Expression of mutant Ets protein at the neuromuscular synapse causes alterations in morphology and gene expression

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Received April 4, 2002; revised September 6, 2002; accepted September 18, 2002

The localized transcription of several muscle genes at the motor endplate is controlled by the Ets transcription factor GABP. To evaluate directly its contribution to the formation of the neuromuscular junction, we generated transgenic mice expressing a general Ets dominant-negative mutant specifically in skeletal muscle. Quantitative RT–PCR analysis demonstrated that the expression of genes containing an Ets-binding site was severely affected in the mutant mice. Conversely, the expression of other synaptic genes, including MuSK and Rapsyn, was unchanged. In these animals, muscles expressing the mutant transcription factor developed normally, but examination of the post-synaptic morphology revealed marked alterations of both the primary gutters and secondary folds of the neuromuscular junction. Our results demonstrate that Ets transcription factors are crucial for the normal formation of the neuromuscular junction. They further show that Ets-independent mechanisms control the synaptic expression of a distinct set of synaptic genes.

INTRODUCTION

The current model for the neuronal control of synaptic gene transcription in skeletal muscle involves two distinct mechanisms. On the one hand, neurally evoked electrical activity in muscle fibres represses transcription of synaptic genes in extra-synaptic areas. On the other, nerve-derived signals specifically activate synaptic gene expression in sub-synaptic myonuclei (for a review see Schaeffer et al., 2001).

We have previously identified a DNA regulatory element, termed the N-box, which is required for targeting the transcription of the acetylcholine receptor (AChR) δ and AChR ε subunit-encoding genes to sub-synaptic nuclei (Koike et al., 1995; Ducler et al., 1996). Interestingly, the utrophin and acetylcholinesterase (AChE) genes have subsequently been shown to contain a functional N-box (Chan et al., 1999; Gramolini et al., 1999; Khurana et al., 1999). Further studies have shown that an N-box is required for neuregulin-stimulated transcription of the utrophin, AChR δ and AChR ε promoters in cultured muscle cells (Fromm and Burden, 1998; Sapru et al., 1998; Schaeffer et al., 1998; Khurana et al., 1999) and of the utrophin promoter in intact skeletal muscle (Gramolini et al., 1999). In these experiments, the contribution of the Ets-related transcription factor GABPαβ was also demonstrated because of its ability to bind the N-box and to transcriptionally activate the utrophin, AChR δ and AChR ε genes in response to ARIA. Recently, Bruguet and Ruegg (2000) highlighted the importance of GABP in vivo by showing its requirement for the formation of agrin-induced ectopic synapses.

Given that the N-box is required for regulating the expression of genes encoding critical components of the motor endplate, we have evaluated the contribution of N-box-dependent transcription to the formation of the neuromuscular junction (NMJ).
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RESULTS AND DISCUSSION

Transgenic line

The DNA-binding domain (DBD) of Ets-2 on its own acts as a classical trans-dominant-negative mutant. The absence of the natural DBD flanking sequences strengthens the binding of the mutant to DNA (Langer et al., 1992). The Ets-2 DBD has already been shown to efficiently block activation of the N-box (Sapru et al., 1998) and, when fused to β-galactosidase, retains the Ets dominant-negative activity (Langer et al., 1992). This fusion protein was used to make the transgene, since this made it possible to detect muscle fibers expressing the trans-dominant-negative mutant. To drive expression of the fusion protein, the myosin light chain 1F...
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Synaptic gene expression

The expression of various components of the post-synaptic apparatus of the NMJ was determined by quantitative real-time RT–PCR on mRNAs isolated from the vastus lateralis of 3-week-old animals. The results obtained with the LacZ-positive transgenic mice were normalized to β-actin, and then compared with those obtained with their LacZ-negative litter mates (Figure 2). Three gene products tested depend on an N-box and GABP for their synaptic expression, i.e. the AChR ε subunit, acetylcholinesterase and utrophin A mRNAs (Duclect et al., 1996; Chan et al., 1999; Gramolini et al., 1999). The promoter of the β2 laminin gene contains Ets-binding sites, but their role has yet to be examined (Brandenberger et al., 1996). Two of the gene products tested do not contain a consensus N-box in their promoters: the AChR α and utrophin B mRNAs, although the AChR α subunit promoter contains putative Ets-binding sites (Merlie and Kornhauser, 1989; Burton et al., 1999). Finally, the murine promoters of rapsyn and MuSK have not been characterized.

In agreement with our hypothesis, expression of the AChR α and AChR ε, AChE, utrophin A, and β2 laminin mRNAs were reduced in mutant muscles (respectively, 64, 37, 35, 45 and 52% relative to the expression levels in control animals; see Figure 2), thus showing that, in vivo, the expression of these genes is under the control of Ets transcription factors. In comparison to genes containing a consensus N-box, the effect of the mutant factor on AChR α gene expression was less pronounced. This could reflect the presence of weak Ets-binding sites in the AChR α promoter.

The levels of utrophin B, rapsyn and MuSK mRNAs were not significantly affected in muscles of mutant animals. To exclude the possibility that extra-synaptic variations of gene expression could interfere with our results, extra-synaptic regions of the vastus lateralis from mutant and control animals were dissected, but no variation could be observed for any of the genes tested (data not shown). In addition, the synaptic/extra-synaptic ratio for the MuSK RNA is higher than for the AChR α RNA, thus showing that in the vastus lateralis, MuSK expression is even more sharply compartmentalized than is AChR expression (data not shown).

The lack of effect of the mutant on the expression of MuSK, rapsyn or utrophin B suggests that the expression of some synaptic genes is controlled by a mechanism that does not involve Ets transcription factors. Interestingly, the transcription factor Sp1 has already been shown to mediate the transcriptional activation of the AChR δ and AChR ε promoters by neuregulins in P-19 teratocarcinoma cells (Alroy et al., 1999). This mechanism does not have to be strictly transcriptional, and could, for example, involve selective RNA stabilization and degradation, in synaptic versus extra-synaptic zones, respectively (for further discussion, see Newey et al., 2001). However, it is also possible that Ets transcription factors participate in MuSK and rapsyn expression, but that other factors also significantly stimulate their expression at the NMJ, thus minimizing the effect of the Ets

(MLC1F) promoter was chosen (Kelly et al., 1997). This promoter possesses several key properties. First, it produces some of the highest expression levels in transgenic mouse skeletal muscle (Rao et al., 1996) and is specific for skeletal muscle. Furthermore, the transgene is active in the myotome by day 9.5 of embryonic development and in forming limb muscles by day 11.5 (Grieshammer et al., 1992 and references therein). Finally, expression of the transgene is 100-fold lower in slow versus fast muscle fibers and occurs in a proximo-distal gradient, thus limiting expression in vital muscles such as the diaphragm (Donoghue et al., 1991).

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mutant. Consistent with this is the fact that although MuSK expression is stimulated by neuregulins/erbB (Ip et al., 2000; Moore et al., 2001), this stimulation is only 3-fold in chick primary myotubes whereas AChR α subunit gene expression is activated 30-fold in the same cells (Altiok et al., 1995; Ip et al., 2000). It is thus conceivable that the contribution of neuregulins to MuSK expression is proportionally less important than in the case of AChR genes, thus rendering it undetectable by our approach. Finally, we cannot exclude the possibility that the perturbation of the NMJ by the Ets mutant induces a compensatory mechanism in order to preserve MuSK expression.

Neuromuscular junction morphology and ultrastructure

To determine whether perturbations in the expression of Ets-binding site-containing genes affect the formation of NMJs, we examined the morphology of wild-type and mutant junctions by confocal microscopy, using rhodamine-labelled α-bungarotoxin. At 1 week of post-natal development, NMJs have not reached their mature shape, and AChRs form a simple patch under the nerve terminal. In comparison with wild-type tissue at this stage of development, AChR patches in mutant animals are slightly smaller (Figure 3A). At 3 weeks of post-natal development, the NMJs have matured and the primary gutters and secondary folds have formed. At this stage, the morphology of NMJs from mutant animals is clearly different from that of wild-type NMJs (Figure 3B). In muscles from mutant mice, two distinct categories of NMJs can be observed. The first represents 30% of all synapses and is characterized by atrophic NMJs whose primary gutters form a single ring. Conversely, the second category represents 70% of the junctions and in these the primary gutters are more ramified than in the wild-type muscles, thus forming more AChR-free sub-domains. In addition, the

![Fig. 3](image-url). Comparison of the wild-type and mutant NMJ morphologies. NMJs were labelled with rhodaminated α-bungarotoxin and observed by confocal microscopy. (A) One week post-natal NMJ. (B) Three week post-natal NMJ. The NMJ surface corresponds to the surface occupied by the NMJ. Surfaces are expressed in arbitrary units. The primary gutter surface corresponds to the surface labelled by, and thus occupied by, the AChR. AChR-free domains correspond to the number of closed surfaces, comprised in the NMJs and not labelled by the α-bungarotoxin. The +/- values correspond to the standard deviation. Mutants 1 and 2 illustrate the two morphological types of mutant NMJ.
primary gutters appear less sharply defined than those seen in normal NMJs. The overall surface of mutant NMJs is only slightly smaller than in wild-type mice, consistent with the notion that the size of the NMJ is mainly determined by the nerve terminal (Sanes and Lichtman, 2001). However, the average surface occupied by AChRs (i.e. the surface of the primary gutters) is twofold smaller at mutant NMJs; this is consistent with the reduction of AChR ε expression. The number of AChR-free sub-domains per NMJ also gives an idea of the organization of the primary gutters. In mutant muscles, the average number of such domains is three times higher than in wild-type muscles.

Utrastructural analysis of thin sections from the NMJs from mice expressing the Ets dominant-negative mutant disclosed severe perturbations in the post-synaptic apparatus (Figure 4). Compared with the post-synaptic membranes of NMJs in control animals (Figure 4A) those of the mutant NMJs showed disorganized secondary folds (arrows in 4B) in some cases. At other junctions, the folds were scarce or even lacking (Figure 4C).

The morphological and ultrastructural alterations of the NMJ by the mutant Ets factor are reminiscent of what is observed in laminin β2- or utrophin-deficient mice (Noakes et al., 1995; Grady et al., 1997). In both cases the formation of synaptic secondary folds is affected.

EM studies also identified two types of mutant NMJs, which possibly correlate with the classes observed by confocal microscopy, but the difficulty in finding a large number of synapses at the EM level precluded the statistical analyses required to directly validate this hypothesis.

Fig. 4. Comparative ultrastructural analysis of mutant versus wild-type NMJs. In agreement with confocal studies, the ultrastructural analysis of NMJs of mutant mice on thin sections disclosed severe perturbations in the post-synaptic apparatus. Compared with control animals (A) the post-synaptic membranes of the NMJs in vastus lateralis muscles showed disorganized secondary folds [arrows in (B)]. In other junctions, the folds were scarce or even lacking (C). Nerve endings (NE) looked normal in all muscles. Magnification = 20 000× in (A–C).
Altogether, our results show that, in vivo, Ets transcription factors are essential for synaptic-specific expression of several synaptic genes and for the proper formation of NMJs. The altered NMJ morphology of muscle fibers expressing the Ets-dominant-negative mutant is most likely a direct result of the reduction in the expression of key components of the NMJ, i.e., the AChR α and AChR ε subunits, acetylcholinesterase, utrophin A and laminin β2, which are known to be involved in the control of neurotransmission, ultrastructure and nerve sprouting, respectively.

Point mutations in the N-box of the AChR ε subunit promoter have been identified in human patients suffering from congenital myasthenia, and are known to reduce expression of the AChR ε gene in these patients (Nichols et al., 1999; Ohno et al., 1999). The transgenic animals generated in the present study could therefore be useful to design protocols for Ets-independent synaptic gene activation.

METHODS

Dominant-negative mutant construction. The Ets-2 DBD (encoding amino acids 336–501) was amplified by RT–PCR and cloned upstream and in-frame with the lacZ gene, in the p1Flac-E plasmid kindly provided by R. Kelly and M. Buckingham (Kelly et al., 1997).

Transgenic animal production. The purified MLC1F promoter/Ets-2 DBD-LacZ/Enhancer fragment was injected into fertilized oocytes of C57BL/6SJL hybrids. To identify the transgenic animals, genomic DNA was isolated from tail biopsies and tested by PCR to detect the presence of the LacZ gene.

Synaptic gene mRNA level analysis. Quantitative real-time PCR analysis. Total RNA of muscles was extracted using the Qiagen RNeasy midi kit (Qiagen, France). Total RNA of muscles was purified from synaptic and extra-synaptic regions of lateralis vastus muscles to compare the sharpness of MuSK mRNA compartmentalization to that of AChR subunit mRNAs. For this purpose, NMJs were stained with the Karnovsky and Roots reaction (Karnovsky and Roots, 1964), and the synaptic and extra-synaptic regions of individual fibres were micro-dissected. Total RNA was then extracted from the isolated synaptic and extra-synaptic regions using the Qiagen RNeasy mini kit (Qiagen, France). The AChR α, ε and MuSK RNAs were quantified using quantitative real-time RT–PCR and the results were normalized to β actin. This allowed a 150- to 1000-fold enrichment of the various mRNAs in synaptic, compared with extra-synaptic, preparations. The AChR α mRNA clearly had a lower synaptic/extra-synaptic ratio than the MuSK and AChR ε mRNAs.

NMJ morphological analysis. Dissected lateralis vastus muscles were either processed for electron microscopy as previously described (Aghulut et al., 2001), or labelled with rhodamine-labelled α-bungarotoxin (Molecular Probes) and observed under a confocal microscope (LSM 510, Zeiss, Germany) as previously described (Altiok et al., 1995). The topological data were calculated from the confocal images using NIH image.

At least 20 NMJs from each mutant individual in a litter were analysed. For the confocal analysis, 50 NMJs from a 3-week-old control animal and 150 NMJs from three 3-week-old mutant animals were analyzed. The morphometric results were pooled and their statistical significance was determined with a one-way ANOVA (P < 0.01).

To ascertain that the phenotype was not due to the perturbation of the expression of a critical gene by the insertion of the transgene in the genome, the expression and NMJ morphology were also measured on diaphragm muscles from transgenic animals. In such animals, the diaphragm muscle contains the transgene but does not express it, and synaptic gene expression and NMJ morphology are normal (data not shown).

ACKNOWLEDGEMENTS

We thank Margaret Buckingham and Robert Kelly for the transgene promoter, Nathalie Duclert, Marie Vandromme and Marie-Anne Ludosky for expert technical assistance. This work was supported by the Association Française contre les Myopathies (AFM), the Centre National de la Recherche Scientifique, the Collège de France, the EEC. A. de K.d’E. is Research Associate of the National Fund for Scientific Research (Belgium).
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DOI: 10.1093/embo-reports/kvf220