European Journal of Neuroscience, Vol. 11, pp. 373–382, 1999

Xenopus muscle-specific kinase: molecular cloning and prominent expression in neural tissues during early embryonic development

Amy K. Y. Fu, Frances D. Smith, Hong Zhou,[1] Apple H. Chu, Karl W. K. Tsim, Benjamin H. Peng[1] and Nancy Y. Ip
Department of Biology and Biotechnology Research Institute, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China
[1]Department of Cell Biology and Anatomy and the Curriculum in Neurobiology, University of North Carolina, Chapel Hill, NC, USA

Keywords: AChR, agrin, neuromuscular junctions, receptor tyrosine kinase, synaptogenesis

Abstract

A muscle-specific receptor tyrosine kinase, designated MuSK, mediates agrin-induced aggregation of acetylcholine receptors at the vertebrate neuromuscular junction. cDNAs encoding *Xenopus* MuSK were isolated from embryonic cDNA libraries. The full-length MuSK cDNA encodes for a polypeptide of 948 amino acids and possesses the features unique to mammalian MuSK, including four Ig-like domains, C6 box, transmembrane region and an intracellular tyrosine kinase domain. Interestingly, *Xenopus* MuSK also contains a kringle domain similar to that previously reported for *Torpedo* MuSK. The overall amino acid sequence identity of *Xenopus* MuSK with mammalian MuSK is ≈ 65%. Northern blot analysis demonstrated the presence of three MuSK transcripts (≈ 1 kb, ≈ 3 kb and ≈ 7 kb) which were differentially expressed during development. The expression of the ≈ 7 kb MuSK transcript remained as the predominant species in adult tissues, e.g. skeletal muscle, spleen and lung. Immunocytochemical analysis with a MuSK-specific antibody revealed that *Xenopus* MuSK was colocalized with AChRs at neuromuscular junctions as well as in spontaneous acetylcholine receptor hot spots of cultured muscle cells. *In situ* hybridization revealed prominent expression of MuSK transcripts in neural tissues and myotomal muscle during the period of neurulation and synaptogenesis. The MuSK transcript detected at abundant levels in the central nervous system (CNS) was localized to the brain, spinal cord and eye vesicles during early embryonic development. In addition, the MuSK protein in the developing eye was found to be prominently expressed during embryonic stages of 32 and 35. These findings raise an intriguing possibility that, in addition to the known function in the formation of the neuromuscular junctions, MuSK may be involved in neural development.

Introduction

Cell to cell communication in the nervous system occurs at the synapses. The most well-studied model of the synapse is the vertebrate neuromuscular junction (NMJ). The NMJ is an intricate structure comprised of the presynaptic motor nerve terminal, postsynaptic muscle membrane and synaptic cleft occupied by basal lamina (Hall & Sanes, 1993). The formation of the NMJ involves an inductive interaction between the presynaptic motor neurons and postsynaptic muscle fibres, resulting in specializations at both the pre- and postsynaptic sites. The presynaptic specialization involves the clustering of synaptic vesicles containing neurotransmitters at the active zones, while the specialization of postsynaptic muscle fibres is indicated by the clustering of a number of postsynaptic proteins, e.g. acetylcholinesterase, rapsyns, α-dystroglycans and acetylcholine receptors (AChRs; Bowe & Fallon, 1995).

Two nerve-derived molecules, ARIA (a member of the neuregulin family; Falls *et al*., 1993) and agrin (McMahan, 1990; Tsim *et al*., 1992) were identified to induce the postsynaptic specializations. Neuregulin increases the local transcription of AChRs at the subsynaptic regions on muscle (Jo *et al*., 1995), while agrin mediates the clustering of pre-existing AChRs and other postsynaptic proteins just beneath the nerve terminals on the muscle fibres. Agrin was originally purified from extracts of the synapse-rich electric organ of the marine ray *Torpedo californica*, based on its ability to induce the aggregation of AChRs on the surface of cultured myotubes (Wallace, 1986; Nitkin *et al*., 1987). The first proposed receptor candidate for agrin is α-dystroglycan, which is a component of the dystrophin–glycoprotein complex and binds agrin (Bowe *et al*., 1994; Campanelli *et al*., 1994; Gee *et al*., 1994; Sugiyama *et al*., 1994). However, the precise role of dystroglycan as a receptor for agrin remains unclear (Sealock & Froehner, 1994). Since tyrosine phosphorylation appears to be required for the agrin signalling cascade, it has been suggested that the actions of agrin at the NMJ may be mediated by a receptor tyrosine kinase (Wallace, 1992, 1995). Consistent with this proposal, a muscle-specific receptor tyrosine kinase, designated MuSK (Valenzuela *et al*., 1995) or Nsk2 (Ganju *et al*., 1995), has recently been suggested to be a required component of the receptor complex for agrin (Glass *et al*., 1996). MuSK is colocalized with AChRs at the synapse of adult rat skeletal muscle, and its expression, similar to AChR, is increased extrasynaptically after denervation (Valenzuela *et al*., 1995). In cultured muscle cells, agrin induces tyrosine phosphorylation of MuSK (Glass *et al*., 1996). Although MuSK does not bind directly to agrin, it is part of the agrin receptor complex. In addition, mutant

Correspondence: N. Y. Ip, as above. E-mail: BOIP@UST.HK

Received 17 March 1998, revised 22 August 1998, accepted 25 August 1998

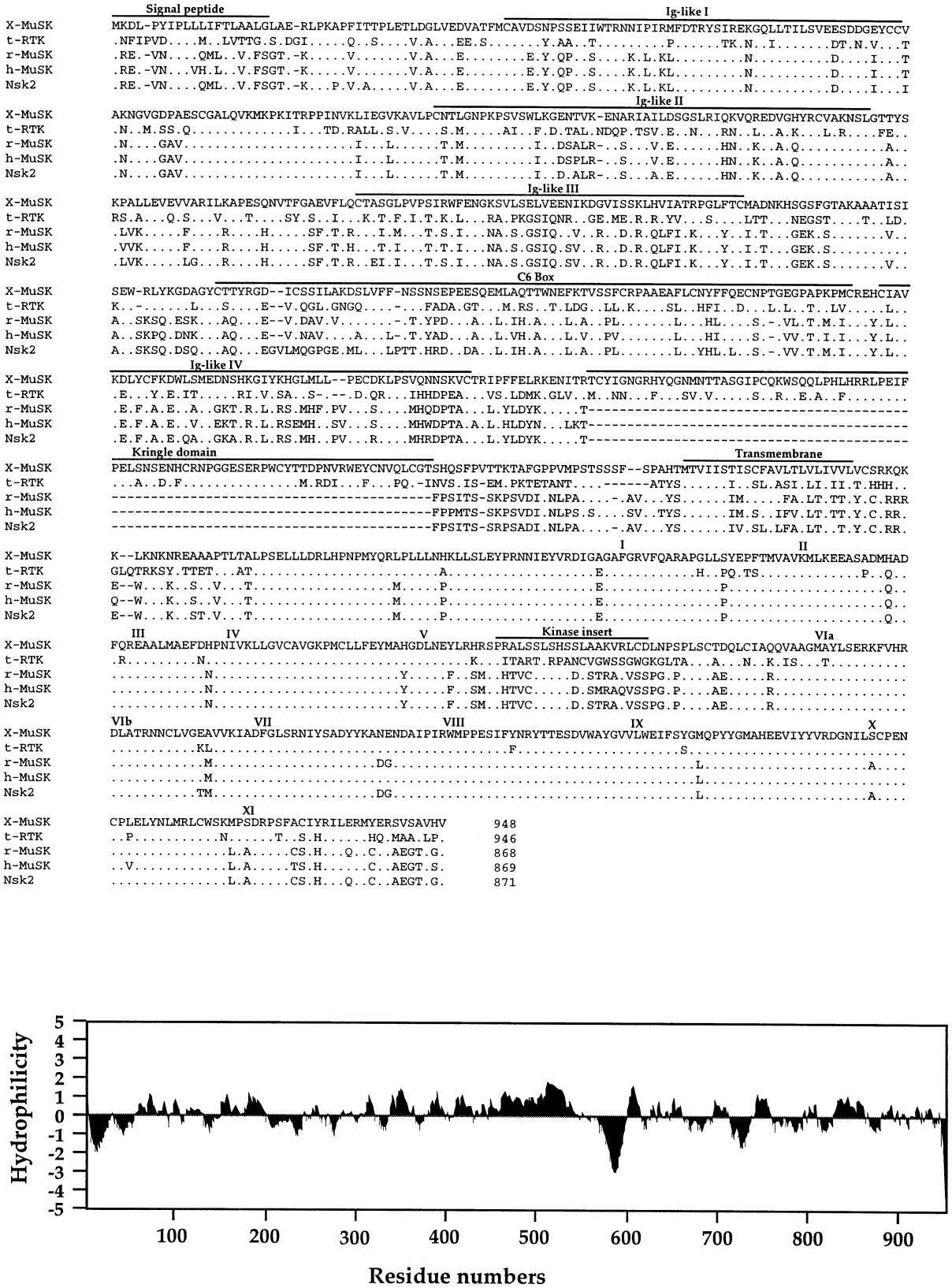

FIG. 1. Sequence of *Xenopus* MuSK. Deduced amino acid sequence of the *Xenopus* MuSK (upper panel). The MuSK full-length sequence encodes for 948 amino acids with a signal peptide, Ig-like domains (I–IV), C-6 box, transmembrane domain and tyrosine kinase domains (I–XI). Comparison of the amino acid sequence of *Xenopus* MuSK (x-MuSK) with *Torpedo* MuSK (t-RTK) and mammalian MuSK (rat, r-MuSK; human, h-MuSK and mouse, Nsk2). Amino acid differences from *Xenopus* MuSK are as indicated; the conserved residues are denoted by periods and the missing residues are indicated by hyphens. Lower panel: hydropathy plot of *Xenopus* MuSK amino acid sequence generated using the MacVector sequence analysis program, with a window size of 19.

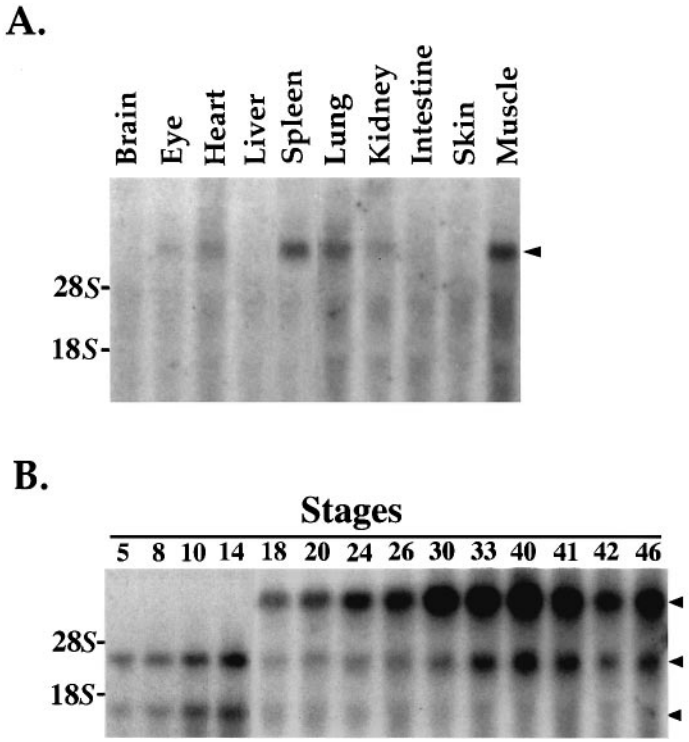

FIG. 2. Expression profile of *Xenopus* MuSK in adult tissues and embryos during development. Northern blot analysis of MuSK was performed with a cDNA fragment encoding the whole extracellular domain and part of the tyrosine kinase domain, as described in Materials and methods. The expression of *Xenopus* MuSK transcripts was examined in different adult tissues (A). The developmental profile of *Xenopus* MuSK expression was examined in embryos from stage 5 to stage 46 (B). Positions of MuSK transcripts are indicated by arrowheads on the right, and ribosomal bands are indicated on the left.

mice lacking MuSK do not exhibit the aggregation of AChRs at the NMJ (DeChiara *et al*., 1996), a phenotype similar to that observed in agrin knockout mice (Gautam *et al*., 1996).

Until now, the study of MuSK has been focused on the mammalian systems. The NMJ in the larvae of the amphibian *Xenopus* has offered a powerful experimental model for understanding the signalling process in the development of this synapse. The larval myotomal muscle cells can be conveniently cultured and innervated with high fidelity by spinal motoneurons (Cohen, 1980; Peng *et al*., 1991). The development of the NMJ can be readily compared with their *in vivo* counterpart in the larvae. It is thus of great interest to elucidate the MuSK signalling in the *Xenopus* system. In the present study, we cloned and characterized cDNAs encoding MuSK from embryonic *Xenopus* cDNA libraries. The expression of *Xenopus* MuSK, unlike its mammalian counterparts, was not confined to the skeletal muscle. Prominent MuSK mRNA expression was detected in the neural tissues during early embryonic development.

Materials and methods

Animals

Xenopus laevis frogs were purchased from Carolina Biological Supply Company (Burlington, NC) and *Xenopus* I (Ann Arbor, MI). Embryos were obtained by artificial fertilization, and the stages were identified and collected according to Nieuwkoop & Faber (1994).

Isolation of Xenopus *MuSK cDNA clones*

Xenopus cDNA libraries (stages 24 and 40) were screened using partial cDNA fragments encoding the extracellular domain and tyrosine kinase domain of rat MuSK (Ip *et al*., 1995, 1996). Individual clones were purified, and phage DNA was recovered and subcloned into pBluescript SK+. Sequence analysis was performed by the dideoxy chain termination method using T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden). The full-length cDNA encoding MuSK was amplified from the RNA of adult muscle by RT-PCR analysis, using oligonucleotide primers corresponding to the 5′ and 3′ end of *Xenopus* MuSK. The DNA sequences were aligned using MacVector software package. The *Xenopus* MuSK sequence has been submitted to the EMBL Nucleotide Sequence Database, with the accession number of AJ222795.

Primary cultures

Xenopus neuron and muscle cocultures were prepared according to a previously published method (Peng *et al*., 1991). Briefly, myotomes from stage 22 embryos were dissociated in Ca^{2+}- and Mg^{2+}-free solution and cultured on a cover glass at 22 °C. To study NMJs, neurons dissociated from neural tubes of stage 22 embryos were seeded into muscle cultures. NMJs, as judged by the development of AChR clusters along the nerve–muscle contact, could be observed 24 h after nerve–muscle coculture. Chick muscle cells were prepared from the limbs of E11 chick embryos. Suspended muscle cells (5×10^5) were plated onto collagen-coated 35 mm dishes, and maintained in Eagle's minimal essential medium (EMEM) containing 10% horse serum, 2% chick embryo extract, 100 units/mL penicillin and streptomycin (Wallace, 1986).

Transfection of chick muscle cultures

The cDNA encoding full-length *Xenopus* MuSK was subcloned into pCMV-Script expression vector (Stratagene, La Jolla, CA, USA), and was transiently transfected into chick muscle cells using calcium phosphate precipitation (Sambrook *et al*., 1989). Ten micrograms of cDNA was used for transfection. Transfection was carried out on 2-day-old cultured myoblasts and the efficiency was ≈ 10%. Mock-transfected chick myotubes were transfected with the expression vector only. Cytosine arabinoside (10^{-5} M) was added ≈ 24 h after the transfection.

Western blot analysis and tyrosine phosphorylation assay

A rabbit polyclonal antiserum raised against a peptide within the extracellular domain (S_{111} to V_{130}) of *Xenopus* MuSK, designated #35241, was generated (Research Genetics, Huntsville, AL, USA). Results obtained with antibody #35241 were confirmed with two commercially available anti-MuSK antibodies, C-19 and N-19, at 1:500 dilution (Santa Cruz Biotech., Santa Cruz, CA, USA). All three antibodies recognized a specific protein band (≈ 140 kDa, corresponding to MuSK) in 293 cells transfected with cDNA encoding *Xenopus* MuSK; this band was not detected in mock-transfected 293 cells. Specificity of the protein bands detected by the MuSK antibodies was confirmed by competition experiment with the appropriate peptides. Following preincubation of the MuSK antibody with a 10-fold (by weight) excess of the appropriate antigen peptide at room temperature for 2 h prior to the Western blot analysis, the protein bands corresponding to MuSK were not observed (data not shown). Tyrosine phosphorylation assay and Western blot analysis of MuSK were performed as previously described (Ip *et al*., 1992; Valenzuela *et al*., 1995). Briefly, chick myotubes were mock transfected or

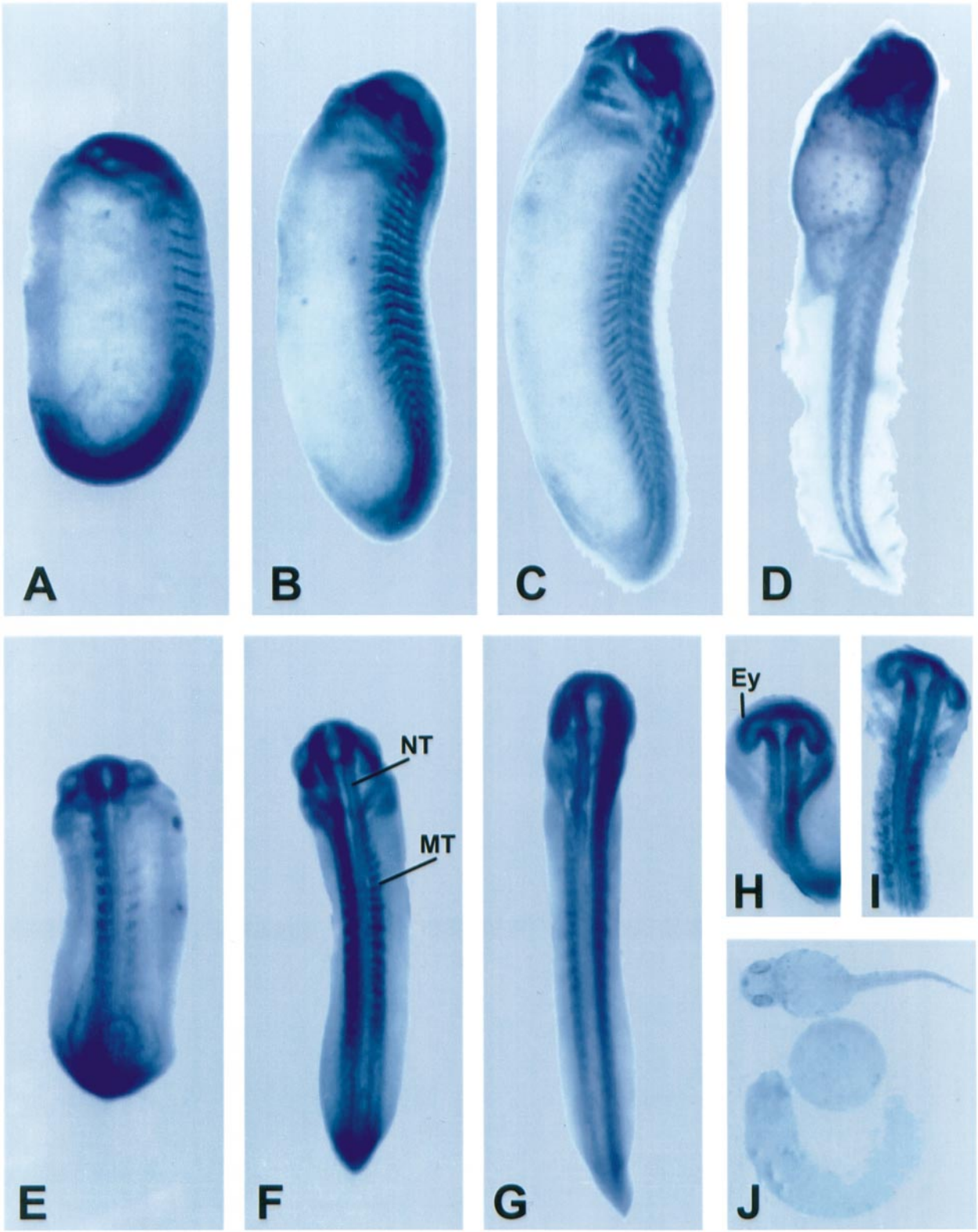

FIG. 3. Localization of MuSK mRNA by whole-mount *in situ* hybridization. (A–I) Anti-sense probe, (J) sense probe as control. (A–D) Lateral view: (A) stage 22, (B) stage 25, (C) stage 28, (D) stage 45. MuSK mRNA was localized in the myotomes as well as in the CNS in embryos during different stages of myotomal NMJ formation (A–C). After this period, mRNA expression is down-regulated in the myotomal musculature (D). (E–I) Dorsal view: (E) stage 22, (F) stage 28, (G) stage 40, (H, I) dorsal part of the embryo only. In this view, the expression of the MuSK mRNA in both neural tube (NT) and myotomes (MT) is clearly seen. Within the CNS, the eye vesicle is a prominent site of mRNA expression. No labelling was seen at all stages when the embryos were labelled with the sense probe (J).

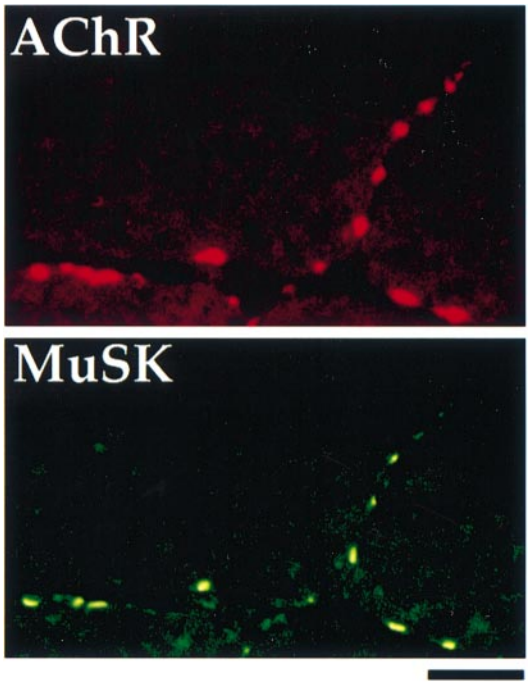

FIG. 4. Immunohistochemical analysis reveals the colocalization of MuSK and AChR in skeletal muscles of adult *Xenopus*. Frozen adult muscle sections were stained with anti-MuSK antibody (MuSK) or rhodamine-conjugated α-bungarotoxin (AChR). Scale bar, 10 μm.

transfected with *Xenopus* MuSK. The cells were treated with ≈ 1 unit chick agrin (CBA-1 as described in Tsim *et al*., 1992) for 5 min; conditioned media collected from 293 stable cell lines expressing an active form of chick agrin were used (Pun & Tsim, 1997). Total cell lysates were immunoprecipitated with anti-MuSK polyclonal antibody followed by immunoblotting with antiphosphotyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY, USA).

Immunohistochemical analysis

For whole-mount immunohistochemical analysis, *Xenopus* embryos (stage 35) were fixed in MEMFA (0.1 M MOPS at pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 2 h at 4 °C. The embryos were then permeabilized with Triton X-100 (0.4%). Staining was performed by incubating the embryos with an anti-MuSK antibody (C-19; 1:200 dilution; Santa Cruz Biotech.) at 4 °C overnight followed by FITC-conjugated rabbit antigoat secondary antibody (1:1000, Cappel, Belgium) for 1 h at room temperature. The embryos were washed and mounted. Projected fluorescent images from serial sections of embryos were analysed using a MRC-600 confocal microscope under two magnifications (10 × and 20 ×; BioRad, Hercules, CA, USA). Results were confirmed with MuSK antibody #35241. For immunohistochemical analysis of the neuromuscular junctions, double immunostaining was performed on gastrocnemious muscle sections or nerve–muscle cocultures using MuSK specific antibody #35241 (1:1000 dilution) and tetramethylrhodamine-conjugated α-bungarotoxin (10^{-7} M; Molecular Probe, Eugene, OR, USA). For the induction of AChR aggregation in chick muscle cultures, myotubes were treated with active agrin (1 unit of CBA-1; Tsim *et al*., 1992) for 12–16 h. Cells were then fixed with 2% paraformaldehyde/5% sucrose in PBS for 15 min at room temperature, washed and incubated with tetramethylrhodamine-conjugated α-bungarotoxin (10^{-7} M) in DMEM/10% FBS for 1 h at 37 °C. The cells were then washed and mounted for fluorescence microscopy. The AChR aggregates were

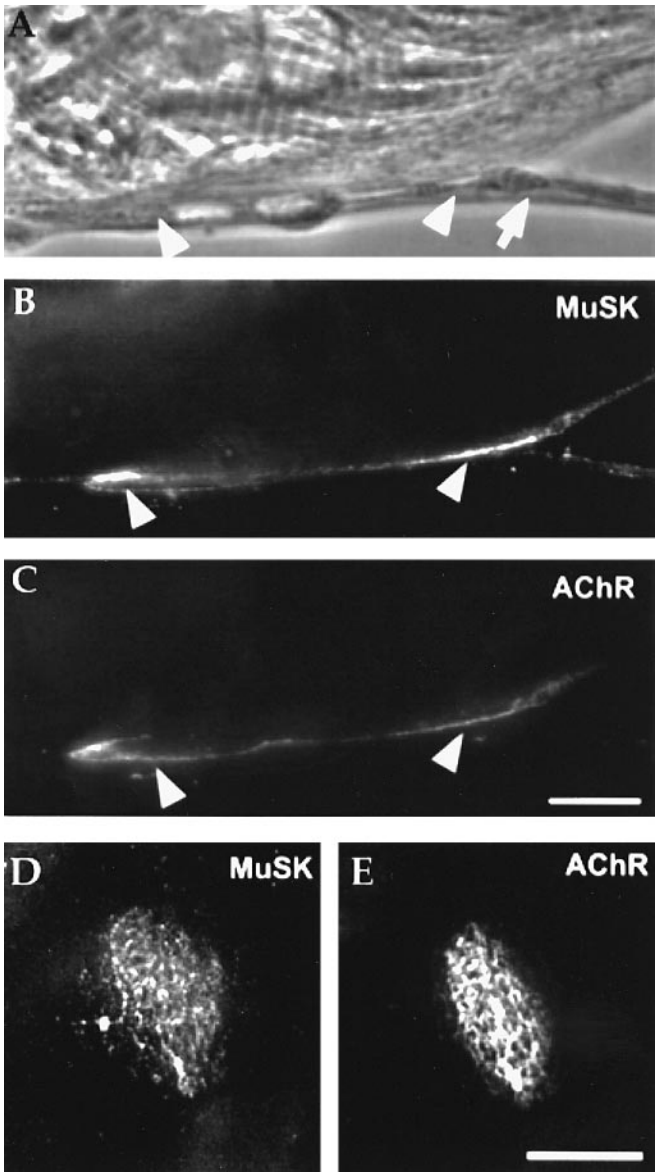

FIG. 5. Localization of MuSK at the NMJ and AChR hot spots. (A) Phase contrast of a neuron muscle coculture; (B) MuSK labelling with the anti-MuSK antibody; (C) rhodamine-conjugated α-bungarotoxin labelling of AChRs. Arrowheads indicate the neuron–muscle contacts as well as the AChR aggregates. MuSK was colocalized with AChR aggregates at the NMJ. The nerve process can be seen in phase contrast as indicated by the arrow in (A). (A–C) represent the same view but with different optics. (D) and (E) shows the colocalization of MuSK and AChR hot spots in uninnervated muscle cells. (D) MuSK labelling; (E) rhodamine-conjugated α-bungarotoxin labelling. (D) and (E) represent the same view. Scale bar, 10 μm.

counted as previously described (Wallace, 1986) under a 40 × objective on a microscope equipped with phase and fluorescence optics. The number of AChR aggregates per myotube was determined by counting 20 myotubes in each culture dish.

Northern blot and RT-PCR analysis

Total RNAs were extracted from adult tissues and embryos by lithium chloride precipitation, as described previously (Ip *et al*., 1995). Twenty micrograms of total RNA was electrophoresed on a 1% agarose–formaldehyde gel. Northern blot analysis was performed using a ≈ 2.3 kb cDNA fragment comprising the extracellular domain

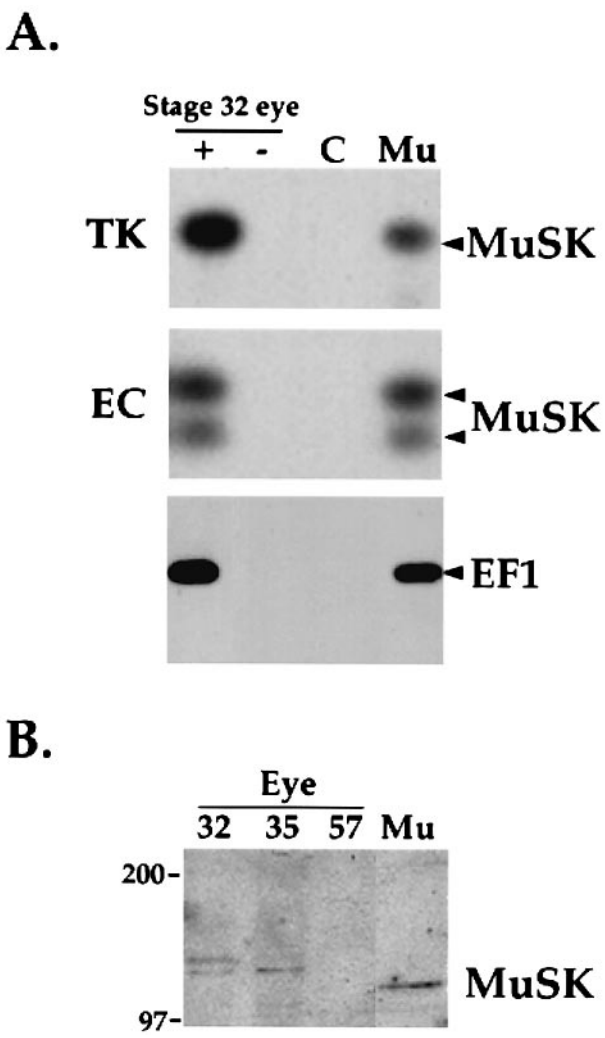

FIG. 6. Expression of MuSK in developing *Xenopus* eyes. (A) Reverse transcription was used to prepare the first strand of cDNA from total RNAs. The cDNAs were amplified by PCR with two sets of primers flanking different regions of *Xenopus* MuSK (TK, tyrosine kinase domain; EC, extracellular domain). Southern blot analysis was performed using the appropriate MuSK cDNA probe. PCR fragments obtained with primers specific for EF1 were used as control for equal loading. + , cDNAs from stage 32 eyes; –, RNAs from stage 32 eyes (without reverse transcription); C, water control; and Mu, adult muscle cDNA. (B) Membrane fractions from eyes (stage 32, 35 or 57) or adult muscle (Mu) were obtained, and proteins (≈ 2 μg) were fractionated by 6% SDS–PAGE. Electrophoresed proteins were transferred onto nitrocellulose membrane, and detected by anti-MuSK antibody (C-19) and peroxidase-conjugated secondary antibody. Positions of molecular markers (97 kDa and 200 kDa) are as indicated.

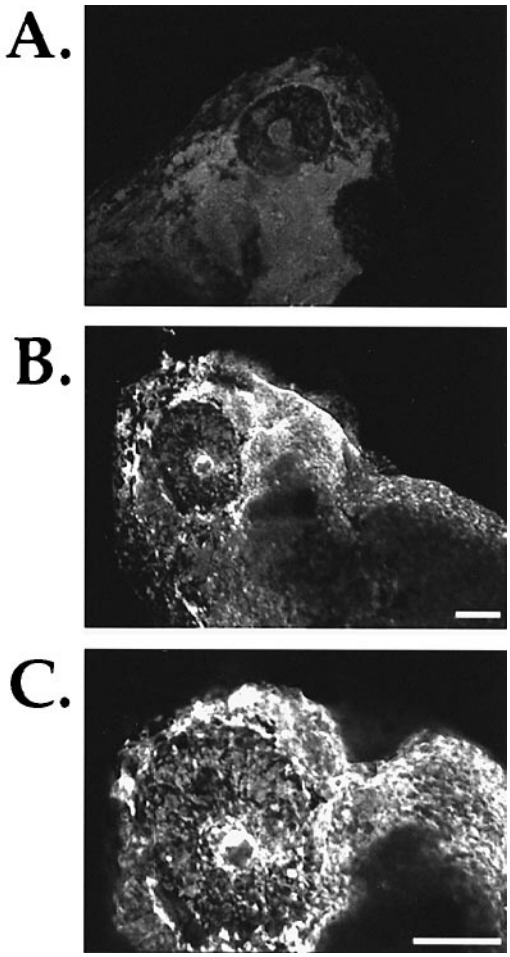

FIG. 7. Immunohistochemical analysis reveals the expression of MuSK protein in the developing eye of stage 35 *Xenopus* embryos. Whole-mount embryos were stained with anti-MuSK antibody (C-19). (A) Control serum, (B) C-19 followed by secondary antibody, (C) higher magnification of (B). Scale bar, 100 μm.

and part of the tyrosine kinase domain of *Xenopus* MuSK (amino acid positions #1–731).

For RT-PCR analysis, 1 μg of total RNAs was reverse transcribed by SuperScript II RNaseH$^-$ reverse transcriptase (GIBCO-BRL, Grand Island, NY, USA) using oligo-dT priming in a 20 μL reaction. One-tenth of the reverse transcription product was used as a template in PCR analysis with primers described below. PCR was carried out for 35 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min in a 25 μL volume containing 0.8 mM dNTPs, 1 × PCR buffer and 0.625 U of *Taq* Polymerase (GIBCO-BRL). PCR products were analysed in a 1% agarose gel and were hybridized with radioactive MuSK cDNA fragments in Southern blot analysis. The PCR primers were designed according to *Xenopus* MuSK cDNA sequence. Two sets of primers were used for the analysis: one set flanking the tyrosine kinase domains (from G_{664} to N_{856}) and the other flanking the extracellular domain (from K_{154} to I_{243}). An equal amount of cDNAs used for the analysis was confirmed using primers specific for elongation factor I (EF1).

Whole-mount in situ *hybridization*

Albino *Xenopus* embryos were collected at different stages, fixed in MEMFA for 2 h, then stored in absolute methanol at –20 °C. The procedure for whole-mount *in situ* hybridization was as previously described (Harland, 1991; Zhou *et al*., 1997).

Results

Cloning and sequence analysis of Xenopus *MuSK*

Xenopus MuSK was identified by screening *Xenopus* cDNA libraries with cDNA fragments encoding the extracellular domain (EC) and tyrosine kinase (TK) domain of rat MuSK. Two overlapping clones, designated as MuSK-1 and MuSK-2, were isolated. The sizes of the cDNA inserts were ≈ 2.3 kb and ≈ 2.0 kb, respectively. Based on the sequence of the isolated cDNAs, two specific oligonucleotide primers were used for RT-PCR analysis. The full-length cDNA encoding *Xenopus* MuSK was amplified from total RNA of adult

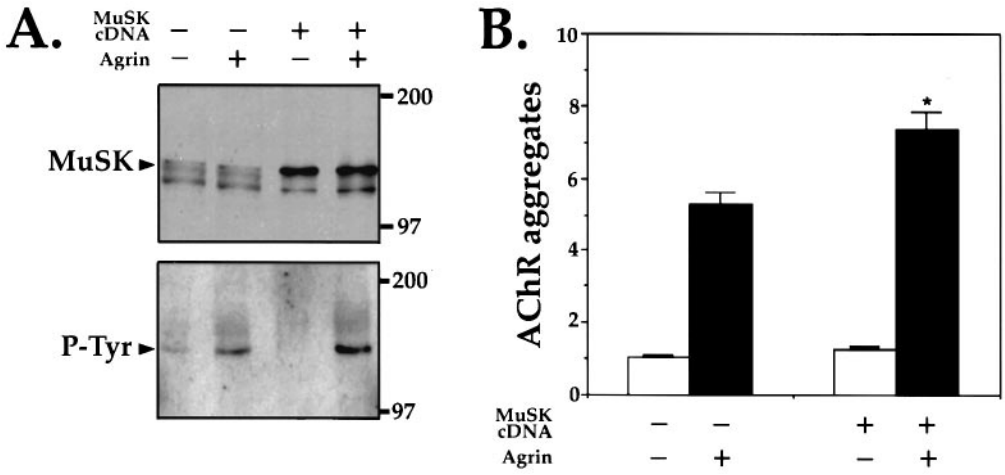

FIG. 8. Functional expression of *Xenopus* MuSK in chick muscle cells. (A) Western blot analysis of crude cell lysates from mock-transfected chick muscle cells or cells over-expressing *Xenopus* MuSK using the MuSK-specific antibody (upper panel). Induction of *Xenopus* MuSK tyrosine phosphorylation by agrin in chick myotubes overexpressing *Xenopus* MuSK (lower panel). Molecular weight markers are in kDa. (B) Overexpression of *Xenopus* MuSK enhanced the aggregation of AChRs in primary chick muscle culture. Chick myotubes were mock transfected or transfected with *Xenopus* MuSK construct, and then treated with active agrin for 12 h. Aggregation of AChRs was assayed by staining with rhodamine-conjugated α-bungarotoxin and counted as described in Materials and methods. Data are presented as the number of AChR aggregates per myotube, mean ± SEM ($n = 5$). The asterisk indicates that the number of agrin-induced AChR aggregates differed significantly from mock-transfected cultures ($P < 0.05$, unpaired *t*-test).

Xenopus muscle. The full-length *Xenopus* MuSK has an open reading frame of 2844 bp that encodes a polypeptide of 948 amino acids with a predicted molecular weight (M_r) of 106.2 kDa (Fig. 1, upper panel). Hydropathy analysis of the deduced protein revealed a signal peptide of 19 amino acids and a putative hydrophobic transmembrane region between M_{573} and L_{594} (Fig. 1, lower panel). Sequence comparison with other species indicated that the isolated cDNA encodes a protein corresponding to MuSK. The ectodomain can be subdivided into four immunoglobulin-like (Ig-like) domains, and a spacer domain containing six conserved cysteines (C6 box) lies between the third and fourth Ig-like domains. Interestingly, *Xenopus* MuSK, as the *Torpedo* MuSK, contains a kringle domain just after the fourth Ig-like domain which was not found in all known mammalian MuSK. It should be noted that the presence of kringle domain in *Xenopus* MuSK with glycosylation sites may account for the larger size of *Xenopus* MuSK protein (≈ 140 kDa; see below) compared to rat MuSK (≈ 100 kDa). The cytoplasmic domain of *Xenopus* MuSK is comprised of a juxtamembrane domain, a TK domain containing a kinase insert and a carboxy terminal domain. The TK domain contains all 12 motifs (I–XI) that are conserved across functional receptor tyrosine catalytic domains (Hanks & Quinn, 1991).

Comparison of the *Xenopus* MuSK sequence with *Torpedo* MuSK and mammalian MuSK reveals that it shares a high degree of conservation to its counterparts. The overall amino acid sequence of *Xenopus* MuSK is 65% identical to that of human and rat MuSK, 66% to *Torpedo* MuSK and 64% to Nsk2 (Fig. 1, upper panel). The amino acid identity observed for the kringle domain of *Xenopus* and *Torpedo* MuSK is ≈ 70%. The highest degree of identity among all species is in the intracellular kinase domain of MuSK, ranging from 85% with mammalian counterparts to 79% with *Torpedo* MuSK. The level of conservation observed for the extracellular domains of MuSK among different species is not particularly high, but appears to be more homologous for the mammalian counterparts than the *Torpedo* ortholog. It is possible that the functional pocket for mediating agrin-induced AChR aggregation is constituted by the tertiary structure of the Ig-like domains.

Expression profile of Xenopus *MuSK mRNA during embryonic development*

In order to examine the spatial expression pattern of *Xenopus* MuSK, Northern blot analysis with different adult tissues was performed. Similar to the mammalian MuSK, a ≈ 7 kb transcript was detected in skeletal muscle (Fig. 2A). However, the restrictive expression pattern of MuSK to the skeletal muscle observed in mammalian species was not found in *Xenopus*. The ≈ 7 kb transcript was also prominently expressed in RNA isolated from spleen. In addition, MuSK mRNA expression was detected in lung, kidney, heart and eye, albeit at a low level. The MuSK transcript was not detectable in stomach, intestine, liver, skin and brain of adult frog.

Analysis of the expression profile of MuSK revealed multiple transcripts of *Xenopus* MuSK (≈ 1 kb, ≈ 3 kb and ≈ 7 kb) in early embryonic development. The ≈ 7 kb MuSK transcript detected in adult muscle was already evident after the completion of neurulation (stage 18; Fig. 2B). The level of MuSK mRNA expression increased during maturation of the embryos from the swimming tadpole stage (stage 30) to stage 40. At the earlier stages (stages 5–14), two smaller MuSK transcripts (≈ 1 kb and ≈ 3 kb), but not the ≈ 7 kb transcript, were detected. The peak of expression of these two transcripts was at stage 14. The presence of the two smaller MuSK transcripts in stage 5 (cleavage stage) implies that they are potentially maternally derived, as zygotic genes are not transcribed until the mid-blastula stage. However, the functional roles of these multiple transcripts in early embryonic development remain to be elucidated.

In situ hybridization of Xenopus *MuSK*

To examine in more detail the spatial expression pattern of *Xenopus* MuSK during embryogenesis, whole-mount *in situ* hybridization for stage 22–45 embryos was performed (Fig. 3). Similar expression profiles of *Xenopus* MuSK were obtained using different probes specific for the EC and TK domains (data not shown). An abundant level of MuSK expression was observed in the embryos from stage 22 to 28 (Fig. 3A–C,E,F). The mRNA expression of MuSK was prominently detected in myotomes as well as in the CNS including

the neural tube. The MuSK expression coincides with the time of postsynaptic differentiation in *Xenopus*, as the innervation of the myotomal muscles starts approximately from stage 20 to stage 30. After the period of NMJ formation, mRNA expression of MuSK was down-regulated in the myotomal musculature (Fig. 3D). Within the CNS, the eye vesicle was a prominent site of MuSK expression (Fig. 3H,I). No expression was detected at any stage when the embryos were labelled with the sense probe of MuSK (Fig. 3J).

Xenopus *MuSK is colocalized with AChRs at the NMJ*

The subcellular localization of MuSK and AChR in skeletal muscle was determined in *Xenopus* muscle and neuron–muscle cocultures. Double-labelling studies with MuSK-specific antibody and rhodamine-conjugated α-bungarotoxin revealed that MuSK was precisely colocalized with AChR at the mature NMJ (Fig. 4). This synaptic expression of MuSK is similar to that observed in other species (Glass *et al*., 1996). In *Xenopus* spinal neuron–muscle cocultures, the AChR aggregates were associated with the innervating neurites (Fig. 5). Moreover, the neuron-induced AChR aggregates were colocalized with MuSK, as revealed by immunocytochemical staining (Fig. 5A–C). This finding suggests that innervation induces MuSK and AChR to cluster at the nerve–muscle contacts. The colocalization of MuSK and AChRs was not only detected at the neuron-induced AChR aggregates, but also in spontaneous hot spots in cultured muscle cells (Fig. 5D,E). The localization of MuSK was more diffuse when compared to the AChR aggregates, i.e. MuSK clusters are consistently larger in area than AChR clusters.

Expression of MuSK in the eyes of developing Xenopus *embryos*

As described above, whole-mount *in situ* hybridization revealed transient mRNA expression of MuSK in tissues other than skeletal muscle during *Xenopus* development. The prominent expression of MuSK transcript in developing eye vesicles prompted us to further analyse the protein expression of MuSK in the eyes of developing embryos. RT-PCR followed by Southern blot analysis revealed the presence of MuSK mRNA in developing *Xenopus* eye (stage 32; Fig. 6A). Results were confirmed using different sets of primers specific for MuSK (Fig. 6A, top and middle panels). The expression of MuSK protein (≈ 150 kDa) was also detected from membrane extract of embryonic eyes prepared from stages 32 and 35 (Fig. 6B). The higher molecular weight of MuSK protein observed in the eyes could be attributed to the difference in protein glycosylation. The level of MuSK protein expression in the eyes decreased during the later stages of development and was below a detectable level at stage 57 (Fig. 6B). Immunohistochemical analysis using MuSK-specific antibody also revealed the presence of MuSK in the developing eyes (stage 35; Fig. 7). Both retina and optic stalk showed prominent MuSK staining.

Overexpression of Xenopus *MuSK in chick myotubes enhances AChR clustering*

To examine whether the cloned *Xenopus* MuSK was effective in enhancing the clustering of AChRs, chick myotubes were transfected with the expression construct encoding full-length *Xenopus* MuSK. Western blot analysis using anti-MuSK antibody revealed a protein of ≈ 140 kDa in the transfected crude cell lysates (Fig. 8A, upper panel). The size of *Xenopus* MuSK (≈ 140 kDa) is similar to that of the chick MuSK, but different from the rat MuSK (Glass *et al*., 1996); the level of expression was increased by the MuSK cDNA transfection (Fig. 8A, upper panel). Addition of agrin to mock-transfected chick myotubes induced the tyrosine phosphorylation of the endogenous chick MuSK (Fig. 8A, lower panel). Chick myotubes transiently transfected with *Xenopus* MuSK cDNA exhibited an increase (≈ twofold) in agrin-induced tyrosine phosphorylation of MuSK, when compared to mock-transfected myotubes (Fig. 8A, lower panel). Overexpression of *Xenopus* MuSK in primary chick myotubes also resulted in ≈ 40% increase in the number of AChR aggregates formed upon agrin application (Fig. 8B). Overexpression of a truncated form of *Xenopus* MuSK lacking the tyrosine kinase domain did not result in an increase in agrin-induced phosphorylation of MuSK (data not shown).

Discussion

In the present study, we have isolated the ortholog of mammalian MuSK from *Xenopus* adult muscle. Similar to its mammalian counterparts with which it shares ≈ 65% identity, *Xenopus* MuSK contains an EC domain with Ig-like regions, a hydrophobic transmembrane domain and an intracellular domain with tyrosine kinase motifs. Analysis of amino acid sequence alignment reveals a high degree of conservation in the tyrosine kinase domains of MuSK among different species (≈ 85% identity with mammalian MuSKs and 79% with *Torpedo* MuSK). Such conservation observed in these evolutionary divergent species suggests that the function of MuSK is conserved across a long evolutionary distance. The full-length *Xenopus* MuSK cloned in the present study was found to be functional, as demonstrated by its ability to enhance the clustering of AChRs in chick myotubes.

Our studies on the expression pattern of MuSK in adult tissues reveal that the expression of *Xenopus* MuSK is not specific to the skeletal muscle lineage. *Xenopus* MuSK expression in muscle can be detected during somitic development and continues throughout the later stages of embryonic muscle formation, similar to that observed in mammalian MuSK. Interestingly, an abundant level of MuSK expression was observed in adult spleen, while a low level of MuSK expression was also detected in other adult tissues. The localization of *Xenopus* MuSK in organs, e.g. eyes and spleen, reported in the present study, is consistent with the possibility that MuSK may be involved in the development of other lineages (Ganju *et al*., 1995; Valenzuela *et al*., 1995). The precise functions of *Xenopus* MuSK present in these tissues, however, remain to be elucidated.

MuSK expression in *Xenopus* was developmentally regulated and detected during neurulation and synaptogenesis until the adult stage. However, different transcripts were expressed during the early stages of development, as shown by Northern blot analysis. The switch in the transcript size of MuSK between stages 14 and 18 is particularly interesting, as it represents the transition stage between neurulation and muscle development. Some of the MuSK transcripts expressed in embryos are different from the transcript expressed in the adult tissues. Recent studies from our laboratory demonstrated that, using probes derived from different regions of MuSK, some of the smaller *Xenopus* MuSK transcripts observed represented forms of MuSK that had partial deletions in the 5′ region (F. D. Smith and N. Y. Ip, unpublished observations). Similarly, multiple transcripts of MuSK were also observed in rat, chick (Ip *et al*., 1996), mouse and *Torpedo* (Jennings *et al*., 1993; Ganju *et al*., 1995) during embryonic development. These multiple transcripts for MuSK suggest that there are differentially spliced forms of MuSK (Valenzuela *et al*., 1995) and they may potentially have different signalling capabilities or ligand specificities.

The subcellular localization of *Xenopus* MuSK at the NMJ of adult muscle was found to be precisely colocalized with AChR, as reported in other species (Valenzuela *et al*., 1995). We have examined the

cellular localization of AChR and MuSK in *Xenopus* neuron–muscle cocultures, and found that MuSK colocalized with the AChR aggregates at the developing NMJ *in vitro*. MuSK was also found to be colocalized with AChR hot spots present in the aneural muscle cultures. Although MuSK and AChR are coclustered at the NMJ and at the hot spots *in vitro*, their distribution is not congruent. It has recently been reported that the synaptic colocalization of MuSK and AChRs is established well before birth, and MuSK is present at the earliest embryonic AChR clusters (Apel *et al*., 1997). In fact, MuSK is clustered at the nerve–muscle contact in the absence of AChR clustering in rapsyn-deficient myotubes (Apel *et al*., 1997). Taken together, these results are consistent with the notion that MuSK activation resulting from nerve–muscle contact triggers the formation of a postsynaptic protein scaffold upon which AChR as well as MuSK itself becomes clustered.

The expression of *Xenopus* MuSK in neural tissues during early embryonic development suggests that this receptor may mediate functions other than that at the NMJ. Our study provides the first demonstration of prominent expression of MuSK in the CNS, particularly in the developing eye, during early stages of development (stages 32–35). The expression of MuSK protein was below a detectable level in stage 57 eyes. Such transient expression of *Xenopus* MuSK in the eye during early development might explain why such an expression profile was not reported for the mammalian counterparts; alternatively, other MuSK-related RTKs might exist in the mammalian CNS. Consistent with the latter possibility is the identification of RTKs that have homology to MuSK, with an expression profile specific for the nervous system in *Drosophila* (Wilson *et al*., 1993; Oishi *et al*., 1997). Detection of prominent MuSK expression in neural tube only during early embryonic development raises the intriguing possibility that this receptor may be involved in processes, e.g. neurogenesis and/or synapse formation, in addition to its well-known role at the NMJ. Interestingly, prominent expression of agrin in developing rat and chick brains (Thomas *et al*., 1993; Ma *et al*., 1994; Kröger & Mann, 1996) has been reported, while a neuronal rapsyn-like molecule was recently isolated from the ciliary ganglion and brain in the chick (Burns *et al*., 1997). It is possible that the dynamic interaction of the synaptic molecules present in the NMJ may be similar in the CNS. Further studies are required to relate the functional roles of agrin, rapsyn and clustering of postsynaptic receptors, e.g. AChRs, in the CNS. Thus, efforts in identifying a brain-specific receptor tyrosine kinase homologous to MuSK are important to help resolve the roles of agrin in the formation and maintenance of synapses in the CNS.

Acknowledgements

We are grateful to Drs Ping Xie and Fanny Ip for their help with tissue preparation and whole-mount immunohistochemical analysis. We also thank Yin King Lee, Janet Cheung and Hiu Yin Choi for their expert technical assistance. This study was supported by grants from the Research Grants Council of Hong Kong (HKUST 524/94 and 568/95 to N.Y.I. and K.W.K.T.) and the Biotechnology Research Institute, and by US NIH grant NS23583 (to H.B.P.).

Abbreviations

AChR, acetylcholine receptor; CNS, central nervous system; EMEM, Eagle's minimum essential medium; NMJ, neuromuscular junction.

References

Apel, E.D., Glass, D.J., Moscoso, L.M., Yancopoulos, G.D. & Sanes, J.R. (1997) Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. *Neuron*, **18**, 623–635.

Bowe, M.A., Deyst, K.A., Leszyk, J.D. & Fallon, J.R. (1994) Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: a heteromeric complex related to the dystroglycans. *Neuron*, **12**, 1173–1180.

Bowe, M.A. & Fallon, J.R. (1995) The role of agrin in synapse formation. *Annu. Rev. Neurosci.*, **18**, 443–462.

Burns, A.L., Benson, D., Howard, M.J. & Margiotta, J.F. (1997) Chick ciliary ganglion neurons contain transcripts coding for acetylcholine receptor-associated protein at synapses (Rapsyn). *J. Neuroscience*, **17**, 5016–5026.

Campanelli, J.T., Roberds, S.L., Campbell, K.P. & Scheller, R.H. (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell*, **77**, 663–674.

Cohen, M.W. (1980) Development of an amphibian neuromuscular junction in vivo and in culture. *J. Exp. Biol.*, **89**, 43–56.

DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., Smith, C., DiStefano, P.S., Glass, D.J., Burden, S.J. & Yancopoulos, G.D. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell*, **85**, 501–512.

Falls, D.L., Rosen, K.M., Confras, G., Lane, W.S. & Fischbach, G.D. (1993) ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell*, **72**, 801–815.

Ganju, P., Walls, E., Brennan, J. & Reith, A.D. (1995) Cloning and developmental expression of Nsk2, a novel receptor tyrosine kinase implicated in skeletal myogenesis. *Oncogene*, **11**, 281–290.

Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P. & Sanes, J.R. (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell*, **85**, 525–535.

Gee, S.H., Montanaro, F., Lindenbaum, M.H. & Carbonetto, S. (1994) Dystroglycan-α, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell*, **77**, 675–686.

Glass, D.J., Bowen, D.C., Stitt, T.N., Radziejewski, C., Bruno, J., Ryan, T.E., Gies, D.R., Shah, S., Mattsson, K., Burden, S.J., DiStefano, P.S., Valenzuela, D.M., DeChiara, T.M. & Yancopoulos, G.D. (1996) Agrin acts via a MuSK receptor complex. *Cell*, **85**, 513–523.

Hall, Z.W. & Sanes, J.R. (1993) Synaptic structure and development: The neuromuscular junction. *Cell*, **72**, 99–121.

Hanks, S.K. & Quinn, A.M. (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Meth. Enz.*, **200**, 38–62.

Harland, R.M. (1991) In situ hybridization: an improved whole-mount method for Xenopus embryos. In Kay, B.K. & Peng, H.B. (eds), *Xenopus laevis: Practical Uses in Cell and Molecular Biology.* Academic Press, San Diego, pp. 685–695.

Ip, F.C.F., Fu, A.K.Y., Tsim, K.W.K., Cheung, J., Valenzuela, D.M., Glass, D.J., Yancopoulos, G.D. & Ip, N.Y. (1996) Developmental and upregulation of muscle specific kinase (MuSK) in denervated skeletal muscle of the chick and rat. *Soc. Neurosci. Abstracts*, **22**, 219.3.

Ip, F.C.F., Fu, A.K.Y., Tsim, K.W.K. & Ip, N.Y. (1995) Cloning of the α component of the chick ciliary neurotrophic factor receptor: developmental expression and downregulation in denervated skeletal muscle. *J. Neurochem.*, **65**, 2393–2400.

Ip, N.Y., Nye, S.H., Boulton, T.G., Davis, S., Taga, T., Li, Y., Birren, S.J., Yasukawa, K., Kishimoto, T., Anderson, D.J., Stahl, N. & Yancopoulos, G.D. (1992) CNTF and LIF act on neuronal cells via shared signalling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell*, **69**, 1121–1132.

Jennings, C.G., Dyer, S.M. & Burden, S.J. (1993) Muscle-specific Trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. *Proc. Natl Acad. Sci. USA*, **90**, 2895–2899.

Jo, S.A., Zhu, X., Marchionni, M.A. & Burden, S.J. (1995) Neuregulins are concentrated in nerve-muscle synapses and activate ACh-receptor gene expression. *Nature*, **373**, 158–161.

Kröger, S. & Mann, S. (1996) Biochemical and functional characterization of basal lamina-bound agrin in the chick central nervous system. *Eur. J. Neurosci.*, **8**, 500–509.

Ma, E., Morgan, R. & Godfrey, E.W. (1994) Distribution of agrin messenger RNA in the chick embryo nervous system. *J. Neuroscience*, **14**, 2943–2952.

McMahan, U.J. (1990) The agrin hypothesis. *Cold Spring Harbor Symp. Quant. Biol.*, **55**, 407–418.

Nieuwkoop, P.D. & Faber, J. (1994) *Normal Table of* Xenopus laevis *(Daudin)*. Garland Publishing, New York.

Nitkin, R.M., Smith, M.A., Magill, C., Fallon, J.R., Yao, Y.-M.M., Wallace, B.G. & McMahan, U.J. (1987) Identification of agrin, a synaptic organizing protein from Torpedo electric organ. *J. Cell Biol.*, **105**, 2471–2478.

Oishi, I., Sugiyama, S., Liu, Z.-J., Yamamura, H., Nishida, Y. & Minami, Y. (1997) A novel Drosophila receptor tyrosine kinase expressed specifically in the nervous system. *J. Biol. Chem.*, **272**, 11 916–11 923.

Peng, H.B., Baker, L.P. & Chen, Q. (1991) Tissue culture of Xenopus neurons and muscle cells as a model for studying synaptic induction. In Kay, B.K. & Peng, H.B. (eds), Xenopus laevis*: Practical Uses in Cell and Molecular Biology. Methods in Cell Biology*, Vol. 36. Academic Press, San Diego, pp. 511–526.

Pun, S. & Tsim, K.W.K. (1997) Antisense agrin cDNA transfection blocks neuroblastoma cell-induced acetylcholine receptor aggregation when co-cultured with myotubes. *Mol. Cell. Neurosci.*, **10**, 87–99.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sealock, R. & Froehner, S.C. (1994) Dystrophin-associated proteins and synapse formation: is α-dytroglycan the agrin receptor? *Cell*, **77**, 617–619.

Sugiyama, J., Bowen, D.C. & Hall, Z.W. (1994) Dystroglycan binds nerve and muscle agrin. *Neuron*, **13**, 103–115.

Thomas, W.S., O'Dowd, D.K. & Smith, M.A. (1993) Developmental expression and alternative splicing of chick agrin RNA. *Dev. Biol.*, **158**, 523–535.

Tsim, K.W.K., Ruegg, M.A., Escher, E., Kroger, S. & McMahan, U.J. (1992) cDNA that encodes active agrin. *Neuron*, **8**, 677–689.

Valenzuela, D.M., Stitt, T.N., DiStefano, P.S., Rojas, E., Mattsson, K., Compton, D.L., Nunez, L., Park, J.S., Stark, J.L., Geis, D.R., Thomas, S., LeBeau, M.M., Fernald, A.A., Copeland, N.G., Jenkins, N.A., Burden, S.J., Glass, D.J. & Yancopoulos, G.D. (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron*, **15**, 573–584.

Wallace, B.G. (1986) Aggregating factor from Torpedo electric organ induces patches containing acetylcholine receptors, acetylcholinesterase and butyrylcholinesterase on cultured myotubes. *J. Cell Biol.*, **102**, 783–794.

Wallace, B.G. (1992) Mechanism of agrin-induced acetylcholine receptor aggregation. *J. Neurobiol.*, **23**, 592–604.

Wallace, B.G. (1995) Regulation of the interaction of nicotinic acetylcholine receptors with the cytoskeleton by agrin-activated protein tyrosine kinase. *J. Cell. Biol.*, **128**, 1121–1129.

Wilson, C., Goberdhan, D.C.I. & Steller, H. (1993) Dror, a potential neurotrophic receptor gene, encodes a Drosophila homolog of the vertebrate Ror family of Trk-related receptor tyrosine kinases. *Proc. Natl Acad. Sci. USA*, **90**, 7109–7113.

Zhou, H., Muramatsu, T., Halfter, W., Tsim, K.W.K. & Peng, H.B. (1997) A role of midkine in development of the neuromuscular junction. *Mol. Cell. Neurosci.*, **10**, 56–70.