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Protein-DNA Array-based Identification of Transcription Factor Activities Differentially Regulated in Skeletal Muscle of Normal and Dystrophin-deficient Mdx Mice

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Abstract

Inactivation of dystrophin gene is the primary cause of Duchenne muscular dystrophy in humans and *mdx* mice. However, the underpinning mechanisms which govern the pathogenesis of dystrophin-deficient skeletal muscle remain poorly understood. We have previously reported activation of MAPK, NF- κ B, and PI3K/Akt signaling pathways in diaphragm muscle of *mdx* mice. In this study, using a protein-DNA array-based approach, we have investigated the activation of 345 transcription factors in diaphragm muscle of 6-week old normal and dystrophin-deficient *mdx* mice. Our data demonstrate increased activation of a number nuclear transcription factors including AP1, HNF-3, PPAR α , c.myc BP, ETF, Fra-1/JUN, kBF-A, N-rasBP, lactoferrin BP, Myb(2), EBP40_45, EKLF (1), p53(2), TFEB, Myc-Max; c-Rel; E2, ISRE; NF- κ B; Stat1 p84/p91, Antioxidant RE, EVI-1, Stat3, AP3, p53, Stat4, AP4, HNF-1, FAST-1, Pax-5, Beta-RE in the diaphragm muscle of *mdx* mice compared to corresponding normal mice. The level of activation for p53 was highest among all the transcription factors studied. Furthermore, higher activation of p53 in diaphragm muscle of *mdx* mice was associated with its increased phosphorylation and nuclear translocation. Collectively, our data suggest that the primary deficiency of dystrophin leads to the aberrant activation of nuclear transcription factors which might further contribute to muscle pathogenesis in *mdx* mice.

Keywords

Protein-DNA array; transcription factors; Dystrophin; *mdx*; NF- κ B

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder caused by the functional inactivation of the dystrophin gene (1–3). Dystrophin has a major structural role in muscle as it links the internal cytoskeleton to the extracellular matrix. The carboxyl terminus of dystrophin binds to the dystrophin-glycoprotein complex (DGC) at the sarcolemma. In the absence of dystrophin, the DGC is destabilized, which results in diminished levels of the membrane proteins (4,5).

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Studies in the recent past have demonstrated that secondary events such as inflammation, regeneration of muscle after necrosis, and fibrosis actively contribute to the pathogenesis of DMD (6–9). Analysis of gene expression differences in the muscle biopsies of DMD patients (10) and in the myofibers of mdx mice (a mouse model of DMD) (11,12) have revealed several differentially regulated genes related to the major characteristics of dystrophic muscles. Accumulating evidence further suggests that the DGC has an important signaling role in striated muscle and is not merely a molecular scaffold serving the mechanical function. Indeed, our group and others have shown that loss of dystrophin leads to the aberrant activation of the mitogen-activated protein kinases (MAPK), the nuclear factor-kappa B (NF- κ B), and phosphoinositide-3 kinase/Akt (PI3K/Akt) signaling pathways in skeletal muscle of mdx mice at both pre-necrotic and necrotic stages (13–15). Although the precise role of these signaling pathways (e.g. MAPK, NF- κ B, and PI3K/Akt) remains enigmatic, it is likely that the anomalous regulation of one or more of these pathways contributes to skeletal muscle pathogenesis in mdx mice. Indeed, several recent studies have shown that the inhibition of NF- κ B signaling pathway ameliorates skeletal muscle pathogenesis and provides functional gain in mdx mice (16–19).

Transcription factors serve as a major link between the activation of various signal transduction pathways and nuclear gene expression (20). Aberrant activation of various transcription factors has been implicated in the manifestation of a wide variety of disease states (21). Activation of transcription factors generally requires specific post-translational modifications by upstream signaling proteins. Although the expression array studies have revealed increased mRNA level of several transcription factors in skeletal muscle of mdx mice (11,12), it remains unclear how the activation (i.e. binding to consensus DNA sequences) of various transcription factors is modulated in myofibers of mdx mice. We hypothesize that the abnormal activation of various signal transduction pathways (13–15) leads to aberrant activation of downstream nuclear transcription factors in the myofibers of mdx mice. To test this hypothesis, we have employed a transcription factor array approach and investigated the activation of 345 transcription factors in diaphragm muscle of normal and mdx mice. Our data suggest atypical activation of several important transcription factors such as p53, signal transducer and activator of transcription (STAT), activator protein -1 (AP-1), nuclear factor-kappa B (NF- κ B), lactoferrin BP etc. in the diaphragm muscle of mdx mice compared to corresponding normal mice. Furthermore, our data suggest that the increased activation of p53 transcription factor in diaphragm muscle of mdx mice is associated with its increased phosphorylation and nuclear translocation.

MATERIAL AND METHODS

Materials

Protein/DNA Spin Combo Array kit (Catalog # MA1215) was purchased from Panomics Inc. (Fremont, CA). Antibodies against phosphorylated p53 (Ser392) protein were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against total p53 and β -actin protein and p53 consensus oligonucleotides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). NF- κ B and AP-1 consensus oligonucleotides were obtained from Promega (Madison, WI). Poly dI-dC was obtained from GE Healthcare (Piscataway, NJ). Protease inhibitor cocktail was obtained from Sigma Chemical Co. (St. Louis, MO). 32 P- γ -ATP was purchased from Perkin-Elmer Life Sciences (Boston, MA).

Mice and Tissue Preparation

Mice (C57BL10/SCSN and DMDMDX/J) originally obtained from Jackson Laboratory (Bar Harbor, ME) were used for these studies. All experimental protocols have been approved by the Institutional Animal Care and Use Committee. Mice were euthanized by 95% CO₂-5% O₂ inhalation and immediately the diaphragm muscle was excised by making an incision in

the thoracic cavity. The excised costal muscles were immediately frozen in dry ice and processed for biochemical analyses.

Preparation of Nuclear and Cytoplasmic Extracts

To investigate how the activation of various transcription factors is affected in dystrophic myofibers of mdx mice, we prepared nuclear extracts from the diaphragm muscle of 6-week old control and mdx mice using a method as described earlier (13). The diaphragm muscle was suspended at 1 mg muscle weight per 18 μ l of low salt lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 μ g/ml leupeptin, 2.0 μ g/ml aprotinin, 0.5 mg/ml benzamidine) followed by mechanical grinding. Cells in the lysis buffer were allowed to swell on ice for 5 min followed by two cycles of freeze-thaw lysis. The tubes containing the lysed muscle cells were then vortexed vigorously for 10 seconds, and the lysate was centrifuged for 10 s at 14,000 rpm. The supernatant (cytoplasmic extracts) was removed and saved at $-80^{\circ}C$ for further biochemical analysis. The nuclear pellet was again washed with 800 μ l of cytoplasmic extraction buffer to remove any residual cytoplasmic proteins. Finally, nuclear pellet was resuspended in 4 μ l of ice-cold high-salt nuclear extraction buffer (20 mM HEPES [pH 7.9], 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 μ g/ml leupeptin, 2.0 μ g/ml aprotinin, 0.5 mg/ml benzamidine) per mg of original muscle weight and was incubated on ice for 30 min with intermittent vortexing. Samples were centrifuged for 5 min at $4^{\circ}C$, and the supernatant (nuclear extract) was either used immediately or stored at $-80^{\circ}C$.

Protein/DNA array Hybridization and Analysis

Protein concentrations were measured using Bio-Rad protein assay reagent (BioRad Laboratories, Hercules, CA). In each group, equal amount (based on protein content) of nuclear extracts were pooled from 5 mice and used for hybridization with biotin-labeled DNA binding oligonucleotides. A set of biotin-labeled DNA binding oligonucleotides were preincubated with the nuclear extracts to allow formation of protein/DNA complexes according to the protocol suggested by the manufacturer of TransSignal Protein/DNA Array System (Panomics). The protein/DNA complexes were separated from the free probes using a spin column separation system (Panomics). The bound probes were extracted and hybridized to an array membrane spotted with 345 different consensus-binding sequences following a protocol suggested by the manufacturer. Signals were detected with luminol and horseradish peroxidase (HRP) and membranes were exposed to X-ray films. Relative spot intensities were determined using ImageQuantTL software (GE Healthcare, Piscataway, NJ).

Electrophoretic Mobility Shift Assay (EMSA)

DNA-binding activity of selected transcription factors was analyzed by EMSA as described earlier (13,22). Briefly, 15 μ g of nuclear extract prepared from diaphragm muscle of normal and mdx mice were incubated with 16 fmol ^{32}P - γ -ATP-end-labeled consensus oligonucleotides for 20 min at $37^{\circ}C$. The incubation mixture included 2–3 μ g of poly dI-dC in a binding buffer (25 mM HEPES [pH 7.9], 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl). The DNA-protein complex thus formed was separated from free oligonucleotide on 7.5% native polyacrylamide gel. The gel was dried, and the radioactive bands were visualized and quantitated by Storm 820 PhosphorImager (GE Healthcare, Piscataway, NJ) and using ImageQuantTL (GE Healthcare, Piscataway, NJ) software.

Western Blotting

Immunoblotting for different proteins was done as described previously (15,23). Briefly, 50–70 μ g of cytoplasmic or nuclear protein extract prepared was resolved on 9% SDS-PAGE gel.

The proteins were then electrotransferred to a nitrocellulose membrane blocked with 3% nonfat milk and probed with suitable antibodies (1:1000 dilution) for overnight. The blot was washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h, and finally detected by chemiluminescence (GE Healthcare, Piscataway, NJ). The X-ray films were digitized to determine the quantitative difference between bands. We used ImageQuantTL software (GE Healthcare, Piscataway, NJ) to determine the quantitative difference between bands on immunoblots.

RESULTS

In this study, we have used diaphragm muscle from 6-week old mdx and corresponding normal C57BL10 mice to study the activation of different transcription factors.

Protein/DNA array hybridization and analysis

Diaphragm muscle from normal and mdx mice were isolated and subjected for nuclear and cytoplasmic extractions. Equal amount of nuclear extracts were pooled from either five normal or five mdx mice and hybridized with the membranes spotted with 345 different oligonucleotides containing consensus-binding sequences for different transcription factors. As shown in Figure 1, a significant increase in the DNA-binding activity of several transcription factors (highlighted in circles in Figure 1) was observed in the diaphragm muscle of mdx mice compared to control mice. The activation of a few transcription factors was also decreased (highlighted in squares in Figure 1) in diaphragm muscle of mdx compared to normal mice. Schematic representation of the consensus sequence corresponding to each spot on the array is presented in the supplementary file and in the online (http://www.panomics.com/downloads/18_4_PDUM_1_V1.pdf) user manual of the Protein/DNA Spin Combo Array kit. These data provide the first evidence that the activities of various transcription factors are differentially regulated in skeletal muscle of normal and mdx mice.

Quantification of Protein/DNA Array Spots

To determine the folds increase in the activity of various transcription factors, we quantified each spot on both array membranes using ImageQuant software (GE Healthcare). Figure 2 show the transcription factors and fold change for only those that were activated by more than 1.5 fold in the diaphragm muscle of mdx mice in two independent experiments. Although the DNA-binding activities of many transcription factors were augmented in mdx mice, p53, Stat1, Stat4, EVI-1, and lactoferrin BP demonstrated the highest increase.

Higher activation of p53, AP-1, and NF- κ B transcription factors in diaphragm muscle confirmed by EMSA

To confirm the results of the array experiment, we also performed EMSA for a select few transcription factors using the same nuclear extracts which were used in Protein/DNA array experiment. As shown in Figure 3, higher activation of NF- κ B, p53, and AP-1 transcription factors was also observed by EMSA. These data are consistent with our previously published report demonstrating increased activation of NF- κ B and AP-1 transcription factors in diaphragm muscle of the mdx mice (13,14). Taken together, we conclude that the data in array experiments represent the differences in the activities of various transcription factors in the diaphragm of normal and mdx mice.

Increased Phosphorylation and Nuclear Translocation of p53 in Diaphragm of Mdx Mice

Since p53 protein undergoes several post-translational modifications including phosphorylation prior to its transcriptional activation (24–26), we studied the level of phosphorylated p53 protein in diaphragm muscle of normal and mdx mice using an antibody

which detects p53 protein that is phosphorylated at Serine 392 residue. The level of phosphorylation at Ser-392 in p53 protein was significantly increased (~ 2.8 fold) in the diaphragm muscle of mdx mice compared to normal mice. In addition, the nuclear level of p53 protein was also increased in the diaphragm muscle of the mdx mice compared to normal mice (Figure 4B). These data provide the evidence that p53 protein is activated in the diaphragm muscle of mdx mice. These data also suggest that the increased DNA-binding in the array experiment is a result of the activation of transcription factors through post-translational modifications possibly by upstream signaling proteins.

DISCUSSION

Although the loss of functional dystrophin protein is the primary cause for DMD, accumulating evidence suggests that the deficiency of dystrophin results in altered activation of a number of cell signaling pathways in skeletal muscle. Modulation of the activation of these signaling pathways and/or their downstream target proteins may serve as potential therapeutics to protect skeletal muscle mass and function and to improve quality and length of life for DMD patients (8,27). Indeed, several genes have been identified which can reduce muscle degeneration at least in animal models of DMD (28). In order to understand the pathophysiological mechanisms in DMD and to identify additional targets, in this study we have determined how the activation of various transcription factors is affected in degenerating diaphragm muscle of dystrophin-deficient mdx mice compared to the controls. We have identified a number of transcription factors that are highly activated in the diaphragm muscle of mdx mice (Figure 1).

Transcription factors represent one of the most powerful drug development targets (29). We have previously reported that the activation of proinflammatory transcription factor NF- κ B was higher in the skeletal muscle of mdx mice compared to normal mice (13). Subsequent reports by other groups showed that NF- κ B was also highly activated in the muscle biopsies of DMD patients (30,31). Furthermore, recent studies have shown that inhibition of NF- κ B in mdx mice using pharmacological and genetic approaches inhibits muscle pathogenesis in mdx mice (16–19). These studies support the contention that modulation of the activity of specific transcription factors could help to improving the conditions of DMD patients.

We have used a commercially available DNA/protein array kit to identify other transcription factors that could be abnormally activated in the myofibers of mdx mice. The major advantages of this approach include that it allows simultaneously determination of the activation of several transcription factors in a single experiment (32). Furthermore, it measures the activities (i.e. actual binding to the consensus DNA sequence) of the transcription factors which is more biologically relevant than merely studying their expression levels. In addition, we were able to validate that the increased activation of transcription factors in array experiment is reproduced by independent EMSA (Figure 3).

Besides NF- κ B, we found that the activation of many other transcription factors was highly up-regulated in diaphragm muscle of the mdx mice (Figure 2). Although the exact role of these transcription factors in pathogenesis of mdx mice remains unknown, many of these activated transcription factors have been shown to be involved in inflammatory (e.g. Stat1, Stat3, Stat4, NF- κ B, and AP-1) and oxidative stress (e. g. PPAR α , NF- κ B, AP-1, Myc) responses. Importantly, both inflammation and oxidative stress contribute significantly to the skeletal muscle pathogenesis in mdx mice and in DMD patients (33–35). It is also possible that some of these transcription factors which are activated in mdx mice (e.g. Pax-5 and AP-1) contribute to the proliferation of satellite cells and regeneration of muscle fibers after initial necrosis. For example, in addition to its role in cellular proliferation and inflammatory responses, AP-1 transcription factor also up-regulates the expression of utrophin, a homologue of dystrophin which can compensates for the loss of dystrophin (36,37).

A striking observation of the present study was that the activation of p53 transcription factor was up-regulated in the diaphragm muscle of mdx mice (Figure 1, 2, and 3) and higher phosphorylation and nuclear localization of p53 protein (Figure 4). The p53 transcription factor is widely known for its tumor suppressor functions and is often referred to as “the guardian of the genome” (38,39). However, accumulating evidence also suggests that p53 plays a critical role in the homeostasis of different tissues including skeletal muscle. Increased activation of p53 is associated with premature senescence and loss of skeletal muscle mass during unloading, denervation, space flight, and aging (40–44). Therefore, it is possible that increased activation of p53 contributes, at least in part to the loss skeletal muscle mass in mdx mice.

Although the exact mechanisms by which loss of dystrophin leads to the activation of various transcription factors in myofibers of mdx mice remains unclear, a few possibilities can be discussed. Accumulating evidence suggests that several cell signaling proteins (e.g. calmodulin kinase II, stress-activated protein kinase-3, nitric oxide synthase, voltage-gated sodium channels, phosphatidylinositol 4,5-bisphosphate, and Grb2) interact with dystrophin-glycoprotein complex (DGC) that is present on skeletal muscle membrane (27). Thus, the loss of this complex in myofibers of mdx mice could lead to aberrant cell signaling and activation of downstream transcription factors. Furthermore, besides having a direct role in cellular signaling, the disruption of DGC could also lead to cell signaling possibly by inducing conformational changes in the membrane proteins or by destabilization of the activity of certain ion channels. Indeed, we have previously reported that the lack of functional dystrophin leads to greater mechanical instability of the sarcolemma with associated increase in the activity of various cell signaling pathways (13–15). Since many of these transcription factors (e.g. AP1, AP2, STATs, NF- κ B, cRel, Fra/Jun etc) are the direct phosphorylation targets of various cell signaling pathways (e.g. MAPK, NF- κ B, PI3K/Akt), the increased activation some of the transcription factors in dystrophin-deficient diaphragm muscle could be a direct result of the activation of upstream signaling pathways. In addition, it is also possible that there is a cooperative interaction between different transcription factors for their activation and to regulate skeletal muscle mass and pathogenesis. For example, under specific conditions p53 protein can either positively or negatively regulates the activation of NF- κ B transcription factor in certain cell types (45–47).

In summary, our study provides a novel piece of information regarding the activation of different transcription factors in degenerating skeletal muscle of mdx mice. Further studies are required to determine the role of individual transcription factor in the pathogenesis of mdx mice and if any of these transcription factors can be used as a molecular target to prevent skeletal muscle pathogenesis in DMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AP-1, activator protein-1; DMD, Duchenne muscular dystrophy; EMSA, electrophoretic mobility shift assay; NF- κ B, nuclear factor-kappa B; PI3K, phosphatidylinositol 3-kinase; STAT, signal transduction and activator of transcription.

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Figure 1. Differential activation of transcription factors in diaphragm muscle of normal and mdx mice

Nuclear extracts made from diaphragm muscle of 6-week old normal and mdx mice were analyzed for the activation of different transcription factors using TransSignal Spin Array kit. Representative array data from two independent experiments are presented here. Each spot on the array corresponds to a specific transcription factor. The name of the transcription factor at the specific site is provided in the user manual

(http://www.panomics.com/downloads/18_4_PDUM_1_V1.pdf) and in supplementary file.

Circled spots represent the transcription factors whose activity was increased and squared spots represent the transcription factors whose activity was decreased. The highlighted spots positions and corresponding transcription factors are as follows: A1:AP-1; A7:HNF-3; A9:PPARα; A13:MTF(1); A17:c.mylb BP; A18:ETF; B11:CTCF; B18:Fra-1/JUN; B19:kBF-A; B21:N-rasBP; C19: lactoferrin BP; C20:Myb(2); D11:EBP40_45; D21:ODC; E11:EKLF (1); E21:p53(2); F23:TFEB; G2:Myc-Max; G5:c-Rel; G23:Thy1BP; H2:NF-1; H4:MRE; I5:E4BP4; J17:E2; K7:ISRE; L2:NF-kB; L3:Stat1 p84/p91; L4:Antioxidant RE; L5:EVI-1; M3:Stat3; M17:AP3; N2:p53; N3:Stat4; N4:AP4; N6:HNF-1; O1:FAST-1; O2:Pax-5; O4:Beta-RE; O6:Afxh; O9:AIC,CBF; O13:PEBP2.

Figure 2. Quantitation of the fold increase in the activities of transcription factors in the protein-DNA array

The fold increase in the activity of various transcription factors in mdx mice compared to normal mice was calculated using ImageQuant software. The transcription factors whose activities were increased more than 1.5 fold are depicted here.

Figure 3. Activation of NF-κB, p53, and AP-1 transcription factors in diaphragm muscle of mdx mice studied by EMSA

Diaphragm muscle from 6-week old normal and mdx mice were processed for cytoplasmic and nuclear protein extraction. Nuclear extracts were subjected to EMSA to determine the DNA-binding activity of NF-κB, p53, and AP-1. Representative EMSA gel presented here shows that the DNA-binding activity of NF-κB, p53, and AP-1 was significantly higher in mdx mice compared to normal mice. These results are consistent with the data in the Protein-DNA array experiment in Figure 1.

A. **B.**

Figure 4. Activation and nuclear translocation of p53 protein in diaphragm muscle of mdx mice
A). The levels of phosphorylated p53 protein was measured in total protein extracts made from diaphragm muscle of 6-week old normal and mdx mice. Data presented here show that the level of phosphorylated p53 (ser 392) was higher in diaphragm muscle of mdx mice compared to normal mice. There was no difference in the level of an unrelated protein actin between normal and mdx mice. **B).** Nuclear and cytoplasmic extracts made from diaphragm muscle of normal and mdx mice were subjected to western blot analysis for determining the level of p53 protein. Data presented here show that the level of p53 was increased in nuclear extracts made from the diaphragm muscle of mdx mice compared to normal mice.