 50kD portion of human agrin 4,8 that has been modified by covalent addition of polyethylene glycol.

DETAILED DESCRIPTION OF THE INVENTION

The MuSK tyrosine kinase molecule is related to the trk, family of tyrosine kinases. The sequence of the protein is set forth in Figure 1 as SEQ. ID NO: 1. The coding region of the mature protein is

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believed to begin on or around the serine-glycine-threonine on or around position 20 of the coded region.

This tyrosine kinase has been found to be induced in denervated skeletal muscle. Accordingly, it has been designated as MuSK (muscle specific kinase). It has also been referred to previously as Dmk (denervated muscle kinase). In addition to being found in skeletal muscle, both normal and denervated, MuSK has also been found to be present in, but not be limited to, the spleen, ovary and retina. It appears to be present during early development, but is also found in adult tissue.

MuSK may be related to the Torpedo RTK identified by Jennings, et al. supra. However, MuSK differs in that it appears to be induced in denervated muscle, whereas no such induction has been reported with regard to the Torpedo is RTK. Furthermore, the Torpedo RTK has an extracellular kringle domain, whereas MuSK does not. However, these kinases may be members of the same or related families.

The gene encoding rat MuSK has been cloned and the DNA sequence determined (Figure 1; SEQ ID NO: 2). The extracellular domain of the mature protein is believed to be encoded by the nucleotide sequence beginning on or around position 192 and ending on or around position 1610. The transmembrane portion of the protein is believed to be encoded by the nucleotide sequence beginning on or around position 1611 and ending on or around position 1697. The intracellular domain is believed to be encoded by the nucleotide sequence beginning on or around position 1698 and ending on or around position 2738. A cDNA clone encoding Dmk (MuSK) was deposited with the American Type Culture Collection on July 13, 1993 and accorded an accession number of ATCC No. 75498.

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The extracellular domain of the protein is believed to be comprised of the amino acids at or around positions 20 through 492 of the coding region set forth as SEQ ID NO: 1.

The similarity between MuSK and the Torpedo RTK suggests the utilization of regions of sequence homologies within these genes to develop primers useful for searching for additional, related RTKs.

The amino acid sequence for the MuSK protein is set forth in Figure 1 (SEQ ID NO: 1). Functionally equivalent molecules include derivatives in which amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

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Musk proteins also include proteins or fragments (portions) or derivatives thereof which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Substantially similar proteins are those from different species or are family members within a species and are identical in at least 40% of positions. Substantial similarity at the protein level includes the ability of a subject protein to compete with MuSK for binding to monoclonal antibodies raised against MuSK epitopes.

The MuSK protein is useful in 1) screening strategies, 2) purification strategies and 3) diagnostic uses. With respect to screening strategies, expression cloning strategies based on cell survival and proliferation assays provide a method of screening for cognate ligands (Class, et al. (1991) Cell 66:405-413). Since ligands that bind MuSK may be membrane bound, other strategies for identification of such receptors may be more well suited (Armitage, et al. 1992, Nature 357:80-82; Smith, et al. 1993, Cell 73:1349-1360). In preferred embodiments, the extracellular domain of MuSK is fused to a marker to create a chimeric protein which enables identification and purification of the extracellular domain when bound to a cognate.

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Further insight into the physiological role of MuSK will come from the further definition of the activating molecule of the present invention. The kinase domain of the MuSK receptor appears to be related to other receptor tyrosine kinases, thus it is likely that the MuSK receptor is involved in signal transduction in cells in which it is expressed. Accordingly, the MuSK activating molecule of the present invention may be used to induce signal transduction not only in naturally occurring MuSK-expressing cells, which include cells found in the muscle tissue, heart, spleen, ovaries and retina, but also in cells engineered to express the MuSK receptor. The MuSK activating molecule of the present invention may be used to promote the growth or survival of such cells.

The term "MuSK activating molecule" as used herein refers to a molecule which is capable of inducing phosphorylation of the MuSK receptor in the context of a differentiated muscle cell. One such activating molecule is agrin as described in the Examples set forth herein.

As used herein, the term "MuSK activating molecule" includes the isolated and purified MuSK receptor activating polypeptides described herein, as well as functionally equivalent molecules in which amino acid residues are substituted for residues within the sequence resulting in a silent change. For

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example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are proteins or fragments (portions) or derivatives thereof which exhibit the same or similar biological activity and derivatives which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

The present invention relates to an isolated and purified polypeptide which activates MuSK receptor. In one embodiment, the polypeptide of the invention is encoded by a nucleotide sequence contained in the vector designated as pBluescript human Agrin-1 (pBL-hAgrin1) that was deposited with the American Type Culture Collection on December 12, 1995 under ATCC Accession No. 97378 which encodes the portion of human agrin which activates the MuSK receptor. The present invention further provides for an isolated polypeptide which is functionally equivalent to this polypeptide.

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The invention further provides for an isolated nucleic acid molecule encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence contained in the vector designated as pBL-hAgrin 1 (ATCC Accession No. 97378) or a fragment thereof which encodes the portion of human agrin which activates the MuSK receptor; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) and which encodes the active portion of human agrin.

The invention also provides for the above-described nucleic acid molecule which additionally contains a nucleotide sequence so that the encoded polypeptide contains the eight amino acids ELANEIPV at the position corresponding to amino acid position 1780 as shown in Figure 14.

The invention further provides for an isolated nucleic acid molecule encoding the portion of human agrin which activates the MuSK receptor, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 15;

(b) a nucleotide sequence that differs from the nucleotide sequence of (a) by the absence of an insert corresponding to the insert at position Y in Figure 15 and/or by the absence of an insert corresponding to the insert at position Z in Figure 15 and/or by the presence of a nucleic acid encoding a further insert having the amino acid sequence VLSASHPLTVSGASTPR immediately following the insert at position Y in Figure 15;

(c) a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of (a) or (b) and which encodes a polypeptide of substantially the same number of amino acids and which activates the MuSK receptor; and

(d) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from any of the nucleotide sequences of (a), (b) or (c) and which

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encodes a polypeptide which activates the MuSK receptor.

The invention further provides for an isolated nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence encoding amino acids 1 to 492 set forth in Figure 15;

(b) a nucleotide sequence encoding amino acids 24 to 492 set forth in Figure 15;

(c) a nucleotide sequence encoding amino acids 60 to 492 set forth in Figure 15;

(d) a nucleotide sequence encoding amino acids 76 to 492 set forth in Figure 15;

(e) a nucleotide sequence encoding amino acids 126 to 492 set forth in Figure 15;

(f) a nucleotide sequence encoding amino acids 178 to 492 as set forth in Figure 15;

(g) a nucleotide sequence encoding amino acids 222 to 492 set forth in Figure 15;

(h) a nucleotide sequence encoding amino acids 260 to 492 set forth in Figure 15; and

(i) a nucleotide sequence encoding amino acids 300 to 492 set forth in Figure 15.

A further embodiment of the invention is an isolated and purified nucleic acid molecule encoding agrin 0-8 comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is as set

forth in Figure 15 with the exception that there is no insert at position Y. Another embodiment of the invention is an isolated and purified nucleic acid molecule encoding agrin 4-0 comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is as set forth in Figure 15 with the exception that there is no insert at position Z.

The present invention provides for an isolated polypeptide encoded by any one of the nucleic acid molecules of the invention as set forth herein. Furthermore, the present invention provides for said polypeptides modified by covalent attachment of a polyethylene glycol molecule.

Thus, the present invention provides truncated forms of the human agrin polypeptide which activate the MuSK receptor. As set forth herein, the invention also provides nucleic acid sequences encoding such truncated forms. These truncated forms retain, the ability to induce phosphorylation of the MuSK receptor.

Referring to Figure 15, starting at the N-terminal end (amino acid 24 - KSPC) these truncated forms of human agrin preferably have deletions of up to 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350 or 400 amino acids. Particularly preferred truncated forms are described herein as delta 3 through delta 9.

The invention also provides for a method of promoting the growth or survival of a MuSK receptor expressing cell in culture comprising administering to the MuSK receptor expressing cell an effective amount of agrin or a derivative of agrin. In one embodiment of this method, the agrin is human agrin. In another embodiment of this method, the MuSK receptor expressing cell is a cell which is normally found in the heart, spleen, ovary or

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retina. In another embodiment, the MuSK receptor expressing cell is a cell which has been genetically engineered to express the MuSK receptor.

The present invention also includes a method of treating a patient suffering from a muscle disease or neuromuscular disorder comprising administering to the patient an effective amount of an active portion of agrin or a derivative thereof. The active portion of the human agrin molecule may be any one of the truncated fragments (portions) of human agrin as described herein that is capable of inducing phosphorylation of the MuSK receptor.

The present invention also includes a method of treating a patient suffering from a muscle disease or neuromuscular disorder comprising administering to the patient an effective amount of an active portion of agrin or derivative thereof in combination with Ciliary Neurotrophic Factor (CNTF) or Modified Ciliary Neurotrophic Factor as described in United States Patent No. 5,349,0,56 issued September 20, 1994 to Panayotatos.

The invention also includes an antibody capable of specifically binding the active portion of human agrin. The antibody may be monoclonal or polyclonal. Such antibodies may be used to detect the presence of human agrin in a sample by:

a) reacting the sample with an antibody capable of specifically binding human agrin under conditions whereby the antibody binds to human agrin present in the sample; and

b) detecting the bound antibody, thereby detecting the presence of human agrin in the sample.

The antibody used may be monoclonal or polyclonal. The sample may be biological tissue or body fluid. The biological tissue may be brain, muscle, or spinal cord. The body fluid may be cerebrospinal fluid, urine, saliva, blood, or a blood fraction such as serum or plasma.

The cDNA clone encoding the active portion of human agrin described herein will facilitate screening of cDNA and genomic libraries in order to clone the full length sequence coding for the entire human agrin molecule. Cells may be genetically engineered to produce the active portion or the full length agrin molecule by, e.g., transfection, transduction, electroporation, microinjection, via a transgenic animal, of a nucleotide sequence encoding the active portion or the full length agrin molecule in a suitable expression vector. The invention also provides for a vector comprising an isolated nucleic acid molecule encoding an active portion or the full length human agrin molecule.

The invention further provides for a host-vector system for the production in a suitable host cell of a polypeptide having the biological activity of human agrin. The suitable host cell may be a bacterial cell such as E. coli., a yeast cell such as Pichia pastoris, an insect cell such as Spodoptera frugiperda or a mammalian cell such as a COS or CHO cell. The invention also provides for a method of producing a polypeptide having the biological activity of human agrin which comprises growing cells of the host-vector system under conditions permitting production of the polypeptide and recovering the polypeptide so produced.

The invention further provides for an expression vector comprising a nucleic acid molecule encoding human agrin or a portion thereof, wherein the nucleic acid molecule is operatively linked to an expression control sequence. The invention also provides a host-vector system for the production of a polypeptide having the biological activity of human agrin which comprises

the expression vector of the invention in a suitable host cell. The suitable host cell may be a bacterial cell such as E. coli a yeast cell such as Pichia pastoris, an insect cell such as Spodoptera frugiperda or a mammalian cell such as a COS or CHO cell. The invention further provides for a method of producing a polypeptide having the biological activity of human agrin which comprises growing cells of the host-vector system of the invention, under conditions permitting production of the polypeptide and recovering the polypeptide so produced.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleotide sequence encoding the polypeptide may be regulated by a second nucleotide sequence so that the polypeptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of polypeptide may be controlled by any promoter/ enhancer element known in the art. Promoters which can be used to control expression include, but are not limited to the long terminal repeat as described in Squinto et al., (1991, Cell 65:1-20);

the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothioein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the ß-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). See also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional

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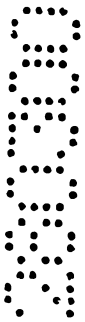
control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the foreign gene is inserted within the

marker gene sequence of the vector, recombinants containing the gene insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant vector. Such assays can be based, for example, on the physical or functional properties of the receptor-encoding gene product, for example, by binding of the receptor to neurotrophic factor or to an antibody which directly recognizes the receptor. Cells of the present invention may transiently or, preferably, constitutively and permanently express the polypeptide of the invention.







As described above, the MuSK tyrosine kinase receptor appears to be expressed in denervated muscle. Probes capable of recognizing these receptors may be used to identify diseases or disorders by measuring altered levels of the receptor in cells and tissues. Such diseases or disorders may, in turn, be treatable using the activating molecule disclosed herein. Such disorders include but are not limited to those in which atrophic or dystrophic change of muscle is the fundamental pathological finding. For example, muscle atrophy can result from denervation (loss of contact by the muscle with its nerve) due to nerve trauma; degenerative, metabolic or inflammatory neuropathy (e.g. Guillian-Barre syndrome), peripheral neuropathy, or damage to nerves caused by environmental toxins or drugs. In another embodiment, the muscle atrophy results from denervation due to a motor neuronopathy. Such motor neuronopathies include, but are not limited to: adult motor neuron disease, including Amyotrophic Lateral Sclerosis (ALS or Lou Gehrig's disease); infantile and juvenile spinal muscular atrophies, and autoimmune motor neuropathy with multifocal conduction block. In another embodiment, the muscle atrophy results from chronic disuse. Such disuse atrophy may stem from conditions including, but not limited to: paralysis due to stroke, spinal cord injury; skeletal immobilization due to trauma (such as fracture, sprain or dislocation) or prolonged bed rest. In yet another embodiment, the muscle atrophy results from metabolic stress or nutritional insufficiency, including,

but not limited to, the cachexia of cancer and other chronic illnesses, fasting or rhabdomyolysis, endocrine disorders such as, but not limited to, disorders of the thyroid gland and diabetes. The muscle atrophy can also be due to a muscular dystrophy syndrome, including but not limited to the Duchenne, Becker, myotonic, Fascioscapulohumeral, Emery-Dreifuss, oculopharyngeal, scapulohumeral, limb girdle, and congenital types, and the dystrophy known as Hereditary Distal Myopathy. In a further embodiment, the muscle atrophy is due to a congenital myopathy, including, but not limited to Benign Congenital Hypotonia, Central Core disease, Nemaline Myopathy, and Myotubular (centronuclear) myopathy. In addition, MuSK and its associated ligand may be of use in the treatment of acquired (toxic or inflammatory) myopathies. Myopathies which occur as a consequence of an inflammatory disease of muscle, include, but not limited to polymyositis and dermatomyositis. Toxic myopathies may be due to agents, including, but are not limited to adiodarone, chloroquine, clofibrate, colchicine, doxorubicin, ethanol, hydroxychloroquine, organophosphates, perihexiline, and vincristine.

Although not wishing to be bound by theory, preliminary mapping of musk in the mouse has revealed that the gene is localized to mouse chromosome 4 in a region of homology with human chromosome 9q. Mutations in mice that are associated with this region of chromosome 4 include the "wi" mutation (whirler), which results in symptoms of the shaker syndrome, including deafness, head-tossing, circling and hyperactivity (Lane, P.W., 963, J. Hered. 54:263-266). Another mutation in mice that is associated with this region of chromosome 4 is the "vc" mutation (vacillans) which is associated with the symptoms of violent tremor when walking and with swaying of the hindquarters (Sirlin, J.L., 1956, J. Genet. 54:42-48).

In humans, the disease known as idiopathic torsion dystonia (ITD) is associated with a gene that has been mapped, through linkage analysis to

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human chromosome 9q band 34. This disease is characterized by sustained, involuntary muscle contractions, frequently causing twisting and repetitive movements or abnormal postures.

The present invention may be used, where indicated, in gene therapy for the replacement of the human agrin gene in situ.

Any of the methods known to one skilled in the art of transferring genes into skeletal muscle tissue may be used in agrin gene therapy protocols. By way of non-limiting example, one skilled in the art may utilize direct injection of naked DNA into muscle tissue, adenovirus-associated gene transfer, primary myoblast transplantation, or cationic liposome: DNA complex gene transfer.

For example, direct injection of DNA into muscle may be employed to optimize vector construction as described by Manthorpe et al., (1993, Hum. Gene Ther. 4: 419-431) in which covalently closed circular plasmid DNA encoding the firefly luciferase reporter gene was injected into adult murine skeletal muscle for the purpose of evaluating the efficacy of various regulatory elements contained in the DNA expression vector. In a biological study, the systemic immunological effects of cytokine genes were evaluated using direct injection into muscle of DNA encoding the genes for IL-2, IL-4, or TGF-β-1 (Raz, et al., 1993, Proc. Natl. Acad. Sci. USA 90: 4523-7). Another study tested the ability of the human kallikrein gene product to reduce blood pressure in spontaneously hypertensive rats following direct DNA injection of the human kallikrein gene into murine skeletal muscle (Mong, et al., 1995,

Hypertension 25: 715-719). In studies aimed at evaluating the expression of muscle-specific proteins following direct injection of DNA various deletion-containing dystrophin gene mutant DNA constructs were injected into mdx mouse skeletal muscle and expression patterns and colocalization studies were performed to investigate dystrophin function (Fritz, et al., 1995, Pediatr. Res. 37: 693-700). Many investigators believe that it may be not only important but advantageous to regulate the timing and level of gene expression following gene transfer through direct DNA injection. For example, Dhawan et al., (1995, Somat. Cell. Mol. Genet. 21: 233-240) have tested a tetracycline-responsive promoter system in which orally or parenterally administered tetracycline can regulate reporter gene expression in mouse skeletal muscle following direct injection of DNA.

Adenovirus-mediated in vivo gene transfer has been studied extensively as a possible method for delivering genes for gene therapy. Recombinant adenovirus vectors containing exogenous genes for transfer are derived from adenovirus type 5 and are rendered replication-deficient by deletion of the E1 region of the viral genome (Brody & Crystal, 1994, Ann. N. Y. Acad. Sci. 716: 90-101). Huard et al., (1995, Gene Ther. 2:107-115) have evaluated the efficiency of viral transduction into rat tissues following various routes of administration (intra-arterial, intravenous, gastric-rectal, intraperitoneal, and intracardiac). The investigators report that route of administration is a major determinant of the transduction efficiency of rat tissue by adenovirus recombinants. In addition to route of administration preferences, it has been shown that vectors carrying U3 region viral long terminal repeats (LTRs) modified in the enhancer region may be used to target tissue- and differentiation-specific gene expression into skeletal muscle (Ferrari et al., 1995, Hum. Gene Ther. 6: 733-742). Many studies have been performed (Ragot, et al., 1993, Nature 361: 647-50; Petrof, et al., 1995, Am. J. Respir. Cell. Mol. Biol. 13: 508-17; Phelps, et al., 1995, Hum. Mol. Genet 4: 1251-1258; Kochanek, et al., 1996, Proc. Natl. Acad. Sci. USA 93: 5731-36) which have

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tested adenovirus-mediated transfer of full length and truncated forms of the dystrophin gene into muscle of normal and mdx mice.

Transplantation into skeletal muscle tissue of retrovirally transformed primary myoblasts expressing recombinant genes has also been extensively studied as a possible approach to gene therapy for muscle as well as non-muscle diseases. It is known that the success of myoblast transplantation for correction of intrinsic muscle defects is dependent on the ability of the transplanted myoblasts to fuse to the host myofibers. To address this issue, Rando & Blau (1994, J. Cell. Biol. 125:1275-87) developed a novel culture system for isolating enriched and clonal populations of primary myoblasts. Myoblasts isolated by this technique were shown to efficiently fuse to host myofibers to form hybrid myofibers persisting for up to six months as evidenced by β-galactosidase reporter gene expression. Because immunorejection of transplanted myoblasts is a potential problem in this gene therapy approach, it has been addressed in studies comparing autologous versus heterologous myoblasts for transplantation (Huard, et al., 1994, Hum. Gene Ther. 5: 949-58) and with Cyclosporin A-induced immunosuppression in adult mice receiving myoblast transplants following muscle injury (Irintchev et al., 1995, J. Neurocytol. 24: 319-331). Representative disease targets for gene therapy using myoblast transplantation include hemophilia B in which circulating human or canine factor IX has been measured in the plasma of mice following transplantation of recombinant myoblasts into skeletal muscles of normal and SCID mice (Roman, et al., 1992, Somat. Cell. Mol. Genet. 18: 247-58; Dai, et al., 1992, Proc. Natl. Acad. Sci. USA 89: 10892-5; Yao, et al., Proc. Natl. Acad. Sci. USA 89: 3357-61; Yao, et al., 1994, Gene Ther. 1: 99-107) and primary myopathies such as Duchenne muscular dystrophy where myoblasts expressing the dystrophin gene have been transplanted into normal and mdx mice (Partridge, et al., Nature 337: 176-9; Sopper, et al., 1994, Gene Ther. 1: 108-113).

Plasmid DNA complexed with cationic lipids has been evaluated for its ability to deliver genes into muscle tissue as well. Trivedi, et al., (1995, J. Neurochem. 64: 2230-38) carried out in vitro studies that utilized polycationic liposomes to successfully deliver the reporter gene LacZ into the cultured mouse myoblast cell line C2C12 and into primary mouse myoblasts derived from normal and mdx mice, forming the basis for adaptation to in vivo gene therapy.

According to the invention, the active portion of agrin, or fragments or derivatives thereof, may be used as an immunogen to generate anti-agrin antibodies. By providing for the production of relatively abundant amounts of protein using recombinant techniques for protein synthesis (based upon the nucleotide sequences of the invention), the problem of limited quantities of protein has been obviated.

To further improve the likelihood of producing an anti-agrin immune response, the amino acid sequence of the active portion of agrin may be analysed in order to

identify portions of the molecule which may be associated with increased immunogenicity. For example, the amino acid sequence may be subjected to computer analysis to identify surface epitopes which present computer-generated plots of hydrophilicity, surface probability, flexibility, antigenic index, amphiphilic helix, amphiphilic sheet, and secondary structure. Alternatively, deduced amino acid sequences from different species could be compared, and relatively non-homologous regions identified; these non-homologous regions would be more likely to be immunogenic across various species.

For preparation of monoclonal antibodies directed toward the active portion of agrin any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

Monoclonal antibodies prepared according to the invention for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

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Various procedures known in the art may be used for the production of polyclonal antibodies. For the production of antibody, various host animals can be immunized by injection with protein, or a fragment or derivative thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

A molecular clone of an antibody to an agrin epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleotide sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

The present invention may also be used to prepare fragments of antibody molecules. Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

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EXAMPLE 1- HOMOLOGOUS RECOMBINATION TO DISRUPT THE MuSK GENE

The tyrosine kinase domain of MuSK is comprised of 11 subdomains that are divided among three coding exons. Subdomain I encoding the ATP-binding domain is located on the first kinase exon, while subdomains 5-11 encoding the catalytic region are located on the third kinase exon (Figure 5). To disrupt MuSK tyrosine kinase activity, a targeting vector was designed that would delete most of the third kinase exon upon homologous recombination into the endogenous mouse MuSK locus (Figure 5); this targeting vector contained a total of 3.8 kb of homology with the mouse MuSK gene.

The MuSK gene targeting vector was constructed from mouse genomic DNA fragments isolated from a lambda FIX II phage library prepared with 129 strain mouse genomic DNA (Stratagene). The 1.7 kb SpeI fragment depicted in Figure 5 was ligated into the compatible ends of a unique XbaI site upstream of the PGK-neo cassette (destroying the SpeI and XbaI sites), while the 2.1 kb BamHI DNA fragment depicted in Figure 5 was blunt-end ligated into the unique HindIII site between the PGK-neo cassette and MC1-tk expression cassettes (destroying the BamHI and HindIII sites). The targeting vector was linearized by digestion with NotI and then electroporated into E14.1 embryonic stem cells, which were subjected to a double selection protocol (gancyclovir addition resulted in a 5-10 fold enrichment compared with selection in G418 alone) and then used to generate chimeric mice as previously described (Conover et al., 1995; DeChiara et al., 1995).

Successful gene targeting using this construct was predicted to result in the generation of a novel 3.8 kb EcoRI fragment from the targeted allele as detected by a 5' probe, as well as loss of two NcoI fragments hybridizing to a kinase probe (Figure 5). Southern blot screening for these fragments revealed

that successful targeting of the mouse MuSK gene was achieved in four of approximately 400 embryonic stem (ES) cell clones obtained using a double selection scheme intended to enhance for selection of targeted clones; the ES cells were derived from the 129 strain of mice. Male chimeras derived from all four of these targeted clones were bred with C57BL/6 females. Chimeras from two of the targeted clones transmitted the mutant allele to the F1 generation. The resulting F1 progeny heterozygous for the MuSK mutation were viable and appeared normal and fertile.

EXAMPLE 2- MuSK GENE DISRUPTION RESULTS IN PERINATAL LETHALITY

The heterozygous F1 progeny were interbred to generate mice homozygous for the MuSK gene disruption (designated MuSK-/- mice). Among the F2 litters derived from these crosses were newborn mice that died perinatally. Genotype analysis of tail DNA mice revealed that the dead pups were homozygous for the mutant MuSK allele (Figure 6); significantly, not a single mouse homozygous for the mutation survived the perinatal period (37 homozygotes were noted among the first 138 pups that were genotyped, corresponding to a 26.8% frequency of homozygotes).

To determine the phenotype of the MuSK-/- newborns immediately at birth, applicants were careful to observe the births of several litters derived from heterozygote crosses. Though normal in their gross anatomy and body weight, the MuSK-/- pups differed in several striking ways from their littermate controls. First, they showed no spontaneous movement and did not respond to a mild tail or leg pinch. Only a strong tail pinch was able to elicit a weak uncoordinated movement. By contrast, littermate controls showed extensive movement and responded vigorously to a mild tail pinch. Second, the MuSK-/- pups were cyanotic at birth and appeared not to breathe, although their hearts continued to beat for a short time after birth. To determine

whether the MuSK-/- pups had ever taken a breath, applicants examined the lungs histologically. Lung alveoli are collapsed in utero, and expand with the first breath of life; even if respiration is then terminated, the alveoli remain expanded.

Histological examination (Figure 7A) revealed that the alveoli of MuSK-/- pups were not expanded, indicating that the pups had never taken a breath. In contrast, the lungs of the littermate controls displayed expanded alveoli (Figure 7B).

EXAMPLE 3- NORMAL SKELETAL MUSCLE IN MuSK -/- MICE

Because MuSK is localized to synaptic sites in skeletal muscle (Valenzuela, D., et al., 1995, Neuron 15: 573-584) and because MuSK-/- mutant mice are immobile at birth and die shortly thereafter, applicants reasoned that neuromuscular synapse formation might be aberrant in MuSK-/- mutants. Applicants first examined the diaphragm muscle because its simple organization and thin structure allows synaptic sites to be visualized in whole-mount preparations. The diaphragm muscle is innervated by the phrenic nerve, which normally enters near the center of the diaphragm muscle. The main intramuscular nerve is oriented perpendicular to the long axis of the muscle fibers and extends through the central region of the muscle.

For whole-mount diaphragm preparations newborn mice were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for several hours and then rinsed briefly in PBS. Diaphragm muscles were dissected out, washed twice for 10 minutes in PBS, incubated in 0.1M glycine in PBS for 15 minutes, rinsed for 5 minutes in PBS, and permeabilized with 0.5% Triton X-100 in PBS (PBT) for 5 minutes. The muscles were then incubated with rabbit antibodies to synaptophysin (kindly provided by Dr. R. Jahn, Yale University Medical School), which were diluted 1/1000 in PBT with 2% BSA,

overnight at 4°C, subsequently rinsed for 5 minutes in PBT, washed three times for one hour in PBT and then incubated simultaneously with flourescein-conjugated sheep anti-rabbit IgG (Boehringer Mannheim) and tetramethlyrhodamine-conjugated α-bungarotoxin (α-BGT) (Molecular Probes, Oregon) overnight at 4°C. The tissues were then washed three times for 1 hour with PBT, rinsed once with PBS for 5 minutes, fixed in 100% methanol at -20°C and mounted in 90% glycerol, 0.1 M Tris, pH 7.5 with 1 mg/ml *p*-phenylenediamine. The whole-mounts were viewed with epiflourescence and filters that were selective for rhodamine or flourescein, and images were recorded either on film or on a CCD camera (Princeton Instruments).

The arrangement and gross structure of the muscle fibers (compare Figure 7C and 7D), as well as of the main intramuscular nerve, appeared to be unaltered in MuSK-/- mutant mice. Thus, although the onset of MuSK expression occurs at about embryonic day 11 in developing mouse somites (within the presumptive myotome), MuSK does not appear to be essential for the generation, proliferation and fusion of myoblasts, or for the growth of motor axons from spinal cord to muscle.

EXAMPLE 4- AGRIN FAILS TO INDUCE AChR CLUSTERING IN MYOTUBES LACKING MuSK

The localization of MuSK to the NMJ inspired us to ask whether MuSK is required for responsivity to agrin. To test this, applicants first isolated myoblasts from newborn MuSK$^{-/-}$ mice or from control pups, attempted to differentiate them into myotubes in culture, and then assayed for their responsiveness to agrin.

Primary myoblast cultures were established from hind limb musculature of newborn MuSK-/- or littermate control pups. This tissue was treated sequentially with collagenase and trypsin, then plated onto plastic tissue culture dishes. After 1 hour, non-adherent cells (principally myoblasts) were removed and plated onto chamber slides coated with poly-D-lysine and fibronectin. Myoblast cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 25% fetal calf serum, 10% horse serum, and 50 µg/ml gentamycin. To induce myotube formation, cultures were switched to a medium consisting of DMEM containing 5% horse serum, L-glutamine and gentamycin to which 20 µM cytosine arabinoside was added after 24 hr. After an additional 2-3 days, contractile myotubes had formed abundantly in cultures from both MuSK-/- and control pups. C2C12 cells were maintained and caused to differentiate in a serum-poor medium as previously described (Ferns, M., et al., 1993, Neuron 11: 491-502).

For agrin-mediated AChR clustering assays on primary myotubes, cultures on chamber slides were treated overnight with c-agrin4,8 at 0.01-100 nM; for evaluating MuSK-Fc as an inhibitor of clustering, differentiated C2C12 cells, on chamber slides coated with fibronectin and poly-D-lysine, were pretreated with MuSK-Fc or a control receptor-body for 1 hr at 37°C before addition of approximately 10 nM agrin4,8 for overnight incubation. Following overnight treatments with agrin, the cells were next incubated in rhodamine-conjugated α-bungarotoxin to label AChRs, then fixed and mounted for fluorescence microscopy. To quantify the extent of AChR clustering, randomly selected myotubes were viewed under fluorescein optics, then switched to rhodamine optics and the number of AChR clusters within a reticule grid aligned along the long axis of the myotube were counted. AChRs on the surface of cultured primary myotubes were quantitated by incubating live cultures with 25mCi 125I-α-BGT for 1 hr at room temperature, washing, and then lysing the cells in 0.1 N NaOH. The protein concentration in aliquots of each extract was

determined using a BCA protein assay kit (Pierce),while the remainder of the extract was counted in a gamma counter.

Myoblasts from both the control and MuSK$^{-/-}$ mice were able to fuse and form long, twitching myotubes in culture. Together with the observation that skeletal muscle appears rather normal in MuSK$^{-/-}$ mice, these findings indicate that MuSK is not critical for early muscle development and myoblast fusion. On the other hand, MuSK appeared to be absolutely required for AChR clustering in response to agrin. After stimulation with the most active form of c-agrin, containing both the four and eight amino acid insertions (c-agrin$_{4,8}$), AChR clusters were evident only in the myotubes from control mice (Figure 8A). While clusters were induced in normal myotubes with as little as 1 nM c-agrin$_{4,8}$, no clustering was observed in MuSK$^{-/-}$ myotubes even after increasing the concentration of c-agrin$_{4,8}$ to as high as 100nM (Figure 8B). Lack of detectable clustering was not due to the absence of AChRs, since myotubes from MuSK$^{-/-}$ mice expressed similar numbers of AChR on their surface as did myotubes from control mice (Figure 8C). Thus MuSK appeared to be absolutely required for AChR clustering in response to agrin.

EXAMPLE 5- AGRIN INDUCES PROMINENT AND RAPID TYROSINE PHOSPHORYLATION OF MuSK

The inability of agrin to induce AChR clustering in myotubes from MuSK$^{-/-}$ mice demonstrates that MuSK is required for agrin responsiveness, and is consistent with the possibility that MuSK serves as the functional agrin receptor. However, since clustering occurs over a period of hours, these results are also consistent with the possibility that MuSK acts much further downstream in the agrin signaling pathway. To begin to distinguish between

these possibilities, applicants took advantage of the fact that RTKs become rapidly autophosphorylated on tyrosine upon challenge with their cognate ligand. Applicants decided to assay four of the known forms of soluble agrin - which exhibit differing AChR clustering activities (Ruegg, M.A. et al., 1992, Neuron 8: 691-699; Ferns, M., et al., 1992, Neuron 8: 1079-1086; Ferns, M., et al., 1993, Neuron 11: 491-502; Hoch, W. et al., 1994, EMBO J. 13: 2814-2821) - for their ability to induce phosphorylation of the MuSK receptor.

The ability of various agrins and growth factors to induce MuSK or ErbB3 tyrosine phosphorylation, for the indicated times and at the indicated concentrations, was evaluated in primary rat myoblasts and in either untransfected C2C12 myoblasts, or in C2C12 myoblasts stably transfected with a chick MuSK-expressing plasmid. The cells were challenged at confluence in an undifferentiated state, or approximately 4-5 days after being induced to differentiate into myotubes in serum-poor media. After challenge, the cells were lysed, the extracts subjected to immunoprecipitation with receptor-specific antibodies, and then immunoblotted with either receptor-specific or phosphotyrosine-specific antibodies, using methods previously described (Stitt, T., et al., 1995, Cell 80: 661-670). Polyclonal antibodies for MuSK were generated as follows: for rat MuSK, rabbits were immunized with a peptide corresponding to the carboxy-terminal 20 amino acids of the rat MuSK protein (Valenzuela, D., et al., 1995, Neuron 15: 573-584; the nomenclature for this antibody is: 41101K); for chick MuSK, rabbits were immunized with a peptide corresponding to the first 19 amino acids of the chick MuSK cytoplasmic domain (Peptide: TLPSELLLDRLHPNPMYQ; the nomenclature for this antibody is 52307K). The specificity of the antibodies was determined on Cos-cell expressed MuSK proteins, by both immune-precipitation and Western, comparing untransfected Cos cell lysates to lysates from rat and chicken-MuSK transfected Cos cells. 41101K immune precipitates and Westerns rodent MuSK, but does not recognize chicken

MuSK. 52307 immune precipitates and Westerns chicken MuSK. Antibodies to ErbB3 were obtained from Santa Cruz Biotechnology, Inc.

Cultures of confluent C2C12 cells, either undifferentiated or differentiated in serum-poor media for four to five days as described above, were transferred to 4°C and incubated for 90 minutes with either MuSK-Fc or TrkB-Fc (at 5 mg/ml), each in the presence of the indicated mock or agrin-containing conditioned media (with 100nM agrin). Agrin levels were determined by Western analysis of the conditioned media with a rat agrin antibody (131, from StressGen, Inc.), using a purified agrin control of known concentration. Following these incubations, the cells were washed four times with PBS containing calcium and magnesium, and then incubated for an additional hour with radio-iodinated goat anti-human IgG (NEN/Dupont; 1 mCi/ml in PBS) to detect surface-bound receptor-Fc. After four additional washes, cells were solubilized in 0.1N NaOH, and bound radioactivity was determined. The assay is similar to that described elsewhere (Davis, S., et al., 1994, Science 266: 816-819).

Transient transfections using either previously described agrin constructs (Ferns, M., et al., 1993, Neuron 11: 491-502) or empty vector controls, or stable transfections of a chick MuSK-expression construct, were performed as described (Glass, D., et al., 1991, Cell 66: 405-413; Ip, N.Y., et al., 1992, PNAS (USA) 89: 3060-3064). Agrin concentrations in conditioned media derived from transient transfections were estimated by immunoblot comparisons with purified agrin of known concentration.

Phosphorylation was assessed on the endogenous MuSK receptor that is highly expressed in myotube cultures, obtained by differentiating either the C2C12 mouse myoblast cell line (Valenzuela, D., et al., 1995, Neuron 15: 573-584) or primary rat myoblasts. Strikingly, soluble agrins containing the eight amino acid insert at position Z (c-agrin$_{4,8}$ and c-agrin$_{0,8}$), which are the forms

capable of inducing AChR clustering, were also the forms that induced prominent tyrosine phosphorylation of MuSK (Figure 9A). The agrin most active in clustering (c-agrin$_{4,8}$) was also most active in inducing MuSK phosphorylation (Figure 9A). In contrast, the soluble agrins lacking the eight amino acid insert (c-agrin$_{4,0}$ and c-agrin$_{0,0}$), which cannot induce AChR clustering, also could not induce MuSK phosphorylation (Figure 9A).

The specificity of action of agrin was further explored by comparing its activity to growth factors known to have receptors on muscle. Of the several such factors tested, including insulin, fibroblast growth factor (FGF) and ARIA/neuregulin, only agrin could induce phosphorylation of MuSK (Figure 9A); since FGF also induces AChR clustering on myotubes (Peng, H.B., et al., 1991, Neuron 6: 237-246), these results also indicate that MuSK phosphorylation is specific to agrin responses and not just to agents capable of inducing clustering. Furthermore, while such factors could be shown to induce phosphorylation of their own RTKs on myotubes (e.g., neuregulin induces phosphorylation of its cognate RTK, erbB3), agrin could only activate MuSK and not other RTKs (Figure 9B).

The activation of a RTK by its cognate ligand typically tends to occur rapidly, and applicants could demonstrate that agrin induces tyrosine phosphorylation of MuSK with kinetics similar to those seen for well-characterized RTK/ligand systems (e.g. Kaplan, D.R., et al., 1991, Nature 350: 158-160); induction was detectable by one minute, peaked within the first five minutes, and remained elevated for over an hour (Figure 9D). The tyrosine phosphorylation of MuSK also occurred using agrin at concentrations similar to those noted for other ligands that act on RTKs (Ip, N.Y., et al., 1993, Neuron 10: 137-149), with phosphorylation detectable using 1 nM agrin (Figure 9C).

The requirement of MuSK for agrin responsiveness, the ability of agrin to induce rapid and prominent MuSK phosphorylation, the specificity of agrin for MuSK as compared to other factors tested, and the precise correlation of agrin forms active in AChR clustering assays and in MuSK phosphorylation assays, together continue to support the notion that MuSK serves as the functional agrin receptor.

EXAMPLE 6 - AGRIN DOES NOT DIRECTLY BIND TO AN ISOLATED MuSK ECTODOMAIN

If MuSK is indeed the functional agrin receptor, applicants would expect to be able to demonstrate binding of agrin to MuSK. In an attempt to demonstrate such binding, applicants first constructed an expression construct encoding a fusion protein between the ectodomain of rat MuSK and the Fc portion of human immunoglobulin G1 (designated MuSK-Fc), and then produced and purified the fusion protein. Similar receptor-Fc fusions have previously been used to characterize binding between RTKs and their ligands (Davis, S., et al., 1994, Science 266: 816-819; Stitt, T., et al., 1995, Cell 80: 661-670).

Baculovirus expression vectors encoding MuSK-Fc, TrkB-Fc, and Ret-Fc produced fusion proteins in which the ectodomains of rat TrkB, rat Ret, or rat MuSK, respectively, were linked to a spacer with the sequence Gly-Pro-Gly, followed by the hinge, CH2, and CH3 regions of human IgG1, beginning with the residues Glu-Pro-Lys, as described (Davis, S., et al., 1994, Science 266: 816-819). Baculovirus infections into Spodoptera frugiperda SF-21AE insect cells were performed by standard methods (Stitt, T., et al., 1995, Cell 80: 661-670). The soluble Fc-containing proteins were purified by protein A-Sepharose (Pharmacia) chromatography.

The binding of agrin to immobilized MuSK-Fc as compared to a monoclonal antibody specific for agrin was evaluated by use of BIAcore biosensor

technology (Pharmacia Biosensor), using approaches previously described (Stitt, T., et al., 1995, Cell 80: 661-670). Heparin and CaCl2 were supplied by Sigma Chemical Co. (St. Louis, MO) and used without further purification. The agrin-specific monoclonal antibody (clone AGR131 generated to rat agrin) was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada).

In a first approach, applicants used MuSK-Fc together with BIAcore biosensor technology. The BIAcore technology allows for the direct and quantitative measure of binding of soluble ligands to receptors coupled onto a sensor chip. Recombinant MuSK-Fc was covalently coupled to a surface on the BIAcore sensor chip, and as a control, a monoclonal antibody specific for rat agrin was also coupled to a separate surface on the sensor chip; media containing c-agrin$_{4,8}$ was then passed over the two surfaces. While robust binding of the agrin to the antibody surface was easily detected, no binding of the agrin to the MuSK surface could be seen (Figure 10A). Furthermore, while binding to the antibody surface was specifically competable by excess soluble antibody added to the agrin-containing media, the binding was not competable by excess soluble MuSK-Fc (Figure 10A). Since agrin activity requires calcium (Bowe and Fallon, 1995, Ann. Rev. Neurosci. 18: 443-462), and because some heparin-binding factors require heparin to bind to their receptors (Goldfarb, M., 1990, Cell Growth & Differentiation 1: 439-445), applicants also attempted binding in the presence of calcium or heparin; in neither case was binding to the MuSK surface observed (Figure 10A).

Next, applicants tried to demonstrate binding of MuSK and agrin by attempting to use MuSK-Fc to detect agrin immobilized onto nitrocellulose. In contrast to our control experiments, in which immobilized brain-derived neurotrophic factor (BDNF) was easily detected by an Fc fusion of its cognate receptor (TrkB-Fc), and in which immobilized agrin was easily detected by the agrin-specific monoclonal antibody, immobilized agrin could not be detected by MuSK-Fc (Figure 10B).



The negative binding results described above demonstrate that the isolated MuSK receptor is not sufficient to bind agrin. Thus, despite the plethora of functional data indicating that agrin acts via MuSK, MuSK may not directly serve as a receptor for agrin. Alternatively, MuSK may require additional components or modifications which are required for it to bind and respond to agrin.

EXAMPLE 7 - AGRIN ACTIVATES MuSK IN A CELL-CONTEXT-DEPENDENT FASHION: REQUIREMENT FOR A MYOTUBE-SPECIFIC ACCESSORY COMPONENT

Based on the results described above, applicants considered the possibility that the agrin-MuSK interaction requires additional components. To further explore this possibility, applicants determined the cell-context dependency for agrin activation of MuSK, reasoning that if an accessory component was required, it might be specifically expressed only on cells normally responding to agrin. Thus applicants ectopically expressed full-length cDNAs encoding rat, human and chicken MuSK in fibroblasts, and assayed for whether these MuSK receptors could be inducibly phosphorylated by agrin. When expressed in fibroblasts, none of the three species of MuSK could be phosphorylated in response to agrin. While this supported the possibility that MuSK requires an accesssory myotube-specific component to respond to agrin, it was also possible that our cDNAs encoded MuSK variants that could not respond to agrin. This was a potentially worrisome possibility since there are multiple differently spliced versions of the MuSK transcript (Valenzuela, D., et al., 1995, Neuron 15: 573-584), applicants did not know which of the forms were normally agrin-responsive, and our cDNAs only accounted for a subset of the variant forms. Thus applicants decided to express our cDNAs in myoblasts to verify that they could mediate responses to agrin when expressed in the right

context. For this purpose applicants chose to express the chicken MuSK in the mouse C2C12 myoblast cell line, since the chicken MuSK could easily be distinguished from the endogenous mouse MuSK based on size and by using particular antibodies. When expressed in undifferentiated myoblasts, the chicken MuSK did not undergo phosphorylation in response to any isoforms of agrin (Figure 11, see lanes indicated "Undif", upper panel), just as it did not undergo phosphorylation in fibroblasts; undifferentiated C2C12 cells do not express appreciable amounts of endogenous MuSK (Figure 11, lanes indicated "Undif", lower panel and also (Valenzuela, D., et al., 1995, Neuron 15: 573-584), so applicants could not compare activation of the endogenous mouse MuSK in myoblasts. Upon differentiation into myotubes, the introduced chicken MuSK was as effectively activated by agrin as was the endogenous mouse MuSK (Figure 11, lanes indicated "Diff", upper panel); both introduced and endogenous MuSK had identical profiles of responsivity to the various forms of agrins, with activations mediated only by forms having the eight amino acid insert at the Z position. Thus our cDNAs encode MuSK proteins that are perfectly competent to undergo agrin-induced phosphorylation, but they can only be activated by agrin in the context of a differentiated myotube, consistent with the notion that agrin activation of MuSK requires a myotube-specific accessory component that is not expressed in fibroblasts or undifferentiated myoblasts.

EXAMPLE 8 - A RECEPTOR COMPLEX CAN BE DEMONSTRATED BETWEEN AGRIN, MuSK AND A MYOTUBE-SPECIFIC ACCESSORY COMPONENT

Altogether, the above data indicate that agrin requires MuSK to mediate clustering and that agrin activates MuSK very rapidly, but that agrin does not directly bind to a purified MuSK ectodomain and can only activate MuSK in the context of a myotube. These findings are consistent with the possibility

that MuSK is a requisite part of an agrin receptor complex, but that although MuSK provides a key signaling function for this complex, it requires another component(s) to bind to agrin. Similar types of receptor complexes have been described for other ligands. Perhaps some of the best characterized examples include the receptor complexes for ciliary neurotrophic factor (CNTF) and its cytokine relatives (Davis, S., et al., 1993, Science 260: 1805-1808; Stahl and Yancopoulos, 1993, Cell 74: 587-590). In order to interact with its two signal transducing "b" receptor components, gp130 and LIFRb, CNTF must first bind to its "a" receptor component, known as CNTFRa. CNTFRa serves no signaling role, and is in fact linked to the surface via a glycosylphosphatidylinositol linkage and thus has no cytoplasmic domain. The receptor complex for CNTF is built in step-wise fashion: CNTF first binds to CNTFRa; this initial complex can then bind to and recruit a single "b" component; finally, a complete complex forms that involves "b" component dimerization, which is required for signal initiation (Figure 12A). In the final complex, CNTF seems to make contacts with all three receptor components. Interestingly, receptor complexes for CNTF can be built in solution using just the soluble ectodomains of the various components. Furthermore, if just one of the receptor components is linked to the surface, a receptor complex can be built around it using soluble versions of the other components, but only in a CNTF-dependent fashion (Figure 12B).

If agrin binds to MuSK in a receptor complex, applicants reasoned that they might be able to manipulate this complex in much the same way the CNTF receptor complex can be manipulated. To explore the possibility that myotubes specifically express an accessory component(s) required for agrin to bind MuSK (Figure 12C), applicants decided to test whether applicants could specifically build a receptor complex on the surface of myotubes, but not on other cells, using agrin together with a soluble version of the MuSK receptor to complex to the putative accessory component(s) on the surface of myotubes. Confirming this possibility, applicants found that the binding of

soluble MuSK-Fc to the surface of cells can be increased using agrin, but only on the surface of differentiated myotubes and not on the surface of fibroblasts or myoblasts (Figure 13A). These data demonstrate that complexes can form between agrin and MuSK, but only in the presence of a myotube-specific component(s) (as suggested in Figure 12C). Interestingly, although forms of c-agrin containing the eight amino acid insert at the Z position are best able to promote agrin-dependent MuSK complex formation, forms of c-agrin without this insert can also form these complexes. The ability of all the soluble forms to promote complex formation, including those lacking the eight amino acid insert for activity, may be related to previous findings that matrix-bound forms of agrins lacking the Z insert can activate clustering (Ferns, M., et al., 1992, Neuron 8: 1079-1086). Thus although soluble agrins lacking inserts at the Z position do not seem capable of signaling, they may be able to form partial complexes, while matrix-associated forms of these same agrins can proceed to form complete signaling-competent complexes. Interestingly, ligands for the EPH family of RTKs provide an example of ligands that bind but do not activate their receptors when presented in soluble form, but which can act as potent activators when bound to the cell surface (Davis, S., et al., 1994, Science 266: 816-819); deliberate clustering of the soluble ligands can allow them to activate as well, suggesting that the role of surface-attachment is to allow for ligand-clustering (Davis, S., et al., 1994, Science 266: 816-819).

In the absence of added agrin, the MuSK-Fc exhibited much higher binding to myotube surfaces than did several control receptor-Fc fusion proteins (Figure 13A, data shown for TrkB-Fc); the MuSK-Fc, however, displayed similar agrin-independent binding to both myoblast and fibroblasts as did control receptor-Fc proteins (Figure 13A). Specific binding of MuSK-Fc to myotube surfaces, in the absence of exogenously provided agrin, may indicate that MuSK has an affinity for its myotube-specific accessory component in the absence of ligand. Alternatively, since myotubes make muscle forms of agrin

(lacking the eight amino acid insert at the Z position), the specific binding of MuSK-Fc to myotubes in the absence of added agrin could be explained by the formation of a complex between the added MuSK-Fc and endogenously expressed muscle agrin along with the accessory component; adding additional exogenous soluble agrin may simply allow for even more MuSK to be recruited into complexes with the myotube-specific accessory component. Although both myoblasts and myotubes make endogenous agrin, myoblasts seemingly cannot form complexes with added MuSK-Fc since they do not express the required accessory component.

To confirm that MuSK directly interacts with agrin as part of its receptor complex, applicants next demonstrated that radiolabelled agrin could be cross-linked to MuSK receptors on the surface of myotubes.

Flg-tagged human agrin protein corresponding to the COOH-terminal 50 kD of human agrin 4,8 was expressed in Cos cells and purified by affinity and size-exclusion chromatography to >95% purity. Twenty µg were iodinated by a modification of the lactoperoxidase method described previously (DiStefano, P., et al, 1992, Neuron 8: 983-993). Incorporation of 125I was greater than 80%; 125I-h-agrin 4,8-flg was separated from free 125I on a 1x3 cm Sephadex G-25 column prior to use in cross-linking assays. Specific activity was ~4000 cpm/fmol (~2400 Ci/mmol). Biological activity of 125I-h-agrin 4,8-flg was monitored by tyrosine phosphorylation of MuSK in C2C12 myotubes and was found to be indistinguishable from its unlabeled counterpart. For cross-linking studies, 10 cm plates of differentiated C2C12 myotubes were incubated in 1 nM of [125I]-agrin$_{4,8}$ in 1.5 ml of PBS containing 1% BSA and 1 mg/ml glucose in the presence or absence of 150-fold excess unlabeled agrin$_{4,8}$ for 75 min at 4°C. The cross-linking agent DSS (disuccinimidyl suberate) was added to a final concentration of 0.2mM and the plates were incubated at room temperature for 30 min, washed 3 times with 50 mM Tris/150mM NaCl pH 7.5, lysed, and subjected to immunoprecipitation with MuSK-specific

antibodies. For peptide competition, peptide antigen was included in the immunoprecipitation at a final concentration of 20 µg/ml. The samples were then electrophoresed and the fixed and dried gels were exposed for autoradiography.

Immunoprecipitations using a MuSK-specific antibody, from lysates of myotubes chemically cross-linked to radiolabelled recombinant human agrin contained complexes corresponding in size to agrin/MuSK complexes (Figure 13B). These agrin/MuSK complexes were not seen in the presence of excess unlabelled agrin, or if a peptide was used to block MuSK precipitation (Figure 13B). Additional radiolabelled species that immunoprecipitated with the MuSK antibody correspond to forms of agrin that are associated with, but not cross-linked to, MuSK, presumably due to the low efficiency of cross-linking (Figure 13B); low levels of additional agrin complexes, perhaps involving MASC, could also be detected in these immunoprecipitations.

Finally, if our findings that soluble MuSK could form complexes with its requisite myotube-specific accessory component are correct, then this soluble receptor should also act as an inhibitor of agrin-mediated responses by sequestering the accessory component and preventing it from interacting with the endogenously-expressed, signaling-competent MuSK. Indeed, addition of increasing amounts of MuSK-Fc did inhibit agrin-mediated clustering of AChRs (Figure 13C) as well as agrin-induced MuSK phosphorylation in a dose-dependent manner, while control receptor-Fc proteins had no inhibitory effect.

EXAMPLE 9- CLONING OF HUMAN AGRIN cDNA

Probes corresponding to human agrin were prepared by PCR based on partial sequences of human agrin available from the Genbank database.

Two pairs of PCR primers were synthesized based on human agrin cDNA sequences obtained from Genbank. The sequences of the oligonucleotide primers were as follows:

Primer pair 18:
h-agrin 18-5' : 5'-GACGACCTCTTCCGGAATTC-3'
h-agrin 18-3' : 5'-GTGCACATCCACAATGGC-3'

Primer pair 35:
h-agrin 35-5' : 5'-GAGCAGAGGGAAGGTTCCCTG-3'
h-agrin 35-3' : 5'-TCATTGTCCCAGCTGCGTGG-3'

The oligonucleotide primers were used for PCR amplification of two segments of DNA of approximately 100 nts (primer pair 18) and 85 nts (primer pair 35) using 300 ngs of human genomic DNA as a template. The PCR amplification was carried out as recommended by the manufacturer (Perkin-Elmer) under the following conditions: 35 cycles at 94°C for 60 sec, 55°C for 50 sec and 72°C for 30 sec. The PCR fragments obtained were purified from an agarose gel and re-amplified for 30 cycles using the same PCR conditions described above.

After amplification, the PCR reactions were electrophoresed in agarose gels, the agarose containing the DNA bands of 100 and 85 nts respectively was excised, purified by QiaEx II (Qiagen), and then cloned into plasmid pCR-script using Stratagene's pCR-Script cloning kit, followed by bacterial transformation and plating onto agar-ampicillin plates as recommended by the manufacturer. Bacterial colonies containing the 100 and 85 nt inserts were identified by PCR using the primers described above. The PCR fragments obtained were radiolabeled for use as probes using a standard PCR reaction (Perkin-Elmer) on 20 ng of DNA template, except that 5 nmoles each of dATP, dGTP and dTTP and 0.2 mCurie of alpha ^{32}P-dCTP (Du Pont 3000 Ci/mmol)

were added to the reaction mixture and then subjected to 7 cycles of PCR. Unincorporated label was separated from the probes on a G50 NICK column (Pharmacia). These probes were used to screen a human fetal brain cDNA library (Stratagene Cat# 936206) using standard library screening procedures (Sambrook, Fritsch and Maniatis, Molecular Cloning, a Laboratory Manual, (1989) Second Edition, Cold Spring Harbor Laboratory Press). One and a half million phage plaques were plated in XL-1 Blue bacteria as recommended by Stratagene, and transferred to nitrocellulose filters in duplicate as previously described (Id.). The filters were processed and each filter replica was used for hybridization with one of the above probes as previously described (Id.). Plaques hybridizing to both probes were isolated and purified and a plasmid containing the cDNA insert was excised from the lambda clone according to Stratagene's recommended procedure (EXASSIST/SOLR System). The pBluescript plasmid containing the human Agrin insert was purified and the insert was then sequenced using an automated sequencing kit (Applied Biosystems).

As a result of this screen, one clone (pBL-hAgrin1) was obtained which contains a nucleotide sequence encoding an amino acid sequence of human agrin. The first amino acid encoded by the cloned nucleotide sequence corresponds approximately to amino acid 424 of rat agrin (See Figure 14). The nucleotide sequence of the clone ends downstream of the stop codon. Clone pBL-hAgrin1 contains a 4 amino acid insert starting at the position which corresponds to position 1643 of Figure 14, a point which was previously described for the rat as position "Y" (Stone, D.M. and Nikolics, K., J. Neurosci. 15: 6767-6778 (1995)). The sequence of the 4 amino acid insert both in clone pBL-hAgrin1 and in the rat is KSRK.

A second clone was obtained from this screen. This second clone (pBL-hAgrin23) also contains a nucleotide sequence encoding an amino acid sequence of human agrin. The first amino acid encoded by the cloned

nucleotide sequence corresponds approximately to amino acid 1552 of the rat agrin (See Figure 14). The nucleotide sequence of the clone ends downstream of the stop codon. Clone pBL-hAgrin23 contains an 8 amino acid insert starting at a position which corresponds to position 1780 of Figure 14, a point which was previously described for the rat as position "Z" (Stone, D.M. and Nikolics, K., J. Neurosci. 15: 6767-6778 (1995)). The sequence of the eight amino acid insert both in clone pBL-hAgrin23 and in the rat is ELANEIPV. As previously discussed, it has been reported that the 8 amino acid insert plays an important role in regulating the AChR clustering activity of different agrin forms. Therefore, by inserting a nucleotide sequence encoding the eight amino acid sequence ELANEIPV into clone pBL-hAgrin1 at the position corresponding to position Z of rat agrin, a human 4-8 agrin clone may be obtained. The addition of the 8 amino acid insert at position Z should confer a high level of biological activity to the human 4-8 clone.

Clone pBL-hAgrin23 also contains the 4 amino acid "Y" insert as described above for clone pBL-hAgrin1. However, clone pBL-hAgrin23 contains 17 extra amino acids at the same "Y" position, such that the sequence of the "Y" insert in clone pBL-hAgrin23 is KSRKVLSASHPLTVSGASTPR. Therefore, in addition to the (4-0) and (4-8) human agrin splice variants described above, human clones corresponding to splice variants containing (Y-Z) inserts of (17-0), (17-8), (21-0), and (21-8) are indicated by these results and are within the scope of the present invention.

EXAMPLE 10- EXPRESSION OF HUMAN AGRIN

Construction of human agrin expression vector

A human agrin Sfi I - Aat II fragment containing the 4 amino acid insert at the position corresponding to the Y-site described for rat agrin (see Figure 14)

was excised from clone pBL h agrin-1. A human agrin Aat II - Not I fragment containing the 8 amino acid insert at the position corresponding to the Z-site described for rat agrin (see Figure 14) was excised from clone pBL h agrin-23. A Xho I - Sfi I fragment was then generated via PCR that contained a preprotrypsin signal peptide, the 8 amino acid flg peptide (from the flag tagging system, IBI/Kodak, Rochester, NY) and the human agrin sequence corresponding to the sequence of amino acids from position 1480 to the Sfi I site located at amino acids 1563-1566 of rat agrin (see Figure 14). The three fragments were then ligated into a Xho I - Not I digested pMT21 expression vector to form the human agrin 4-8 expression vector pMT21-agrin 4-8. The sequence of human agrin 4-8 that was encoded in the expression vector is shown in Figure 15. Expression vectors for the human clones corresponding to splice variants containing (Y-Z) inserts of (0-8) and (4-0) were also constructed.

Expression of human agrin (4-8) in E. coli

The gene for human agrin 4-8 was PCR amplified from pMT21-agrin 4-8 with the primer pair AG5' (5'-GAGAGAGGTTTAAACATGAGCCCCTGCCAGCCCAACCCCTG-3') and AG3' (5'-CTCTGCGGCCGCTTATCATGGGGTGGGGCAGGGCCGCAG-3'). The PCR product was digested with the restriction enzymes Pme I and Not I and cloned into the Pme I and Not I sites of the vector pRG501, a pMB1 replicon that confers kanamycin resistance and is designed to express cloned genes from the phage T7 promoter. One isolate was characterized and named pRG531. The 1315 base pair Nco I - Nae I fragment internal to agrin in pRG531 was then replaced with the corresponding fragment from pMT21-agrin 4-8. The resulting plasmid, pRG451, was transformed into the expression strain RFJ209 [IN(rrnD-rrn/E)1 $lacI^Q$ lacZpL8 fhuAD322-405 $rpoS_{(MC4100)}$ ara::(lacUV5-T7 gene 1)8]. Cultures of RFJ209 / pRG541 induced with IPTG express human agrin to about 5% of total cellular protein and

fractionates with soluble protein upon cell disruption. The crude soluble protein fraction containing human agrin 4-8, as well as human agrin 4-8 purified by Q-Sepharose chromatography was determined to be active in phosphorylation of MuSK receptor.

Expression of human agrin (4-8) in Pichia pastoris

The 50kD active fragment (portion) of human agrin 4-8 was cloned by PCR using a primer containing a portion of the S. cerevisiae α mating factor pre-pro secretion signal and the 5' end of the region encoding the 50kD agrin fragment (GTATCTCTCGAGAAAAGAGAGGCTGAAGCT AGCCCCTGCCAGCCCAACC), and a primer containing sequences from the region 3' of the agrin coding region and a NotI site (AATAGTGCGGCCGCCAACACTCAGGCAAGAAAATCATATC). After PCR the fragment was digested with XhoI, which recognizes sequences in the 5' primer, and NotI, and was cloned into pPIC9 (Invitrogen) digested with XhoI and NotI. The resulting clone was digested with NotI and partially digested with NcoI to remove most of the PCRed agrin sequences. This region was replaced by a NotI-NcoI fragment of agrin from pRG541. PCRed regions were sequenced and shown to be wild-type. This clone, pRG543 was digested with SalI and transformed into Pichia pastoris by electroporation. Transformants were selected for a His+ Mut+ phenotype. Induction of the AOX1 promoter driving the expression of hAgrin was done by growing the cells in buffered glycerol-complex medium containing 0.5% glycerol, pH=6.0, for 24 hrs until the glycerol was exhausted, at which point methanol was added to a final concentration of 0.5%. The culture was centrifuged and the supernatant was dialyzed against PBS. The concentration of hAgrin was approximately 10ug/ml and was determined to be active in phosphorylation of MuSK receptor.

Production of Human Agrin 4,8 from Baculovirus Infected Insect Cells

Virus Production

The flg-tagged gene for human agrin 4-8 was engineered into a baculovirus expression plasmid and recombined with viral DNA to generate recombinant baculovirus, amplified and harvested using methods previously described (O'Reilly, D.R., L.K. Miller, and V.A. Luckow, Baculovirus Expression Vectors - A Laboratory Manual 1992, New York: W.H. Freeman). SF21 insect cells (Spodoptera frugiperda) obtained from Invitrogen were adapted and expanded at 27°C in Gibco SF900 II serum-free medium. Uninfected cells were grown to a density of $1x10^6$ cells/mL. Cell density was determined by counting viable cells using a hemacytometer. The virus stock for FLAG-agrin was added to the bioreactor at a low multiplicity 0.01-0.1 PFU/cell to begin the infection. The infection process was allowed to continue for 3-4 days allowing maximum virus replication without incurring substantial cell lysis. The cell suspension was aseptically aliquoted into sterile centrifuge bottles and the cells removed by centrifugation (1600 RPM, 30 min). The cell-free supernatant was collected in sterile bottles and stored at 4°C in the absence of light until further use.

The virus titer was determined by plaque assay as described by O'Reilly, Miller and Luckow. The method is carried out in 60mm tissue-culture dishes which are seeded with $1.5x10^6$ cells. Serial dilutions of the virus stock are added to the attached cells and the mixture incubated with rocking to allow the virus to adsorb to individual cells. An agar overlay is added and plates incubated for 5 days at 27°C. Viable cells were stained with neutral red revealing circular plaques which were counted to give the virus titer expressed in plaque forming unit per milliliter (PFU/mL).

Infection of Cells for Protein Production

Uninfected SF21 cells were grown in a 60L ABEC bioreactor containing 40L of Gibco SF900 II medium with gentamicin sulfate (25 mg/L) and amphotericin B (1 mg/L). Temperature was controlled at 27C and the dissolved oxygen level was maintained at 50% of saturation by controlling the flowrate of oxygen in the inlet gas stream. When a density of $2x10^6$ cells/mL was reached, the cells were concentrated within the bioreactor to 20L, using a low shear steam sterilizable pump and a tangential flow filtration device with Millipore Prostak 0.45 micron membranes. After concentration, fresh sterile growth medium was slowly added to the bioreactor while the filtration system continued to remove the spent growth medium by diafiltration. After two volume exchanges an additional 20L of fresh medium was added to the bioreactor to resuspend the cells to the original volume of 40L.

The amount of virus stock required was calculated based on the cell density, virus titer and the desired multiplicity of infection (MOI). Multiplicity ratios between 1 and 10 pfu/cell have been effectively used. The virus stock was added aseptically to the bioreactor and the infection was allowed to proceed for three to four days.

Recovery and Chromatographic Purification

At the conclusion of the infection phase of the bioreactor process the cells were concentrated in the bioreactor using a 30 ft^2 Millipore Prostak filter (0.45 micron) pore size. The cell-free permeate passing through the filter was collected in a clean process vessel. The protein was diafiltered into a low conductivity buffer (20 mM citrate, pH 5.5) using Millipore Pellicon ultrafiltration membrane cassettes totaling 20 ft^2 with a nominal 10 kiloDalton cutoff. The protein in the retentate was loaded onto a cation exchange column (Pharmacia SP Sepharose FF) equilibrated with 20 mM citrate buffer, pH 5.5. After loading the protein was washed first with 20 mM citrate, 200 mM sodium chloride, pH 5.5 then with 20 mM Bicine, pH 8.0 to remove contaminating proteins. The protein was eluted with a 0-750 mM

sodium chloride linear gradient over 7.5 column volumes. The eluted agrin was buffer exchanged into 20 mM Tris, pH 8.5 buffer to remove salt for subsequent binding to an anion exchange column.

The agrin was then bound to a Pharmacia Q Sepharose FF column equilibrated with 20 mM Tris, pH 8.5. After loading the column was washed with the same buffer to remove contaminating proteins and the protein eluted with a 0-250 mM sodium chloride gradient. The fractions containing agrin were pooled and concentrated and dialyzed into PBS containing calcium and magnesium.

Expression of human agrin (4-8) in COS-7 cells

Lipofectamine reagent (GIBCO-BRL, Inc.) and recommended protocols were used to transfect COS-7 cells with the human agrin cDNA clone pMT21-agrin 4-8 containing a nucleotide sequence encoding the eight amino acid sequence ELANEIPV at the position corresponding to position Z of rat agrin. COS media containing secreted ligand was harvested after three days and concentrated 20-fold by diafiltration (DIAFLO ultrafiltration membranes, Amicon, Inc.). The quantity of active human agrin present in the media was determined and expressed as the amount (in resonance units, R.U.) of MuSK receptor specific binding activity measured by a BIAcore binding assay.

EXAMPLE 11 - PREPARATION OF TRUNCATED MOLECULES CONTAINING THE MUSK ACTIVATING PORTION OF HUMAN AGRIN

It has recently been reported that a 21kD fragment of chick agrin is sufficient to induce AChR aggregation (Gesemann, M., et al., 1995, J. Cell. Biol. 128: 625-636). Applicants therefore decided to investigate the properties of various portions of human agrin and to test the ability of each to induce phosphorylation of the MuSK receptor.

As set forth in Figure 15, the amino acid sequence of the 50 kD active portion of human agrin 4,8 is 492 amino acids long. A preprotrypsin signal sequence (Stevenson et al., 1986. Nucleic Acids Res. 21: 8307-8330) precedes a FLAG tag sequence (Hopp etal. 1988. Bio/Technology 6: 1204-1210); together, they constitute the first 23 amino acids. Thus the agrin 4,8 sequence begins with amino acid 24. Truncated molecules were created, each of which contained the signal sequence and FLAG tag (23 amino acids) followed by the agrin 4,8 sequence to which N-terminal deletions had been made to create portions of agrin (designated delta 3 through 9) as follows:

delta 3: agrin sequence starts with amino acid #60: QTAS...
delta 4: agrin sequence starts with amino acid #76: NGFS...
delta 5: agrin sequence starts with amino acid #126: VSLA...
delta 6: agrin sequence starts with amino acid #178: GPRV...
delta 7: agrin sequence starts with amino acid #222: GFDG...
delta 8: agrin sequence starts with amino acid #260: ASGH...
delta 9: agrin sequence starts with amino acid #300: AGDV...
All of the sequences continue to the terminal amino acids PCPTP, as with the 50kD agrin.

The truncated molecules were made as follows: PCR primers were designed consistent with the nucleotide sequences encoding the first and last ten amino acids of each construct. Included in the 5' primer was sequence data to append the preprotrypsin signal sequence and "FLAG-tag" to the amino terminus of each agrin fragment. Thus, the shortest truncated molecule (delta 9) contains the signal sequence and FLAG tag and the human agrin sequence from amino acid 300 to 492 of human c-agrin 4,8. DNA encoding the "delta" forms of truncated c-agrin 4,8 was then cloned into a eukaryotic expression vector, and transient transfections were performed as previously described (Glass, D., et al., 1991, Cell 66: 405-413; Ip, N.Y., et al., 1992, PNAS (USA) 89: 3060-3064).

Agrin levels in the COS-transfected supernatants were determined by Western analysis of the conditioned media with an agrin-specific antibody (Stressgen 131), using a purified agrin control of known concentration. Each of the truncated molecules was expressed and shown to be capable of inducing tyrosine phosphorylation of MuSK receptor.

Figure 16 shows that the delta 9 truncated molecule can induce phosphorylation of the MuSK receptor, though with less efficiency than the 50kD agrin 4,8 molecule. Thus, each of the truncated molecules created exhibited the biological activity of human agrin 4,8 with respect to the MuSK receptor.

EXAMPLE 12 - PEGylation of Agrin 4,8 and Pharmacokinetics Study

After intravenous administration, the 50kD agrin 4,8 was cleared rapidly from the systemic circulation with a half-life of <10 minutes. (See Figure 17). It was known that the properties of certain proteins can be modulated by attachment of polyethylene glycol (PEG) polymers, which increases the hydrodynamic volume of the protein and thereby slows its clearance by kidney filtration. (See, e.g. Clark, R., et al., 1996, J. Biol. Chem. 271: 21969-21977). Therefore, in an attempt to increase its circulating half-life, agrin 4,8 was modified by covalent attachment of a polyethylene glycol molecule and the effect on the protein's serum half-life was studied.

A solution of 500 mg of human agrin 4,8 at 3.0 mg/mL was added to a reaction vessel and the PEGylation reaction was initiated by addition of monomethoxypoly-ethyleneglycol succinimidyl propionate (PEG) (approximate molecular weight = 20,000 daltons). A ratio of 1.75 moles PEG reagent to agrin was used for the reaction which was carried out at a pH of 7.3

to 8.5 in phosphate buffered saline over a period of 2 hours. The reaction was stopped by addition of 50 mM Tris hydrochloride.

The PEGylated agrin was diluted with buffer at a pH of 8.2 to lower both the conductivity and the concentration of Tris, before loading onto a cation exchange column (Pharmacia SP High Performance). The column was eluted using a gradient from 0 to 600 mM sodium chloride in Tris buffer at a pH of 8.2. Numerous distinct forms of PEGylated agrin eluted along the gradient and unmodified agrin eluted at the high salt end of the gradient. Monopegylated forms were selectively pooled for subsequent in vivo testing.

Adult Sprague-Dawley rats (male or female) weighing 300-500 g were anesthetized with ketamine/xylazine (50/10 mg/kg) and the right hind limb muscles were denervated by transecting the sciatic nerve at mid thigh level. After 10-14 days (when MuSK expression was substantially elevated in denervated muscle) rats were re-anesthetized and the right jugular vein was exposed by cut down surgery. Rats were then administered doses of agrin 4,8 or PEG-agrin 4,8 ranging from 1-10 mg/kg into the jugular vein with a 27 gauge needle. After injection, the wound was sutured and tail blood samples were taken at 0 (pre-injection), 5, 10, 15, 30 minutes, and at 1, 2, 4, 6, 8, 16, 24, and 48 hours after the injection. Serum samples were harvested from centrifuged blood and assayed using an ELISA. Serum levels are expressed as µg/ml of serum.

As shown in Figure 17, at a dose of 10 mg/kg, i.v., agrin 4,8 was rapidly cleared from the blood with a half-life of ~10 minutes. In contrast, the half-life of PEG-agrin 4,8 (also at 10 mg/kg, i.v.) was dramatically increased by ~10-fold. These results show that modification of agrin 4,8 with PEG greatly increases its apparent half-life in the blood. Thus, PEGylated agrin may have prolonged activity on MuSK in denervated skeletal muscle and may thus be a more effective treatment for muscular disorders and conditions such as muscle

atrophy. It is expected that the PEGylation of the above-described truncated agrin molecules would similarly increase their apparent half-lives in the blood.

DEPOSIT OF MICROORGANISMS

A clone designated pBluescript SK-containing dmk was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on July 13, 1993 under ATCC Accession No. 75498. Recombinant Autographa californica baculovirus encoding the rat Dmk RB (i.e., rat MuSK-IgG1 receptorbody) was designated "vDmk receptorbody" and deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on May 16, 1995 under ATCC Accession No. VR-2507. The cDNA clone pBL-hAgrin1 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on December 12, 1995 under ATCC Accession No. 97378.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Throughout the description and claims of the specification the word "comprise" and variations of the word, such as "comprising" and "comprises" is not intended to exclude other additives, components, integers or steps.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated nucleic acid molecule having a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence contained in the vector designated as pBL-hAgrin 1 (ATCC Accession No. 97378) or a fragment thereof which encodes the portion of human agrin which activates the MuSK receptor;

(b) a nucleotide sequence that differs from the nucleotide sequence of (a) by the presence of an insert corresponding to the insert at position Z in Figure 15 and/or by the absence of an insert corresponding to the insert at position Y in Figure 15 and/or by the presence of a nucleic acid sequence encoding a further insert having the amino acid sequence VLSASHPLTVSGASTPR immediately following the insert at position Y in Figure 15 and which encodes a polypeptide which activates the MuSK receptor;

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes a polypeptide which activates the MuSK receptor.

2. An isolated nucleic acid molecule encoding the portion of human agrin which activates the MuSK receptor, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 15 or a fragment thereof encoding at least amino acids 300 to 492;

(b) a nucleotide sequence that differs from the nucleotide sequence of (a) by the absence of an insert corresponding to the insert at position Y in Figure 15 and/or by the absence of an insert corresponding to the insert at position Z in Figure 15 and/or by the presence of a nucleic acid encoding a further insert having the amino acid sequence VLSASHPLTVSGASTPR immediately following the insert at position Y in Figure 15;

(c) a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of (a) or (b) and which encodes a polypeptide of

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substantially the same number of amino acids and which activates the MuSK receptor; and

(d) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from any of the nucleotide sequences of (a), (b) or (c) and which encodes a polypeptide which activates the MuSK receptor.

3. A nucleic acid according to claim 2 which is selected from:

(a) a nucleotide sequence encoding amino acids 1 to 492 set forth in Figure 15

(b) a nucleotide sequence encoding amino acids 24 to 492 set forth in Figure 15;

(c) a nucleotide sequence encoding amino acids 60 to 492 set forth in Figure 15;

(d) a nucleotide sequence encoding amino acids 76 to 492 set forth in Figure 15;

(e) a nucleotide sequence encoding amino acids 126 to 492 set forth in Figure 15;

(f) a nucleotide sequence encoding amino acids 178 to 492 set forth in Figure 15;

(g) a nucleotide sequence encoding amino acids 222 to 492 set forth in Figure 15;

(h) a nucleotide sequence encoding amino acids 260 to 492 set forth in Figure 15; and

(i) a nucleotide sequence encoding amino acids 300 to 492 set forth in Figure 15;

4. A nucleic acid according to claim 3 which is selected from:

(a) the nucleotide sequence set forth in Figure 15;

(b) the nucleotide sequence encoding amino acids 24 to 492 set forth in Figure 15;

(c) the nucleotide sequence encoding amino acids 60 to 492 set forth in Figure 15;

(d) the nucleotide sequence encoding amino acids 76 to 492 set forth in Figure 15;

(e) the nucleotide sequence encoding amino acids 126 to 492 set forth in Figure 15;

(f) the nucleotide sequence encoding amino acids 178 to 492 set forth in Figure 15;

(g) the nucleotide sequence encoding amino acids 222 to 492 set forth in Figure

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15;

(h) the nucleotide sequence encoding amino acids 260 to 492 set forth in Figure 15; and

(i) the nucleotide sequence encoding amino acids 300 to 492 set forth in Figure 15;

5. An isolated polypeptide encoded by a nucleic acid molecule of claim 1, 2, 3 or 4.

6. A polypeptide of claim 5, modified by covalent attachment of a polyethylene glycol molecule.

7. A vector which comprises the isolated nucleic acid molecule of claim 1, 2, 3 or 4.

8. An expression vector comprising a nucleic acid molecule of claim 1, 2, 3 or 4 wherein the nucleic acid molecule is operatively linked to an expression control sequence.

9. A host-vector system for the production of a polypeptide which activates the MuSK receptor which comprises the vector of claim 8, in a suitable host cell.

10. The host-vector system of claim 9, wherein the host cell is a bacterial cell, yeast cell, insect cell, or mammalian cell.

11. A host-vector system of claim 10, the host cell is a bacterial cell which is E.coli, a yeast cell which is Pichia pastoris, an insect cell which is Spodoptera frugiperda, or a mammalian cell which is a COS cell or a CHO cell.

12. A method of producing a polypeptide which activates the MuSK receptor which comprises growing cells of the host-vector system of claim 9, 10 or 11, under

conditions permitting production of the polypeptide and recovering the polypeptide so produced.

13. A method of promoting the growth, differentiation or survival of a MuSK receptor expressing cell comprising administering to the cell an effective amount of agrin or a portion thereof effective to activate the MuSK receptor.

14. The method of claim 13, wherein the MuSK receptor expressing cell is a cell which is normally found in muscle, heart, spleen, ovary or retina.

15. The method of claim 13 or 14, wherein the MuSK receptor expressing cell is a cell which has been genetically engineered to express the MuSK receptor.

16. A method of producing a monoclonal or polyclonal anti-agrin antibody or fragment thereof wherein a polypeptide of claim 5 or 6 or a fragment or derivative thereof is employed as an immunogen.

17. A method of treating a patient suffering from a disease or disorder affecting muscle comprising administering to the patient an effective amount of the polypeptide of claim 5 or 6, or a derivative thereof.

18. The method of claim 17, wherein the disease or disorder is muscle atrophy resulting from denervation due to nerve trauma, degenerative, metabolic or inflammatory neuropathy, peripheral neuropathy, or damage to nerves caused by environmental toxins or drugs.

19. The method of claim 17, wherein the disease or disorder is muscle atrophy due to a motor neuronopathy, chronic disuse, metabolic stress or nutritional insufficiency, muscular dystrophy syndrome or congenital myopathy.

20. The method of claim 17, wherein the disease or disorder is an acquired (toxic or inflammatory) myopathy.

21. Use of a polypeptide as defined in claim 5 or 6 in the manufacture of a medicament for the treatment of a disease or disorder affecting muscle.

22. Use according to claim 21 wherein the disease or disorder is muscle atrophy resulting from denervation due to nerve trauma, degenerative, metabolic or inflammatory neuropathy, peripheral neuropathy, or damage to nerves caused by environmental toxins or drugs.

23. Use according to claim 21 wherein the disease or disorder is muscle atrophy due to a motor neuronopathy, chronic disuse, metabolic stress or nutritional insufficiency, muscular dystrophy syndrome or congenital myopathy.

24. Use according to claim 21 wherein the disease or disorder is an acquired (toxic or inflammatory) myopathy.

25. A pharmaceutical composition comprising a polypeptide as defined in claim 5 or 6 and a pharmaceutically acceptable carrier.

26. A method of treating a patient suffering from a disease or disorder affecting muscle comprising administering to the patient an effective amount of the nucleic acid molecule of claim 1, 2, 3 or 4, or a derivative thereof.

27. Use of a nucleic acid molecule as defined in claim 1, 2, 3 or 4, or a derivative thereof, in the manufacture of a medicament for the treatment of a disease or disorder affecting muscle.

28. A pharmaceutical composition comprising a nucleic acid molecule as defined in claim 1, 2, 3 or 4, or a derivative thereof, and a pharmaceutically acceptable

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carrier.

29. The vector pBL-hAgrin 1 (deposited as ATCC 97378).

30. A nucleic acid molecule according to claim 1, 2, 3 or 4, or a derivative thereof, substantially as hereinbefore described.

31. A polypeptide according to claim 5 substantially as hereinbefore described.

32. A vector according to claim 7 or an expression vector according to claim 8, substantially as hereinbefore described.

33. A host vector system according to claim 9, substantially as hereinbefore described.

34. A method according to claim 12, 13, 17 or 26 substantially as hereinbefore described.

35. Use according to claim 21 or 27 substantially as hereinbefore described.

36. A pharmaceutical composition according to claim 25 or 28 substantially as hereinbefore described.

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Attorneys for:
REGENERON PHARMACEUTICALS, INC.

Fig.1.

```
 5   10   15   20   25   30   35   40   45   50   55   60   65   70
GAATTCGGCA CGAGCAAACA GTCATTAGTG GACGACTCTA TTGTAATAAA CTGTGCTTTA AAATGTAAAC

 75   80   85   90   95  100  105  110  115  120  125  130  135
CAGGGAGCGT TTTTTTTCCT CACATTGTCC AGAAGCAACC TTTCTTCCTG AGCCTGGATT AATC ATG
                                                                       M>

140   145   150   155   160   165   170   175   180   185   190
AGA GAG CTC GTC AAC ATT CCA CTG TTA CAG ATG CTC ACC CTG GTT GCC TTC AGC GGG
 R   E   L   V   N   I   P   L   L   Q   M   L   T   L   V   A   F   S   G>

195   200   205   210   215   220   225   230   235   240   245   250
ACC GAG AAA CTT CCA AAA GCC CCT GTC ATC ACC ACG CCT CTT GAA ACT GTA GAT GCC
 T   E   K   L   P   K   A   P   V   I   T   T   P   L   E   T   V   D   A>

255   260   265   270   275   280   285   290   295   300   305
TTA GTT GAA GAA GTG GCG ACT TTC ATG TGC GCC GTG GAA TCC TAC CCT CAG CCT GAA
 L   V   E   E   V   A   T   F   M   C   A   V   E   S   Y   P   Q   P   E>

310   315   320   325   330   335   340   345   350   355   360   365
ATT TCT TGG ACC AGA AAT AAA ATT CTC ATC AAG CTG TTT GAC ACC CGC TAC AGC ATC
 I   S   W   T   R   N   K   I   L   I   K   L   F   D   T   R   Y   S   I>

370   375   380   385   390   395   400   405   410   415   420
CGA GAG AAC GGT CAG CTC CTC ACC ATC CTG AGT GTG GAG GAC AGT GAT GAT GGC ATC
 R   E   N   G   Q   L   L   T   I   L   S   V   E   D   S   D   D   G   I>

425   430   435   440   445   450   455   460   465   470   475
TAC TGC TGC ACA GCC AAC AAT GGA GTG GGA GGA GCG GTG GAA AGT TGT GGC GCC CTG
 Y   C   C   T   A   N   N   G   V   G   G   A   V   E   S   C   G   A   L>

480   485   490   495   500   505   510   515   520   525   530   535
CAA GTG AAG ATG AAG CCT AAA ATA ACT CGT CCT CCC ATC AAT GTA AAA ATA ATT GAG
 Q   V   K   M   K   P   K   I   T   R   P   P   I   N   V   K   I   I   E>

540   545   550   555   560   565   570   575   580   585   590
GGA TTG AAA GCA GTC CTA CCG TGC ACT ACG ATG GGT AAC CCC AAG CCA TCC GTG TCC
 G   L   K   A   V   L   P   C   T   T   M   G   N   P   K   P   S   V   S>

595   600   605   610   615   620   625   630   635   640   645   650
TGG ATT AAG GGG GAC AGT GCT CTC AGG GAA AAT TCC AGG ATT GCA GTT CTT GAA TCT
 W   I   K   G   D   S   A   L   R   E   N   S   R   I   A   V   L   E   S>

655   660   665   670   675   680   685   690   695   700   705
GGG AGT TTA AGG ATC CAT AAT GTG CAA AAG GAA GAC GCA GGA CAG TAC CGA TGT GTG
 G   S   L   R   I   H   N   V   Q   K   E   D   A   G   Q   Y   R   C   V>

710   715   720   725   730   735   740   745   750   755   760
GCA AAA AAC AGC CTG GGC ACA GCT TAC TCC AAA CTG GTG AAG CTG GAA GTG GAG GTT
 A   K   N   S   L   G   T   A   Y   S   K   L   V   K   L   E   V   E   V>

765   770   775   780   785   790   795   800   805   810   815   820
TTT GCA AGA ATC CTG CGT GCT CCT GAA TCC CAC AAT GTC ACC TTT GGT TCC TTT GTA
 F   A   R   I   L   R   A   P   E   S   H   N   V   T   F   G   S   F   V>
```

Fig.1 (Cont 1).

```
 825  830  835  840  845  850  855  860  865  870  875
ACC CTA CGC TGC ACA GCA ATA GGC ATG CCT GTC CCC ACC ATC AGC TGG ATT GAA AAC
 T   L   R   C   T   A   I   G   M   P   V   P   T   I   S   W   I   E   N>

 880  885  890  895  900  905  910  915  920  925  930  935
GGA AAT GCT GTT TCT TCA GGT TCC ATT CAA GAG AAT GTG AAA GAC CGA GTG ATT GAC
 G   N   A   V   S   S   G   S   I   Q   E   N   V   K   D   R   V   I   D>

  940  945  950  955  960  965  970  975  980  985  990
TCA AGA CTC CAG CTC TTT ATC ACA AAG CCA GGA CTC TAC ACA TGC ATA GCT ACC AAT
 S   R   L   Q   L   F   I   T   K   P   G   L   Y   T   C   I   A   T   N>

 995  1000 1005 1010 1015 1020 1025 1030 1035 1040 1045
AAG CAT GGA GAG AAA TTC AGT ACC GCA AAG GCT GCA GCC ACT GTC AGT ATA GCA GAA
 K   H   G   E   K   F   S   T   A   K   A   A   A   T   V   S   I   A   E>

1050 1055 1060 1065 1070 1075 1080 1085 1090 1095 1100 1105
TGG AGC AAA TCA CAG AAA GAA AGC AAA GGC TAC TGT GCC CAG TAC AGA GGG GAG GTG
 W   S   K   S   Q   K   E   S   K   G   Y   C   A   Q   Y   R   G   E   V>

 1110 1115 1120 1125 1130 1135 1140 1145 1150 1155 1160
TGT GAT GCC GTC CTG GTG AAA GAC TCT CTT GTC TTC TTC AAC ACC TCC TAT CCC GAC
 C   D   A   V   L   V   K   D   S   L   V   F   F   N   T   S   Y   P   D>

1165 1170 1175 1180 1185 1190 1195 1200 1205 1210 1215 1220
CCT GAG GAG GCC CAA GAG CTG CTG ATC CAC ACT GCG TGG AAT GAA CTC AAG GCT GTG
 P   E   E   A   Q   E   L   L   I   H   T   A   W   N   E   L   K   A   V>

  1225 1230 1235 1240 1245 1250 1255 1260 1265 1270 1275
AGC CCA CTC TGC CGA CCA GCT GCC GAG GCT CTG CTG TGT AAT CAC CTC TTC CAG GAG
 S   P   L   C   R   P   A   A   E   A   L   L   C   N   H   L   F   Q   E>

1280 1285 1290 1295 1300 1305 1310 1315 1320 1325 1330
TGC AGC CCT GGA GTG CTA CCT ACT CCT ATG CCC ATT TGC AGA GAG TAC TGC TTG GCA
 C   S   P   G   V   L   P   T   P   M   P   I   C   R   E   Y   C   L   A>

1335 1340 1345 1350 1355 1360 1365 1370 1375 1380 1385 1390
GTA AAG GAG CTC TTC TGT GCA AAG GAA TGG CTG GCA ATG GAA GGG AAG ACC CAC CGC
 V   K   E   L   F   C   A   K   E   W   L   A   M   E   G   K   T   H   R>

  1395 1400 1405 1410 1415 1420 1425 1430 1435 1440 1445
GGA CTC TAC AGA TCC GGG ATG CAT TTC CTC CCG GTC CCG GAG TGC AGC AAG CTT CCC
 G   L   Y   R   S   G   M   H   F   L   P   V   P   E   C   S   K   L   P>

1450 1455 1460 1465 1470 1475 1480 1485 1490 1495 1500 1505
AGC ATG CAC CAG GAC CCC ACA GCC TGC ACA AGA CTG CCG TAT TTA GAT TAT AAA AAA
 S   M   H   Q   D   P   T   A   C   T   R   L   P   Y   L   D   Y   K   K>

 1510 1515 1520 1525 1530 1535 1540 1545 1550 1555 1560
GAA AAC ATA ACA ACA TTC CCG TCC ATA ACG TCC TCC AAG CCG AGC GTG GAC ATT CCA
 E   N   I   T   T   F   P   S   I   T   S   S   K   P   S   V   D   I   P>

1565 1570 1575 1580 1585 1590 1595 1600 1605 1610 1615
AAC CTG CCT GCC TCC ACG TCT TCC TTC GCC GTC TCG CCT GCG TAC TCC ATG ACT GTC
 N   L   P   A   S   T   S   S   F   A   V   S   P   A   Y   S   M   T   V>
```

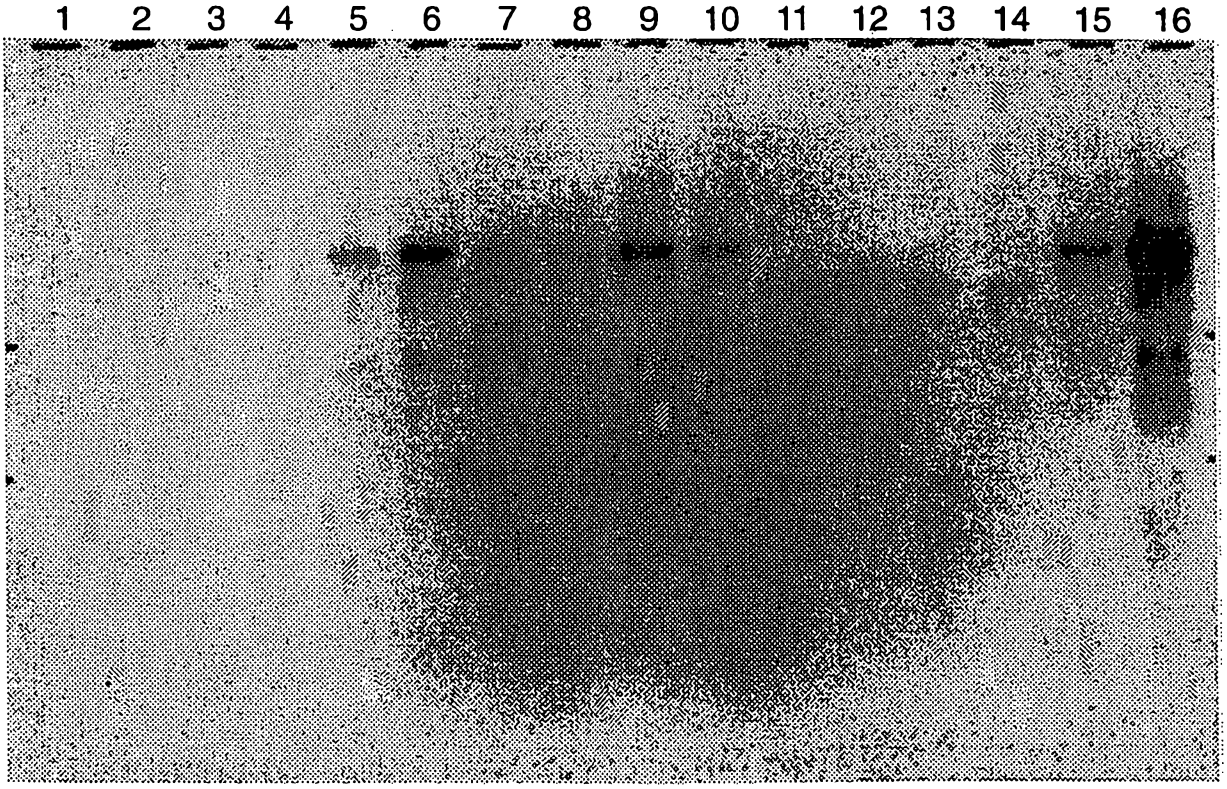
Fig.1 (Cont 2).

```
1620 1625 1630 1635 1640 1645 1650 1655 1660 1665 1670 1675
ATC ATC TCC ATC ATG TCC TGC TTT GCG GTG TTT GCT CTC CTC ACC ATC ACT ACT CTC
 I   I   S   I   M   S   C   F   A   V   F   A   L   L   T   I   T   T   L>

1680 1685 1690 1695 1700 1705 1710 1715 1720 1725 1730
TAT TGC TGC CGA AGG AGG AGA GAG TGG AAA AAT AAG AAA AGA GAG TCG GCA GCG GTG
 Y   C   C   R   R   R   R   E   W   K   N   K   K   R   E   S   A   A   V>

1735 1740 1745 1750 1755 1760 1765 1770 1775 1780 1785 1790
ACC CTC ACC ACA TTG CCT TCC GAG CTC CTG CTG GAC AGG CTG CAT CCC AAC CCC ATG
 T   L   T   T   L   P   S   E   L   L   L   D   R   L   H   P   N   P   M>

1795 1800 1805 1810 1815 1820 1825 1830 1835 1840 1845
TAC CAG AGG ATG CCA CTC CTT CTG AAT CCC AAG TTG CTC AGC CTG GAG TAT CCG AGG
 Y   Q   R   M   P   L   L   L   N   P   K   L   L   S   L   E   Y   P   R>

1850 1855 1860 1865 1870 1875 1880 1885 1890 1895 1900
AAT AAC ATC GAG TAT GTC AGA GAC ATC GGA GAG GGA GCG TTT GGA AGG GTC TTT CAA
 N   N   I   E   Y   V   R   D   I   G   E   G   A   F   G   R   V   F   Q>

1905 1910 1915 1920 1925 1930 1935 1940 1945 1950 1955 1960
GCG AGG GCC CCA GGC TTG CTT CCT TAT GAA CCC TTC ACT ATG GTG GCT GTG AAG ATG
 A   R   A   P   G   L   L   P   Y   E   P   F   T   M   V   A   V   K   M>

1965 1970 1975 1980 1985 1990 1995 2000 2005 2010 2015
CTG AAG GAG GAG GCC TCC GCA GAT ATG CAG GCA GAC TTT CAG AGG GAG GCA GCC CTC
 L   K   E   E   A   S   A   D   M   Q   A   D   F   Q   R   E   A   A   L>

2020 2025 2030 2035 2040 2045 2050 2055 2060 2065 2070 2075
ATG GCG GAG TTT GAC AAC CCC AAC ATT GTG AAG CTC TTA GGT GTG TGT GCT GTT GGG
 M   A   E   F   D   N   P   N   I   V   K   L   L   G   V   C   A   V   G>

2080 2085 2090 2095 2100 2105 2110 2115 2120 2125 2130
AAG CCA ATG TGC CTG CTC TTT GAA TAT ATG GCC TAT GGT GAC CTC AAT GAG TTC CTC
 K   P   M   C   L   L   F   E   Y   M   A   Y   G   D   L   N   E   F   L>

2135 2140 2145 2150 2155 2160 2165 2170 2175 2180 2185
CGA AGC ATG TCC CCT CAC ACT GTG TGC AGC CTC AGC CAC AGT GAC CTG TCC ACG AGG
 R   S   M   S   P   H   T   V   C   S   L   S   H   S   D   L   S   T   R>

2190 2195 2200 2205 2210 2215 2220 2225 2230 2235 2240 2245
GCT CGG GTG TCC AGC CCT GGT CCT CCA CCC CTG TCT TGT GCG GAA CAG CTC TGT ATT
 A   R   V   S   S   P   G   P   P   P   L   S   C   A   E   Q   L   C   I>

2250 2255 2260 2265 2270 2275 2280 2285 2290 2295 2300
GCC AGG CAA GTG GCA GCT GGC ATG GCC TAC CTG TCG GAG CGC AAG TTT GTC CAT CGG
 A   R   Q   V   A   A   G   M   A   Y   L   S   E   R   K   F   V   H   R>

2305 2310 2315 2320 2325 2330 2335 2340 2345 2350 2355 2360
GAC TTA GCT ACC AGG AAC TGC CTG GTT GGA GAG AAC ATG GTG GTG AAA ATT GCA GAC
 D   L   A   T   R   N   C   L   V   G   E   N   M   V   V   K   I   A   D>

2365 2370 2375 2380 2385 2390 2395 2400 2405 2410 2415
TTT GGC CTC TCT AGG AAC ATC TAC TCC GCA GAC TAC TAC AAA GCT GAT GGA AAC GAT
 F   G   L   S   R   N   I   Y   S   A   D   Y   Y   K   A   D   G   N   D>
```

Fig.1 (Cont 3).

```
2420   2425   2430  2435   2440   2445  2450   2455   2460  2465   2470
 *      *      *     *      *      *     *      *      *     *      *
 GCT ATA CCT ATC CGC TGG ATG CCA CCC GAG TCT ATC TTC TAC AAC CGC TAC ACC ACG
  A   I   P   I   R   W   M   P   P   E   S   I   F   Y   N   R   Y   T   T>

2475  2480   2485   2490  2495   2500   2505  2510   2515   2520  2525   2530
       *             *            *             *             *             *
 GAG TCA GAT GTG TGG GCT TAT GGC GTG GTC CTC TGG GAG ATC TTC TCC TAT GGA CTG
  E   S   D   V   W   A   Y   G   V   V   L   W   E   I   F   S   Y   G   L>

 2535  2540   2545   2550  2555   2560   2565  2570   2575   2580  2585
        *             *            *             *             *
 CAG CCC TAC TAT GGA ATG GCC CAT GAG GAG GTC ATT TAC TAT GTG AGA GAT GGT AAC
  Q   P   Y   Y   G   M   A   H   E   E   V   I   Y   Y   V   R   D   G   N>

2590   2595  2600   2605  2610  2615   2620  2625   2630  2635   2640  2645
 *      *     *      *     *     *      *     *      *     *      *
 ATC CTT GCC TGC CCT GAG AAC TGT CCC TTG GAA CTG TAC AAC CTT ATG CGC CTA TGT
  I   L   A   C   P   E   N   C   P   L   E   L   Y   N   L   M   R   L   C>

 2650   2655  2660   2665  2670  2675   2680  2685   2690  2695   2700
  *      *     *      *     *     *      *     *      *     *      *
 TGG AGC AAG CTG CCT GCA GAC AGA CCC AGC TTC TGC AGT ATC CAC CGG ATC CTG CAG
  W   S   K   L   P   A   D   R   P   S   F   C   S   I   H   R   I   L   Q>

2705   2710   2715  2720   2725   2730  2735   2740   2745  2750   2755  2760
        *             *            *             *             *             *
 CGC ATG TGC GAG AGA GCA GAG GGA ACG GTA GGC GTC TAA GGTTGACCA TGCTCAAACA
  R   M   C   E   R   A   E   G   T   V   G   V   *>

 2765  2770   2775  2780   2785  2790   2795  2800   2805  2810   2815  2820   2825  2830
        *             *            *             *             *             *
 ACACCCAGGA GGATCTTTTC AGACTGCGAG CTGGAGGGAT CCTAAAGCAG AGGGCGNATA AGNNCAGATA

 2835  2840   2845  2850   2855  2860   2865
        *             *            *
 GGAAGAGTTT ATCTCAGGCA GCACGTNCAG TTGGTTGTT
```

Fig.2.



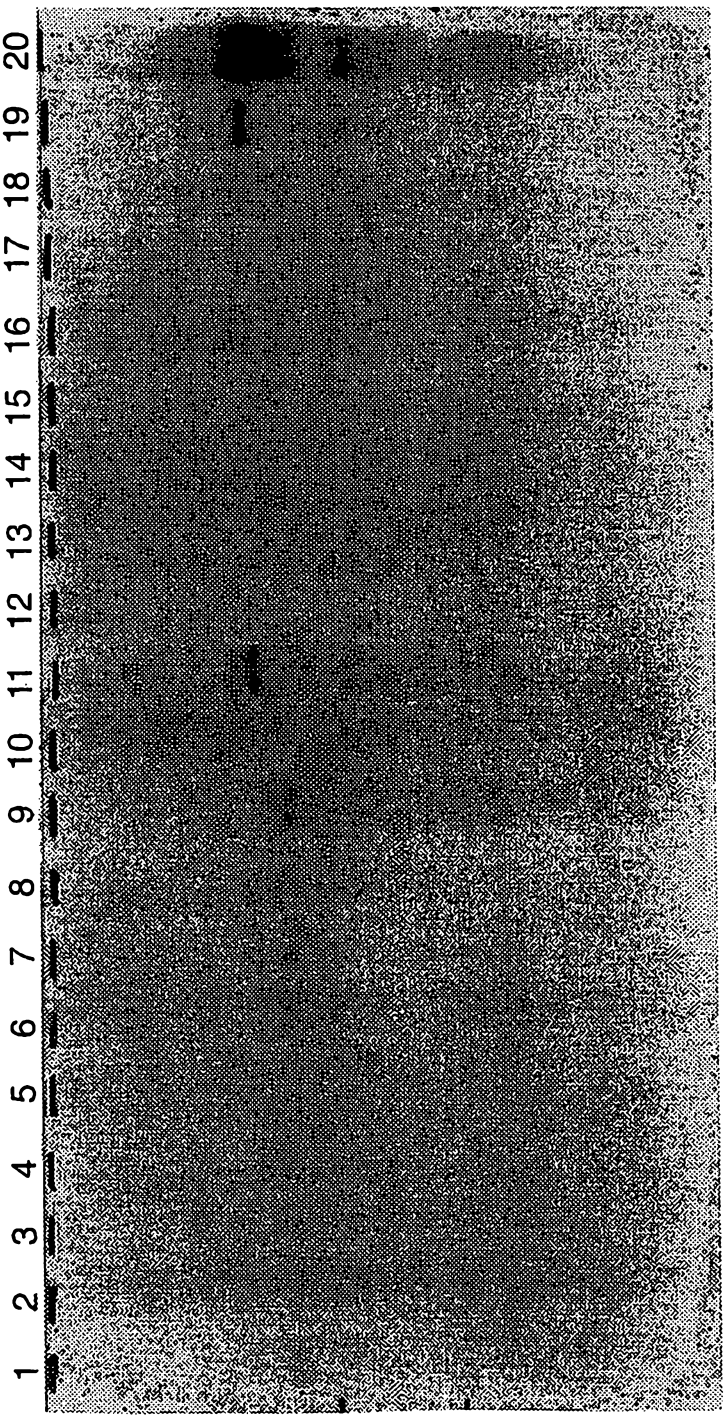


Fig.3.

Fig.4.

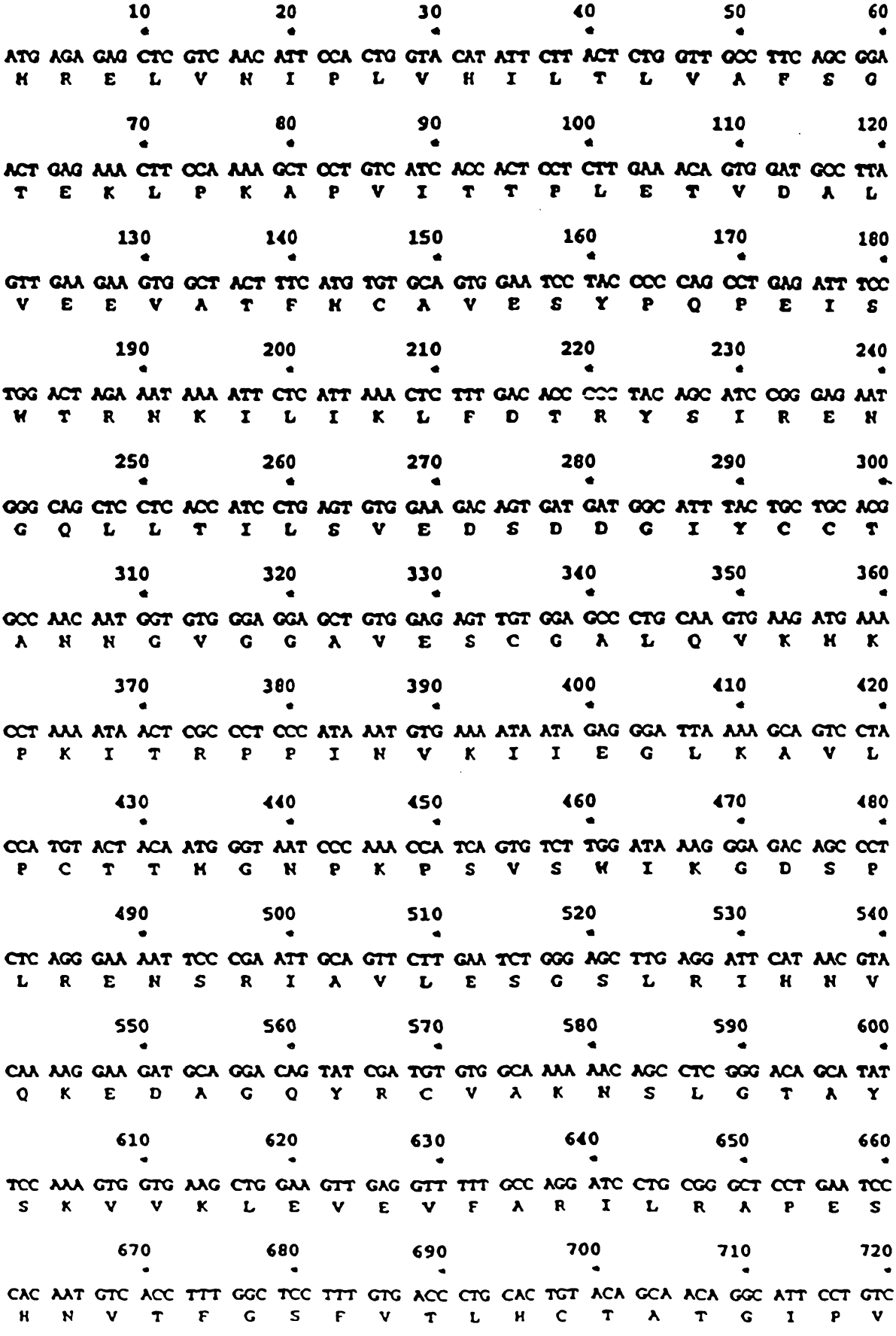

10 20 30 40 50 60
ATG AGA GAG CTC GTC AAC ATT CCA CTG GTA CAT ATT CTT ACT CTG GTT GCC TTC AGC GGA
M R E L V N I P L V H I L T L V A F S G
70 80 90 100 110 120
ACT GAG AAA CTT CCA AAA GCT CCT GTC ATC ACC ACT CCT CTT GAA ACA GTG GAT GCC TTA
T E K L P K A P V I T T P L E T V D A L
130 140 150 160 170 180
GTT GAA GAA GTG GCT ACT TTC ATG TGT GCA GTG GAA TCC TAC CCC CAG CCT GAG ATT TCC
V E E V A T F M C A V E S Y P Q P E I S
190 200 210 220 230 240
TGG ACT AGA AAT AAA ATT CTC ATT AAA CTC TTT GAC ACC CCC TAC AGC ATC CGG GAG AAT
W T R N K I L I K L F D T R Y S I R E N
250 260 270 280 290 300
GGG CAG CTC CTC ACC ATC CTG AGT GTG GAA GAC AGT GAT GAT GGC ATT TAC TGC TGC ACG
G Q L L T I L S V E D S D D G I Y C C T
310 320 330 340 350 360
GCC AAC AAT GGT GTG GGA GGA GCT GTG GAG AGT TGT GGA GCC CTG CAA GTG AAG ATG AAA
A N N G V G G A V E S C G A L Q V K M K
370 380 390 400 410 420
CCT AAA ATA ACT CGC CCT CCC ATA AAT GTG AAA ATA ATA GAG GGA TTA AAA GCA GTC CTA
P K I T R P P I N V K I I E G L K A V L
430 440 450 460 470 480
CCA TGT ACT ACA ATG GGT AAT CCC AAA CCA TCA GTG TCT TGG ATA AAG GGA GAC AGC CCT
P C T T M G N P K P S V S W I K G D S P
490 500 510 520 530 540
CTC AGG GAA AAT TCC CGA ATT GCA GTT CTT GAA TCT GGG AGC TTG AGG ATT CAT AAC GTA
L R E N S R I A V L E S G S L R I H N V
550 560 570 580 590 600
CAA AAG GAA GAT GCA GGA CAG TAT CGA TGT GTG GCA AAA AAC AGC CTC GGG ACA GCA TAT
Q K E D A G Q Y R C V A K N S L G T A Y
610 620 630 640 650 660
TCC AAA GTG GTG AAG CTG GAA GTT GAG GTT TTT GCC AGG ATC CTG CGG GCT CCT GAA TCC
S K V V K L E V E V F A R I L R A P E S
670 680 690 700 710 720
CAC AAT GTC ACC TTT GGC TCC TTT GTG ACC CTG CAC TGT ACA GCA ACA GGC ATT CCT GTC
H N V T F G S F V T L H C T A T G I P V

Fig.4 (Cont 1).

```
          730         740         750         760         770         780
           .           .           .           .           .           .
CCC ACC ATC ACC TGG ATT GAA AAC GGA AAT GCT GTT TCT TCT GGG TCC ATT CAA GAG AGT
 P   T   I   T   W   I   E   N   G   N   A   V   S   S   G   S   I   Q   E   S

          790         800         810         820         830         840
           .           .           .           .           .           .
GTG AAA GAC CGA GTG ATT GAC TCA AGA CTG CAG CTG TTT ATC ACC AAG CCA GGA CTC TAC
 V   K   D   R   V   I   D   S   R   L   Q   L   F   I   T   K   P   G   L   Y

          850         860         870         880         890         900
           .           .           .           .           .           .
ACA TGC ATA GCT ACC AAT AAG CAT GGG GAG AAG TTC AGT ACT GCC AAG GCT GCA GCC ACC
 T   C   I   A   T   N   K   H   G   E   K   F   S   T   A   K   A   A   A   T

          910         920         930         940         950         960
           .           .           .           .           .           .
ATC AGC ATA GCA GAA TGG AGT AAA CCA CAG AAA GAT AAC AAA GGC TAC TGC GCC CAG TAC
 I   S   I   A   E   W   S   K   P   Q   K   D   N   K   G   Y   C   A   Q   Y

          970         980         990         1000        1010        1020
           .           .           .           .           .           .
AGA GGG GAG GTG TGT AAT GCA GTC CTG GCA AAA GAT GCT CTT GTT TTT CTC AAC ACC TCC
 R   G   E   V   C   N   A   V   L   A   K   D   A   L   V   F   L   N   T   S

          1030        1040        1050        1060        1070        1080
           .           .           .           .           .           .
TAT GCG GAC CCT GAG GAG GCC CAA GAG CTA CTG GTC CAC ACG GCC TGG AAT GAA CTG AAA
 Y   A   D   P   E   E   A   Q   E   L   L   V   H   T   A   W   N   E   L   K

          1090        1100        1110        1120        1130        1140
           .           .           .           .           .           .
GTA GTG AGC CCA GTC TGC CGG CCA GCT GCT GAG GCT TTG TTG TGT AAC CAC ATC TTC CAG
 V   V   S   P   V   C   R   P   A   A   E   A   L   L   C   N   H   I   F   Q

          1150        1160        1170        1180        1190        1200
           .           .           .           .           .           .
GAG TGC AGT CCT GGA GTA GTG CCT ACT CCT ATT CCC ATT TGC AGA GAG TAC TGC TTG GCA
 E   C   S   P   G   V   V   P   T   P   I   P   I   C   R   E   Y   C   L   A

          1210        1220        1230        1240        1250        1260
           .           .           .           .           .           .
GTA AAG GAG CTC TTC TGC GCA AAA GAA TGG CTG GTA ATG GAA GAG AAG ACC CAC AGA GGA
 V   K   E   L   F   C   A   K   E   W   L   V   M   E   E   K   T   H   R   G

          1270        1280        1290        1300        1310        1320
           .           .           .           .           .           .
CTC TAC AGA TCC GAG ATG CAT TTG CTG TCC GTG CCA GAA TGC AGC AAG CTT CCC AGC ATG
 L   Y   R   S   E   M   H   L   L   S   V   P   E   C   S   K   L   P   S   M

          1330        1340        1350        1360        1370        1380
           .           .           .           .           .           .
CAT TGG GAC CCC ACG GCC TGT GCC AGA CTG CCA CAT CTA GAT TAT AAC AAA GAA AAC CTA
 H   W   D   P   T   A   C   A   R   L   P   H   L   D   Y   N   K   E   N   L

          1390        1400        1410        1420        1430        1440
           .           .           .           .           .           .
AAA ACA TTC CCA CCA ATG ACG TCC TCA AAG CCA AGT GTG GAC ATT CCA AAT CTG CCT TCC
 K   T   F   P   P   M   T   S   S   K   P   S   V   D   I   P   N   L   P   S
```

Fig.4 (Cont 2).

```
        1450        1460        1470        1480        1490        1500
TCC TCC TCT TCT TCC TTC TCT GTC TCA CCT ACA TAC TCC ATG ACT GTA ATA ATC TCC ATC
 S   S   S   S   S   F   S   V   S   P   T   Y   S   M   T   V   I   I   S   I

        1510        1520        1530        1540        1550        1560
ATG TCC AGC TTT GCA ATA TTT GTG CTT CTT ACC ATA ACT ACT CTC TAT TGC TGC CGA AGA
 M   S   S   F   A   I   F   V   L   L   T   I   T   T   L   Y   C   C   R   R

        1570        1580        1590        1600        1610        1620
AGA AAA CAA TGG AAA AAT AAG AAA AGA GAA TCA GCA GCA GTA ACC CTC ACC ACA CTG CCT
 R   K   Q   W   K   N   K   K   R   E   S   A   A   V   T   L   T   T   L   P

        1630        1640        1650        1660        1670        1680
TCT GAG CTC TTA CTA GAT AGA CTT CAT CCC AAC CCC ATG TAC CAG AGG ATG CCG CTC CTT
 S   E   L   L   L   D   R   L   H   P   N   P   M   Y   Q   R   M   P   L   L

        1690        1700        1710        1720        1730        1740
CTG AAC CCC AAA TTG CTC AGC CTG GAG TAT CCA AGG AAT AAC ATT GAA TAT GTG AGA GAC
 L   N   P   K   L   L   S   L   E   Y   P   R   N   N   I   E   Y   V   R   D

        1750        1760        1770        1780        1790        1800
ATC GGA GAG GGA GCG TTT GGA AGG GTG TTT CAA GCA AGG CCA CCA GGC TTA CTT CCC TAT
 I   G   E   G   A   F   G   R   V   F   Q   A   R   A   P   G   L   L   P   Y

        1810        1820        1830        1840        1850        1860
GAA CCT TTC ACT ATG GTG GCA GTA AAG ATG CTC AAA GAA GAA GCC TCG GCA GAT ATG CAA
 E   P   F   T   M   V   A   V   K   M   L   K   E   E   A   S   A   D   M   Q

        1870        1880        1890        1900        1910        1920
GCG GAC TTT CAG AGG GAG GCA GCC CTC ATG GCA GAA TTT GAC AAC CCT AAC ATT GTG AAG
 A   D   F   Q   R   E   A   A   L   M   A   E   F   D   N   P   N   I   V   K

        1930        1940        1950        1960        1970        1980
CTA TTA GGA GTG TGT GCT GTC GGG AAG CCA ATG TGC CTG CTC TTT GAA TAC ATG GCC TAT
 L   L   G   V   C   A   V   G   K   P   M   C   L   L   F   E   Y   M   A   Y

        1990        2000        2010        2020        2030        2040
GGT GAC CTC AAT GAG TTC CTC CGC AGC ATG TCC CCT CAC ACC GTG TGC AGC CTC AGT CAC
 G   D   L   N   E   F   L   R   S   M   S   P   H   T   V   C   S   L   S   H

        2050        2060        2070        2080        2090        2100
AGT GAC TTG TCT ATG AGG GCT CAG GTC TCC AGC CCT GGG CCC CCA CCC CTC TCC TGT GCT
 S   D   L   S   M   R   A   Q   V   S   S   P   G   P   P   P   L   S   C   A

        2110        2120        2130        2140        2150        2160
GAG CAG CTT TGC ATT GCC AGG CAG GTG GCA GCT GGC ATG GCT TAC CTC TCA GAA CGT AAG
 E   Q   L   C   I   A   R   Q   V   A   A   G   M   A   Y   L   S   E   R   K
```

Fig.4 (Cont 3).

```
          2170        2180        2190        2200        2210        2220
            *           *           *           *           *           *
TTT GTT CAC CGA GAT TTA GCC ACC AGG AAC TGC CTG GTG GGC GAG AAC ATG GTG GTG AAA
 F   V   H   R   D   L   A   T   R   N   C   L   V   G   E   N   M   V   V   K

          2230        2240        2250        2260        2270        2280
            *           *           *           *           *           *
ATT GCC GAC TTT GGC CTC TCC AGG AAC ATC TAC TCA GCA GAC TAC TAC AAA GCT AAT GAA
 I   A   D   F   G   L   S   R   N   I   Y   S   A   D   Y   Y   K   A   N   E

          2290        2300        2310        2320        2330        2340
            *           *           *           *           *           *
AAC GAC GCT ATC CCT ATC CGT TGG ATG CCA CCA GAG TCC ATT TTT TAT AAC CGC TAC ACT
 N   D   A   I   P   I   R   W   M   P   P   E   S   I   F   Y   N   R   Y   T

          2350        2360        2370        2380        2390        2400
            *           *           *           *           *           *
ACA GAG TCT GAT GTG TGG GCC TAT GGC GTG GTC CTC TGG GAG ATC TTC TCC TAT GGC CTG
 T   E   S   D   V   W   A   Y   G   V   V   L   W   E   I   F   S   Y   G   L

          2410        2420        2430        2440        2450        2460
            *           *           *           *           *           *
CAG CCC TAC TAT GGG ATG GCC CAT GAG GAG GTC ATT TAC TAC GTG CGA GAT GGC AAC ATC
 Q   P   Y   Y   G   M   A   H   E   E   V   I   Y   Y   V   R   D   G   N   I

          2470        2480        2490        2500        2510        2520
            *           *           *           *           *           *
CTC TCC TGC CCT GAG AAC TGC CCC GTG GAG CTG TAC AAT CTC ATG CGT CTA TGT TGG AGC
 L   S   C   P   E   N   C   P   V   E   L   Y   N   L   M   R   L   C   W   S

          2530        2540        2550        2560        2570        2580
            *           *           *           *           *           *
AAG CTG CCT GCA GAC AGA CCC AGT TTC ACC AGT ATT CAC CGA ATT CTG GAA CGC ATG TGT
 K   L   P   A   D   R   P   S   F   T   S   I   H   R   I   L   E   R   M   C

          2590        2600        2610
            *           *           *
GAG AGG GCA GAG GGA ACT GTG AGT GTC TAA
 E   R   A   E   G   T   V   S   V   *
```

Fig.5.

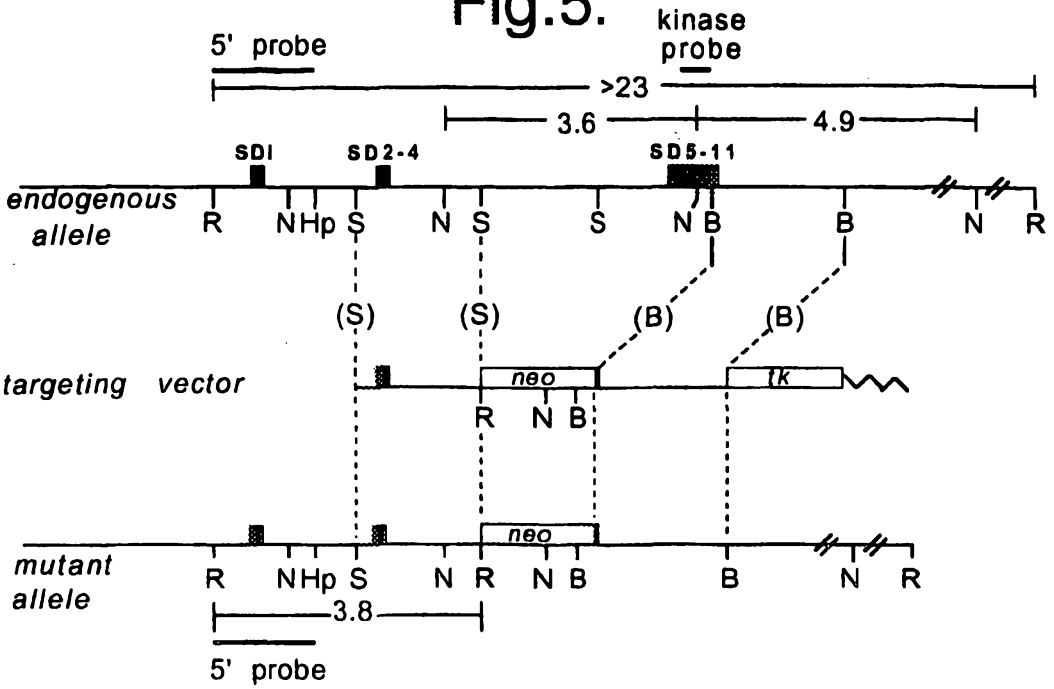

5' probe
kinase probe
>23
3.6
4.9
SD1
SD2-4
SD5-11
endogenous allele
R NHp S N S S N B B N R
(S) (S) (B) (B)
targeting vector
neo
tk
R N B
neo
mutant allele
R NHp S N R N B B N R
3.8
5' probe

Fig.6.

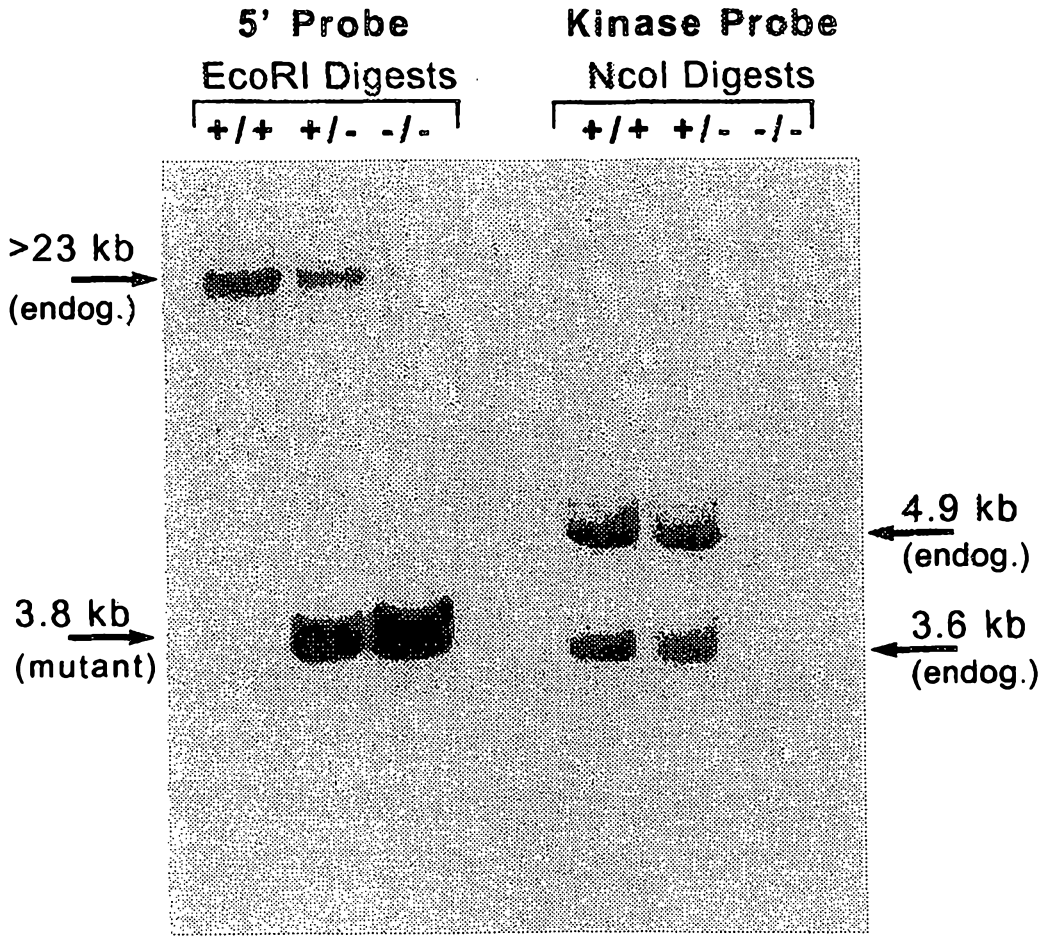

5' Probe
EcoRI Digests
+/+ +/- -/-
Kinase Probe
NcoI Digests
+/+ +/- -/-
>23 kb
(endog.)
4.9 kb
(endog.)
3.8 kb
(mutant)
3.6 kb
(endog.)

Fig.7A.

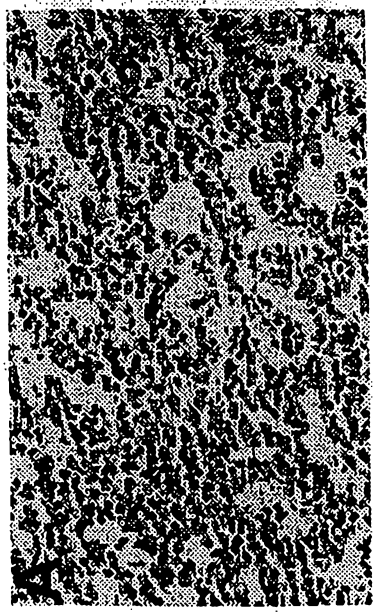


Fig.7B.



Fig.7C.



MuSK -/-

Fig.7D.



CONTROL

Fig.8A.

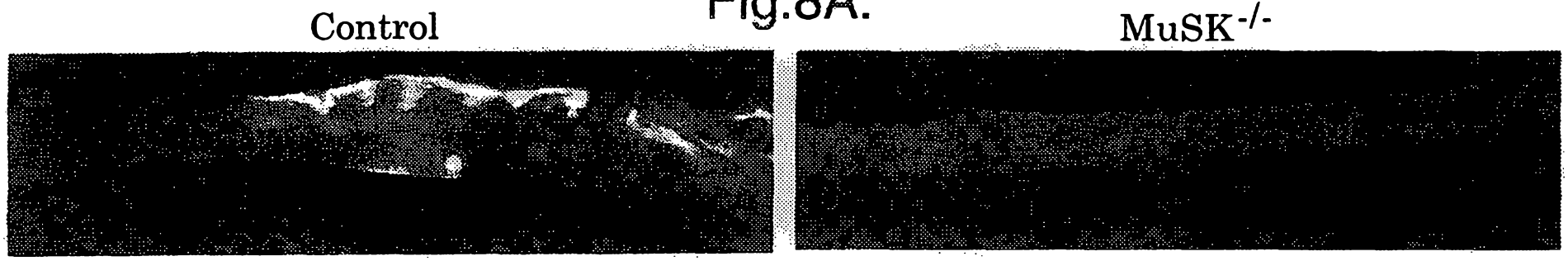


Fig.8B.

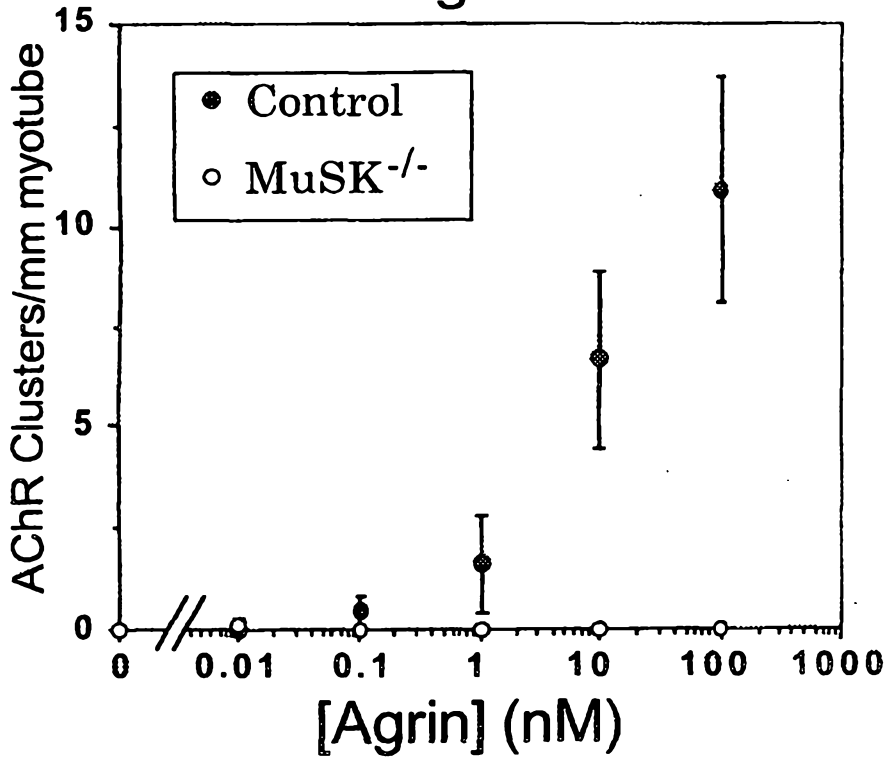


Fig.8C.

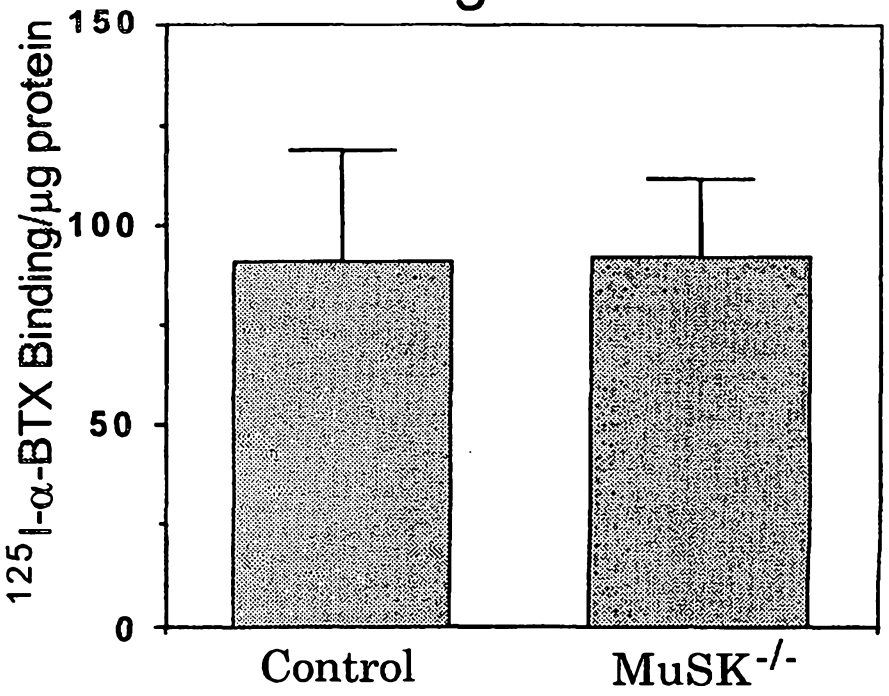


Fig.9A.

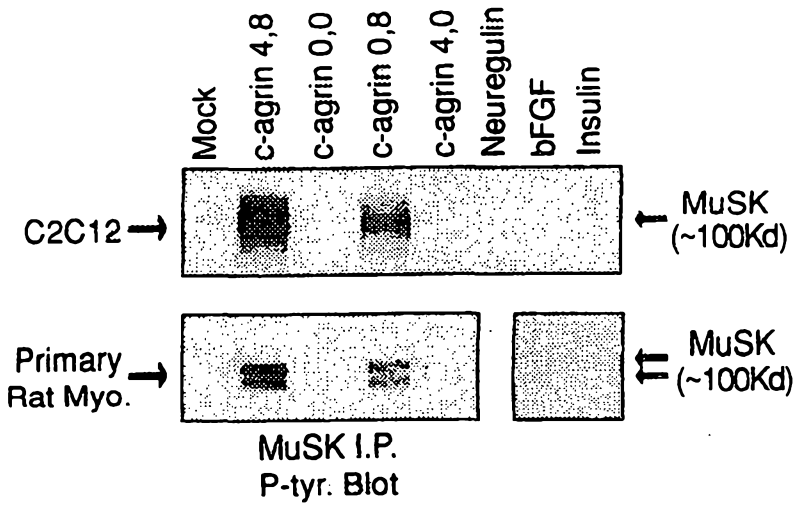


Fig.9B.

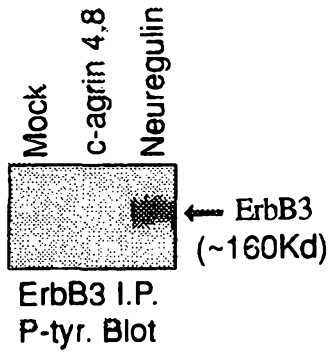


Fig.9C.

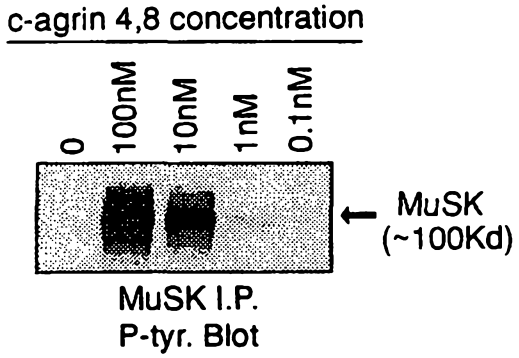


Fig.9D.

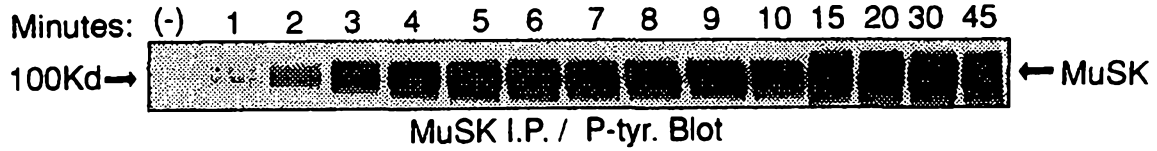


Fig.10A.

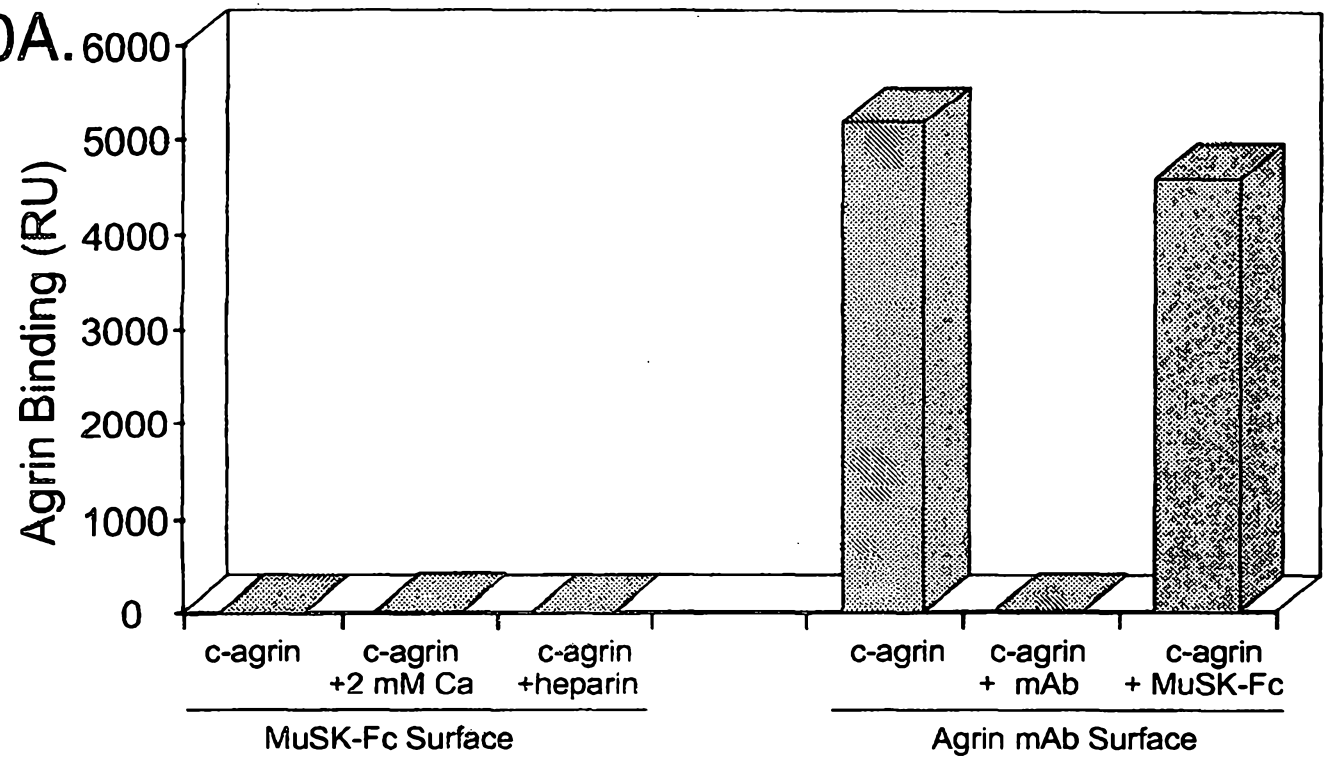


Fig.10B.

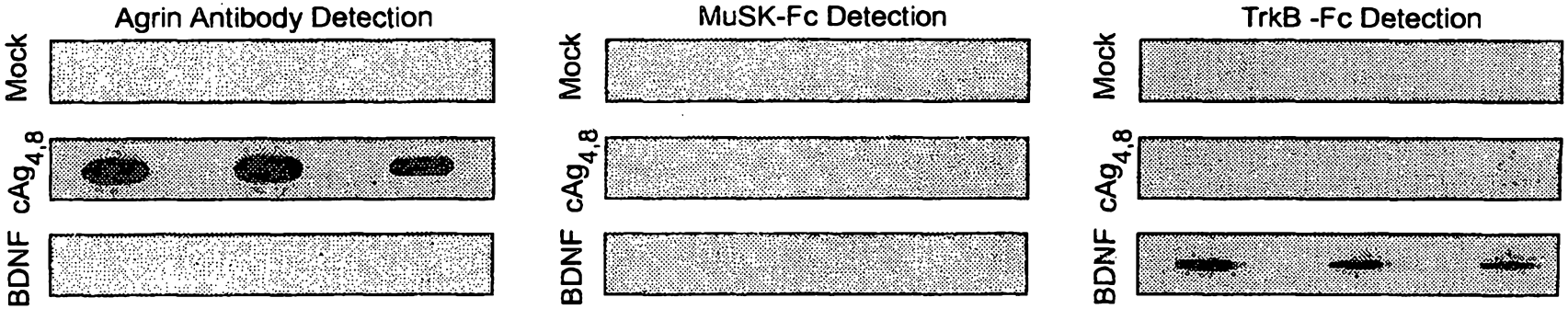


Fig.11.

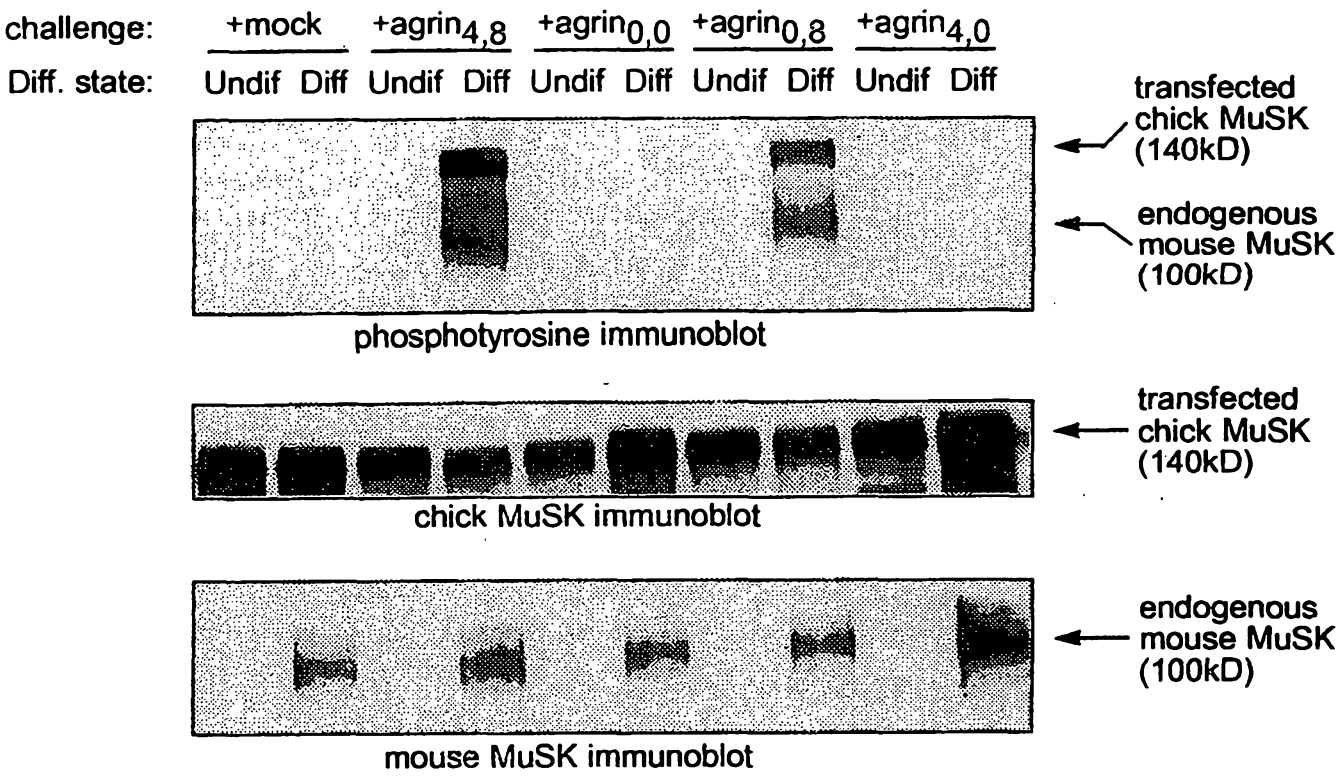
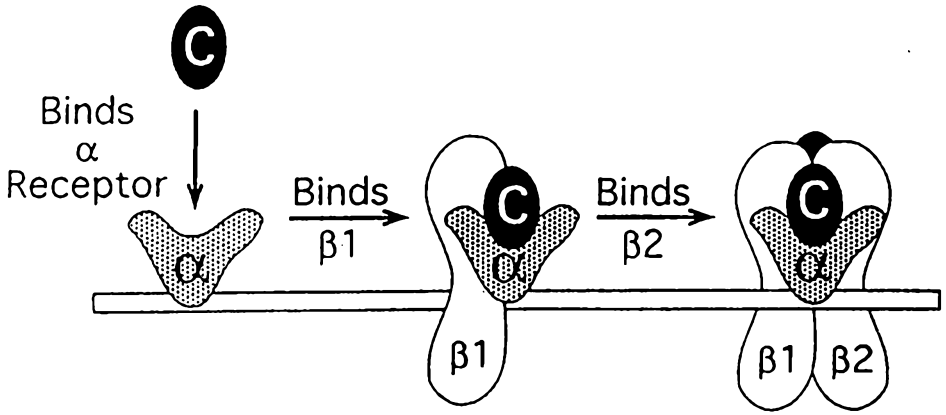
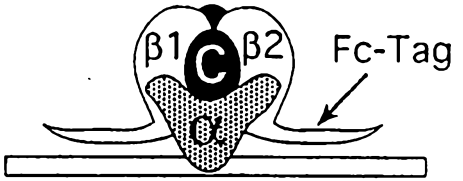

challenge:
+mock
+agrin4,8
+agrin0,0
+agrin0,8
+agrin4,0
Diff. state:
Undif Diff Undif Diff Undif Diff Undif Diff Undif Diff
transfected chick MuSK (140kD)
endogenous mouse MuSK (100kD)
phosphotyrosine immunoblot
transfected chick MuSK (140kD)
chick MuSK immunoblot
endogenous mouse MuSK (100kD)
mouse MuSK immunoblot

Fig.12.

A. Formation of CNTF Receptor Complex



B. CNTF Receptor Complex With Soluble β Components



C. Formation of Receptor Complex For Agrin

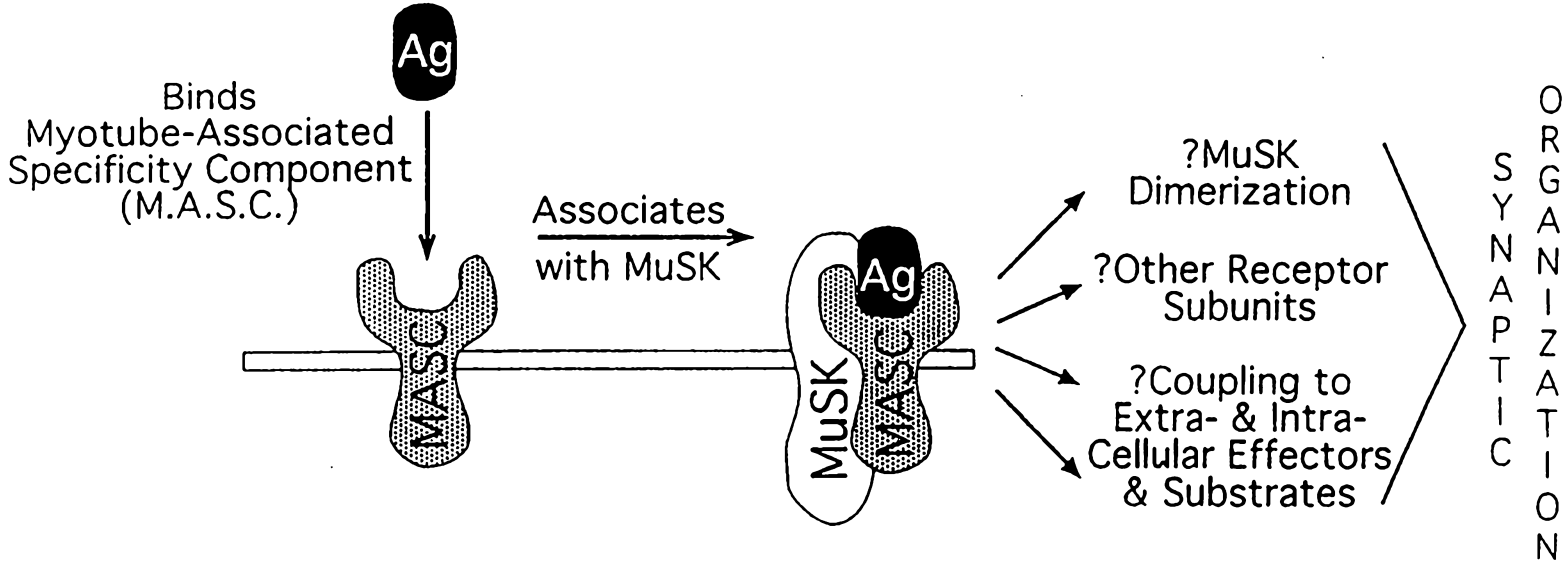


Fig.13A.

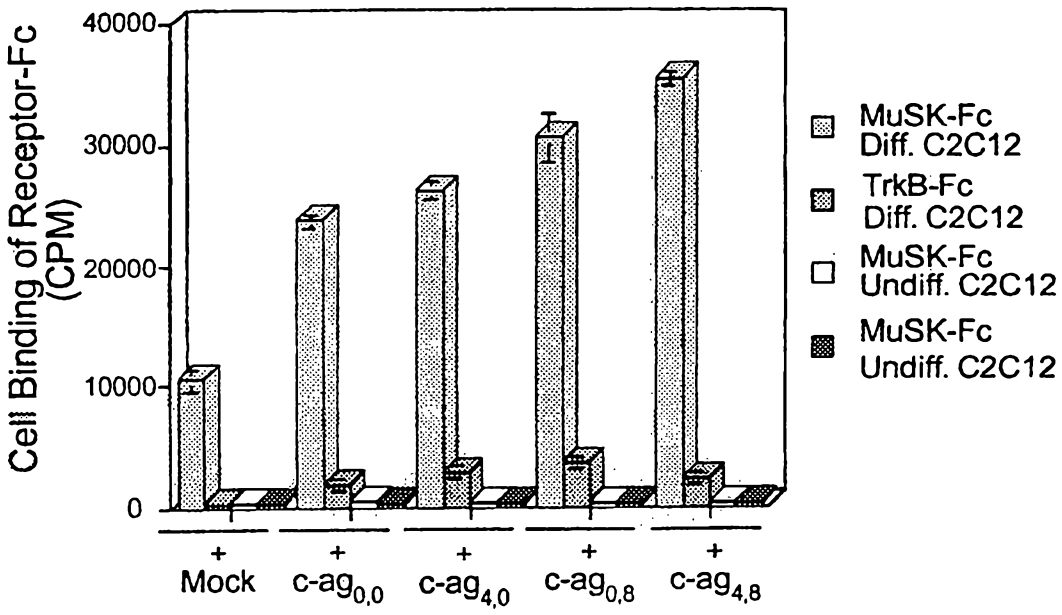


Fig.13B.

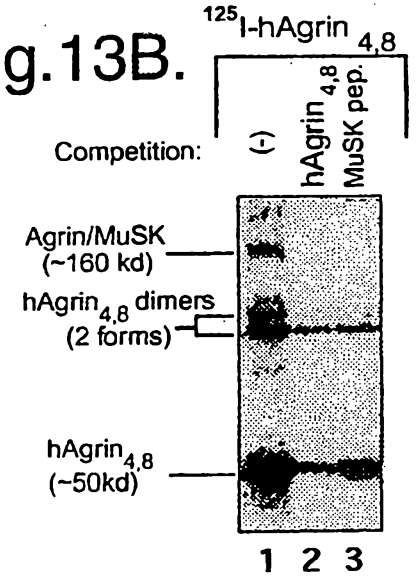


Fig.13C.

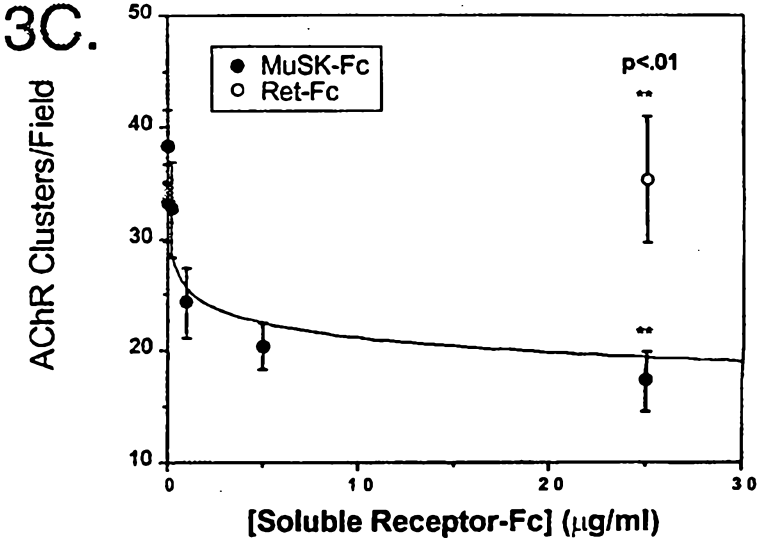


Fig.14.

```
        10         20         30         40         50         60
MPPLPLEHRP RQEPGASMLV RYFMIPCNIC LILLATSTLG FAVLLFLSNY KPGIHFTPAP

        70         80         90        100        110        120
PTPPDVCRGM LCGFGAVCEP SVEDPGRASC VCKKNACPAT VAPVCGSDAS TYSNECELQR

       130        140        150        160        170        180
AQCNQQRRIR LLRQGPCGSR DPCANVTCSF GSTCVPSADG QTASCLCPTT CFGAPDGTVC

       190        200        210        220        230        240
GSDGVDYPSE CQLLSHACAS QEHIFKKFNG PCDPCQGSMS DLNHICRVNP RTRHPEMLLR

       250        260        270        280        290        300
PENCPAQHTP ICGDDGVTYE NDCVMSRIGA TRGLLLQKVR SGQCQTRDQC PETCQFNSVC

       310        320        330        340        350        360
LSRRGRPHCS CDRVTCDGSY RPVCAQDGHT YNNDCWRQQA ECRQQRAIPP KHQGPCDQTP

       370        380        390        400        410        420
SPCHGVQCAF GAVCTVKNGK AECECQRVCS GIYDPVCGSD GVTYGSVCEL ESMACTLGRE

       430        440        450        460        470        480
IQVARRGPCD PCGQCRFGSL CEVETGRCVC PSECVESAQP VCGSDGHTYA SECELHVHAC

       490        500        510        520        530        540
THQISLYVAS AGHCQTCGEK VCTFGAVCSA GQCVCPRCEH PPPGPVCGSD GVTYLSACEL

       550        560        570        580        590        600
REAACQQQVQ IEEAHAGPCE PAECGSGGSG SGEDDECEQE LCRQRGGIWD EDSEDGPCVC

       610        620        630        640        650        660
DFSCQSVPRS PVCGSDGVTY GTECDLKKAR CESQQELYVA AQGACRGPTL APLLPVAFPH

       670        680        690        700        710        720
CAQTPYGCCQ DNFTAAQGVG LAGCPSTCHC NPHGSYSGTC DPATGQCSCR PGVGGLRCDR

       730        740        750        760        770        780
CEPGFWNFRG IVTDGHSGCT PCSCDPRGAV RDDCEQMTGL CSCRPGVAGP KCGQCPDGQV

       790        800        810        820        830        840
LGHLGCEADP MTPVTCVEIH CEFGASCVEK AGFAQCICPT LTCPEANSTK VCGSDGVTYG
```

Fig.14 (Cont 1).

```
       850        860        870        880        890        900
     *    *     *    *     *    *     *    *     *    *     *    *
NECQLKAIAC RQRLDISTQS LGPCQESVTP GASPTSASMT TPRHILSKTL PFPHNSLPLS

       910        920        930        940        950        960
     *    *     *    *     *    *     *    *     *    *     *    *
PGSTTHDWPT PLPISPHTTV SIPRSTAWPV LTVPPTAAAS DVTSLATSIF SESGSANGSG

       970        980        990       1000       1010       1020
     *    *     *    *     *    *     *    *     *    *     *    *
DEELSGDEEA SGGGSGGLEP PVGSIVVTHG PPIERASCYN SPLGCCSDGK TPSLDSEGSN

      1030       1040       1050       1060       1070       1080
     *    *     *    *     *    *     *    *     *    *     *    *
CPATKAFQGV LELEGVEGQE LFYTPEMADP KSELFGETAR SIESTLDDLF RNSDVKKDFW

      1090       1100       1110       1120       1130       1140
     *    *     *    *     *    *     *    *     *    *     *    *
SVRLRELGPG KLVRAIVDVH FDPTTAFQAS DVGQALLRQI QVSRPWALAV RRPLQEHVRF

      1150       1160       1170       1180       1190       1200
     *    *     *    *     *    *     *    *     *    *     *    *
LDFDWFPTFF TGAATGTTAA MATARATTVS RLPASSVTPR VYPSHTSRPV GRTTAPPTTR

      1210       1220       1230       1240       1250       1260
     *    *     *    *     *    *     *    *     *    *     *    *
RPPTTATNMD RPRTPGHQQP SKSCDSQPCL HGGTCQDQDS GKGFTCSCTA GRGGSVCEKV

      1270       1280       1290       1300       1310       1320
     *    *     *    *     *    *     *    *     *    *     *    *
QPPSMPAFKG HSFLAFPTLR AYHTLRLALE FRALETEGLL LYNGNARGKD FLALALLDGR

      1330       1340       1350       1360       1370       1380
     *    *     *    *     *    *     *    *     *    *     *    *
VQFRFDTGSG PAVLTSLVPV EPGRWHRLEL SRHWRQGTLS VDGETPVVGE SPSGTDGLNL

      1390       1400       1410       1420       1430       1440
     *    *     *    *     *    *     *    *     *    *     *    *
DTNLYVGGIP EEQVAMVLDR TSVGVGLKGC IRMLDINNQQ LELSDWQRAA VQSSGVGECG

      1450       1460       1470       1480       1490       1500
     *    *     *    *     *    *     *    *     *    *     *    *
DHPCLPNPCH GGALCQALEA GMFLCQCPPG RFGPTCADEK SPCQPNPCHG AAPCRVLSSG

      1510       1520       1530       1540       1550       1560
     *    *     *    *     *    *     *    *     *    *     *    *
GAKCECPLGR SGTFCQTVLE TAGSRPFLAD FNGFSYLELK GLHTFERDLG EKMALEMVFL

      1570       1580       1590       1600       1610       1620
     *    *     *    *     *    *     *    *     *    *     *    *
ARGPSGLLLY NGQKTDGKGD FVSLALHNRH LEFCYDLGKG AAVIRSKEPI ALGTWVRVFL

      1630       1640       1650       1660       1670       1680
     *    *     *    *     *    *     *    *     *    *     *    *
ERNGRKGALQ VGDGPRVLGE SPKSRKVPHT MLNLKEPLYI GGAPDFSKLA RGAAVSSGFS
```

Y-site

Fig.14 (Cont 2).

```
      1690        1700        1710        1720        1730        1740
    *   *   *   *   *   *   *   *   *   *   *   *
GVIQLVSLRG HQLLTQEHVL RAVDVSPFAD HPCTQALGNP CLNGGSCVPR EATYECLCPG

      1750        1760        1770        1780        1790        1800
    *   *   *   *   *   *   *   *   *   *   *   *
GFSGLHCEKG LVEKSVGDLE TLAFDGRTYI EYLNAVIESE KALQSNHFEL SLRTEATQGL
                                          ▲Z-site
      1810        1820        1830        1840        1850        1860
    *   *   *   *   *   *   *   *   *   *   *   *
VLWIGKAAER ADYMALAIVD GHLQLSYDLG SQPVVLRSTV KVNTNRWLRI RAHREHREGS

      1870        1880        1890        1900        1910        1920
    *   *   *   *   *   *   *   *   *   *   *   *
LQVGNEAPVT GSSPLGATQL DTDGALWLGG LQKLPVGQAL PKAYGTGFVG CLRDVVVGHR

      1930        1940
    *   *   *   *
QLHLLEDAVT KPELRPCPTP *
```

Fig.15.

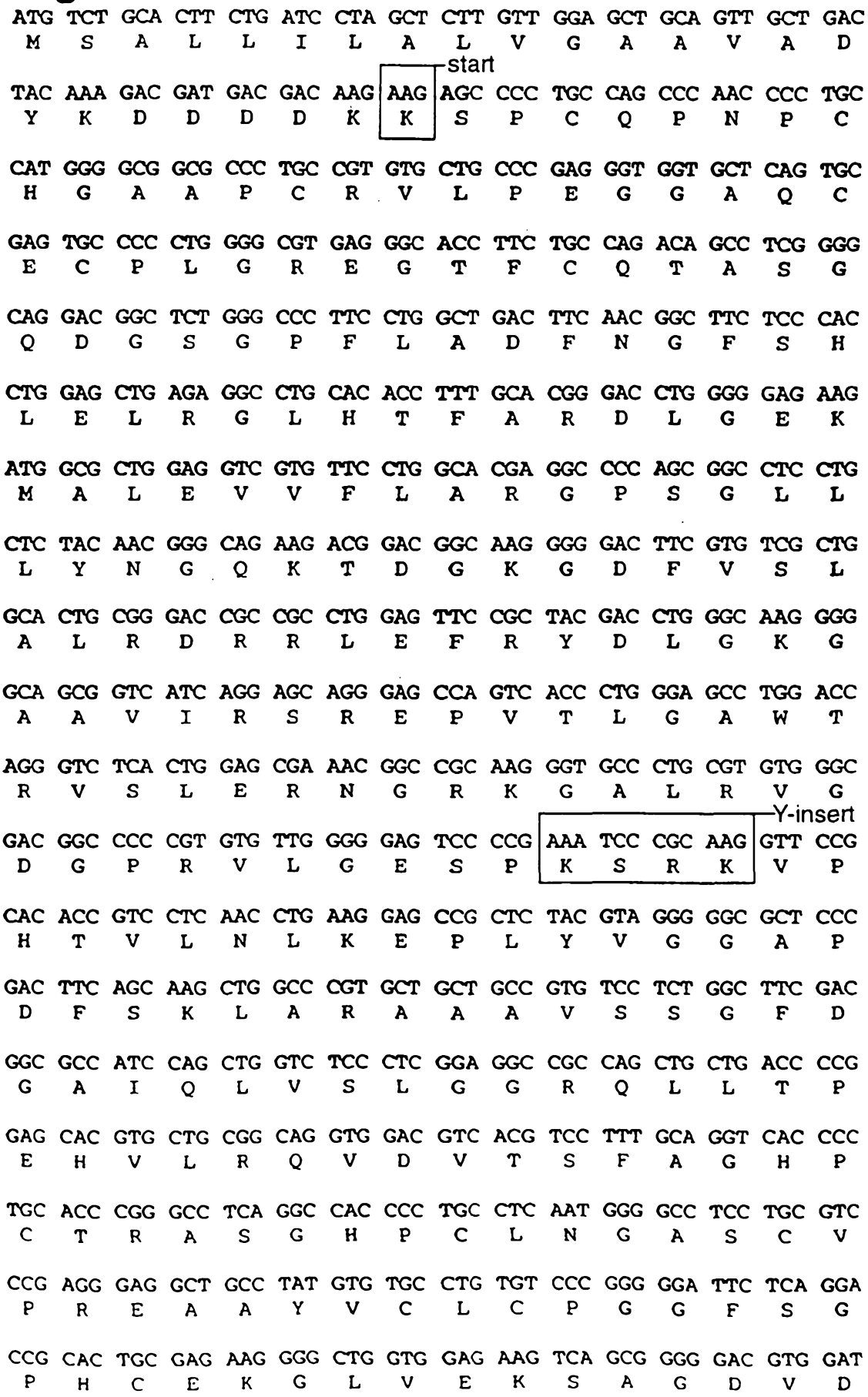
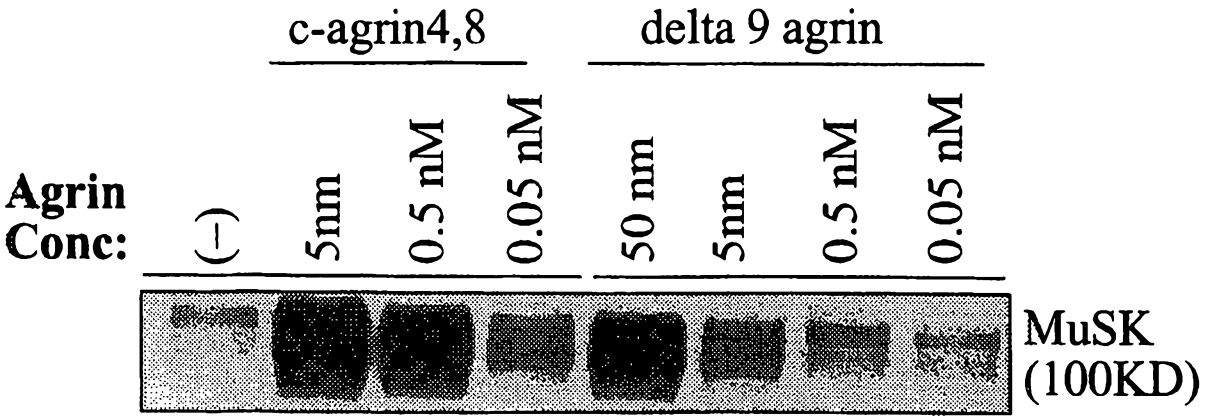


Fig.15 (Cont).

```
ACC TTG GCC TTT GAC GGG CGG ACC TTT GTC GAG TAC CTC AAC GCT GTG
 T   L   A   F   D   G   R   T   F   V   E   Y   L   N   A   V

                                                        Z-insert
ACC GAG AGC GAA CTG GCC AAT GAG ATC CCC GTC GAG AAG GCA CTG CAG
 T   E   S   E   L   A   N   E   I   P   V   E   K   A   L   Q

AGC AAC CAC TTT GAA CTG AGC CTG CGC ACT GAG GCC ACG CAG GGG CTG
 S   N   H   F   E   L   S   L   R   T   E   A   T   Q   G   L

GTG CTC TGG AGT GGC AAG GCC ACG GAG CGG GCA GAC TAT GTG GCA CTG
 V   L   W   S   G   K   A   T   E   R   A   D   Y   V   A   L

GCC ATT GTG GAC GGG CAC CTG CAA CTG AGC TAC AAC CTG GGC TCC CAG
 A   I   V   D   G   H   L   Q   L   S   Y   N   L   G   S   Q

CCC GTG GTG CTG CGT TCC ACC GTG CCC GTC AAC ACC AAC CGC TGG TTG
 P   V   V   L   R   S   T   V   P   V   N   T   N   R   W   L

CGG GTC GTG GCA CAT AGG GAG CAG AGG GAA GGT TCC CTG CAG GTG GGC
 R   V   V   A   H   R   E   Q   R   E   G   S   L   Q   V   G

AAT GAG GCC CCT GTG ACC GGC TCC TCC CCG CTG GGC GCC ACG CAG CTG
 N   E   A   P   V   T   G   S   S   P   L   G   A   T   Q   L

GAC ACT GAT GGA GCC CTG TGG CTT GGG GGC CTG CCG GAG CTG CCC GTG
 D   T   D   G   A   L   W   L   G   G   L   P   E   L   P   V

GGC CCA GCA CTG CCC AAG GCC TAC GGC ACA GGC TTT GTG GGC TGC TTG
 G   P   A   L   P   K   A   Y   G   T   G   F   V   G   C   L

CGG GAC GTG GTG GTG GGC CGG CAC CCG CTG CAC CTG CTG GAG GAC GCC
 R   D   V   V   V   G   R   H   P   L   H   L   L   E   D   A

GTC ACC AAG CCA GAG CTG CGG CCC TGC CCC ACC CCA TGA
 V   T   K   P   E   L   R   P   C   P   T   P   *
```

Fig.16.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delta 9 agrin
Agrin Conc:
(-)
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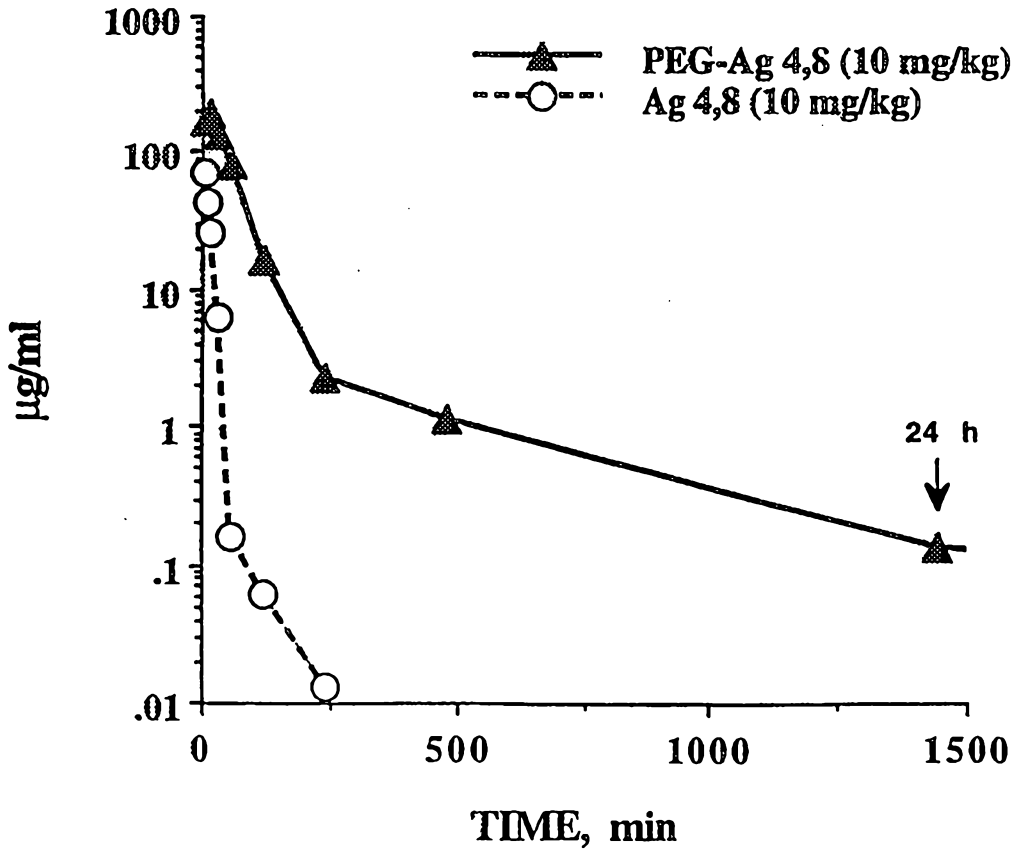
0.5 nM
0.05 nM
50 nm
5nm
0.5 nM
0.05 nM
MuSK (100KD)

Fig.17.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Ag 4,8 (10 mg/kg)
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100
10
1
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.01
µg/ml
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0
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1500
TIME, min