Cloning and Characterization of a Novel Gene, striamin, That Interacts with the Tumor Suppressor Protein p53*

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Expression analysis of a novel cDNA isolated from immortal murine fibroblasts revealed a single transcript of 3.0 kilobase pairs that was highly expressed in mouse and human striated muscle and in mouse heart. The gene has therefore been named striamin. Its expression was confined to skeletal muscle types with a fast glycolytic (2B) contractile phenotype. It was also detected in C2C12 mouse myoblasts and was down-regulated during in vitro myogenesis. The cDNA has a single open reading frame encoding a predicted 16.8-kDa protein of 149 amino acids with no homology to known proteins. Microinjection and transfection of green fluorescence protein-tagged striamin demonstrated that it localizes to the nucleus. Coimmunoprecipitations revealed that it can interact with p53 (a positive marker for myoblast differentiation) in vivo and in vitro. Furthermore, it repressed p53 activity in p53-mediated reporter assays. Fluorescence in situ hybridization with a mouse P1 genomic clone localized the gene to chromosome 12C3, which is syntenic to human chromosome 14q21-22.

Several genes, such as muscle creatine kinase, troponins, caveolin-3, α-actin, and myosin, have been reported to be predominantly expressed in skeletal muscle. A family of muscle-specific transcription factors such as myoD, myogenin, myf-5, and MRF-4/herculin/myf-6 that regulate muscle-specific gene expression has also been cloned. These are phosphorylated nuclear proteins, containing helix-loop-helix motifs required for dimerization and DNA binding, that can determine a specific cellular differentiation program. The myoD family of transcription factors has been shown to direct myogenesis, repress proliferation, activate differentiation, and induce contractile phenotypes. The introduction of any one of these into nonmyogenic cells induces their differentiation into mature muscle cells. The MyoD and myf-5 are expressed in proliferating myoblasts whereas myogenin and MRF-4 are not expressed until myoblasts exit the cell cycle in response to mitogen depletion. Therefore, myoD and myf-5 have been implicated as having a role in proliferating myoblasts whereas myogenin and MRF-4 have been shown to activate and maintain muscle gene expression. In addition, the cell cycle regulatory proteins such as RB (4, 5), p21 (6), cyclin D, cdk2, cdk4 (7), and p53 (8) have been implicated in the muscle differentiation program. Recently, caveolin-3, α-dystroglycan, and DNA methyltransferase (9–11) have also been assigned a positive role in myogenic differentiation.

While looking for genes involved in senescence and immortalization, we fortuitously cloned a novel gene that is specifically expressed in fast twitch skeletal muscles. The gene is named “striamin” because of its specific expression in striated muscle. Cloning of the cDNA, expression analyses, subcellular localization, chromosomal assignment, its interactions with the tumor suppressor p53, and its possible significance during muscle differentiation are reported herein.

EXPERIMENTAL PROCEDURES

Cell Culture—Normal mouse embryonic fibroblasts from the CD1-ICR strain of mouse (CMEF), an immortal clone (RS-4) established from CMEF, and NIH 3T3 cells, initially used for comparison of proteins and cloning studies, were cultured as described (12). C2 cells originally isolated by Yaffe and Saxel (13) and subcloned by Blau et al. (14) were grown in DMEM (Life Technologies, Inc., Melbourne, Australia) supplemented with 20% FCS (Commonwealth Serum Labs, Melbourne, Australia) and 0.5% chick embryo extract (Flow Laboratories, North Ryde, Australia). Cells were induced to differentiate by replacing growth medium with mitogen-poor medium, DMEM plus 2% horse serum. COS7 and Rat-1 cells used for transfection and microinjection studies were cultured in DMEM supplemented with 10% FCS.

cDNA Cloning and Sequencing—A cDNA library from RS-4 cells was constructed in the AZAP II vector and was screened with a polyclonal anti-p33 antibody raised against a protein identified from P-100 fractions of NIH 3T3 cells (12). cDNA clones were sequenced by the dideoxy chain termination method, and the reactions were analyzed on an ABI 377 automated sequencing machine. Full sequence of a 2.4-kb cDNA clone designated 336 was derived by generation of nested deletions from the 3′ end by exonuclease III (Deletion kit, Takara, Tokyo, Japan) and primer walking. Subsequently, the 5′ end of the cDNA was obtained by Marathon® RACE polymerase chain reaction (PCR) on mouse skeletal muscle cDNA by using three antisense gene-specific primers SP1 (5′-TGT CAC TGC CAC GCC TTC TCG GTG CGC AG -3′), SP2 (5′-TCC CGG CTC CCC TTT GCC GCA TCT TGT TCC -3′) and SP3 (5′-TGA GAA GCT GTG AGC TGC TCT CAG ACC CTT-3′). 5′ Marathon® RACE PCR was performed as described (CLONTECH).

RNA Isolation—Total RNA was prepared from C2C12 cultures grown in DMEM supplemented with 20% FCS or 2% horse serum (differentiation medium, for 24–96 h) using Trizol (Life Technologies, Inc.). Skeletal muscles were excised from B6D2F1 male embryos (P1 progeny of C57BL/6J female × DBA/2J male matings), frozen in liquid N2, and homogenized in denaturant. Total cellular RNA was isolated from all muscle samples using the Trizol reagent.

Northern Blot and RT-PCR Analyses—Mouse and human multiple tissue Northern blots containing 2 μg of poly(A)+ RNA per lane were purchased from CLONTECH. Total cellular RNA from C2C12 cultures

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF031663.

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and B6D2 muscles were denatured and size fractionated on 1% agarose gels containing 2.2% formaldehyde and transferred to Hybond N membrane (Amersham Pharmacia Biotech). A 1.4-kb 3'-untranslated region fragment obtained by BanHI digestion of pBSKIII plasmid was used as a probe. Hybridization was performed at 65 °C in SSC-Denhardt’s-SDS (50 mM NaCl, 0.5% SDS) for 16 h at 1.0 × SSC and 0.1% SDS, then washed in 1× SSC and 0.1% SDS twice, and autoradiographed. RNA loading on the blots was determined by hybridization with 18 S ribosomal probe. RT-PCR was performed on total RNA from mouse tissues and C2C12 cells cultured in normal and mitotin poor medium using primers from 5′ and 3′ of striamin open reading frame (ORF). Control RT-PCR was performed with glyceraldehyde-3-phosphate dehydrogenase-specific primers.

**Cellular Localization of striamin**—The striamin ORF was ligated in frame C-terminal to the GFP-ORF in pEGFP-C1 vector (CLONTECH). The plasmid encoding the GFP-striamin fusion protein was transfected into COS7 cells growing on coverslips using LipofectAMINE™ (Life Technologies, Inc.). Coverslips were incubated with nuclear dye, Hoechst 33258 (Sigma) (5–10 μg/ml in culture medium for 10 min before cell fixation), and fixed with methanol:acetic acid (1:1). After three washings in phosphate-buffered saline, the coverslips were mounted with Fluoromount (Difco). The cells were examined using an Olympus BH-2 microscope with epifluorescence optics or 40× Plan-Neofluor objective on a Zeiss Axioshot microscope (Carl Zeiss, Germany) equipped with a Zeiss Microscopy Scanning System (Carl Zeiss, Germany) and a sensitive charge-coupled device camera (CCD) connected to a computer. Immunofluorescence images were captured using a CELLscan system (Scanalytics, Billerica, MA). Microinjections of plasmids were performed using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Cells were fixed and examined for cellular localization of striamin as described above.

**p53-mediated Reporter Assays**—p53−/− mouse embryonic fibroblasts were transfected with a p53-responsive luciferase reporter plasmid, PG-13uc (kindly provided by Dr. Bert Vogelstein). A temperature-sensitive p53 expression plasmid, pMSVp53Al35 (a kind gift from Dr. Paul Jackson) that results in wild-type p53 conformation at 32.5 °C was used for exogenous p53 expression. Control expression plasmid pLK444 (a kind gift from Dr. Paul Jackson) that results in wild-type p53 conformation at 32.5 °C was used for exogenous p53 expression. Luciferase reporter assays were performed 48 h after transfection. Luciferase values were calculated per microgram of protein as determined by Bradford protein assay.

**RESULTS**

### Cloning of a Striated Muscle-specific Gene, striamin

*Cloning and Characterization of striamin cDNA*—A comparison of plasma membrane Triton X-100-insoluble fractions from normal (CMEF) and immortal (NIH 3T3) murine cells revealed a protein of nearly 33 kDa (called p33) present in NIH 3T3 but not in CMEF (12). The protein was isolated from SDS-polyacrylamide gels and was used for raising polyclonal antibody. The anti-p33 antibody thus raised was used for cDNA cloning by immunoscreening of a RS-4 cDNA library as described previously (16). Five cDNA clones were obtained and were characterized by partial sequencing. Three clones showed high identity to known genes, namely FusCHOP, G-utrophin, and dystrophin, while two clones had no matches in the DNA sequence databases. The sequence thus obtained had no homology to any sequence in the database. The 3′-untranslated region of the gene, following which it was used for chromosomal localization by fluorescence in situ hybridization (FISH).

DNA from the mouse p33 clone was labeled with digoxigenin-dUTP by nick translation. Labeled probe was combined with sheared mouse DNA (40–60 kb chromosomal reads) and rotated at 32 °C for 1 h. Primers were pelleted for centrifugation, washed three times with TBS, boiled in SDS sample buffer, and analyzed by Western blotting with anti-His tag antibody.

**Chromosomal Assignment**—A mouse P1 genomic clone was obtained by PCR screening of a P1 bacteriophage mouse genomic library with clone 336-specific primers (sense: 5′-TGGTATTCTTATATTGTTGTAACATCTAATC-3′; antisense: 5′-GGGAGGCCTGACCTAATTGTCTGTCATGCA-3′). The isolated p53 clone was seen to hybridize with the 3′-untranslated region of the gene, indicating that they were not related to the p33. The 3′-untranslated region was amplified by PCR of pBSSK/striamin C′ DNA and 3′-mercaptoethanol, sonicated for 2 min on ice, and ligated into pEGFP-C1. Microinjection of pEGFP-C1/striamin was co-injected for the identification of the injected cells. After overnight incubation at 32.5 °C, the cells were fixed with 4% formaldehyde, permeabilized with phosphate-buffered saline containing 0.1% Triton X-100 for 5 min on ice, washed three times with phosphate-buffered saline, and then stained with fluorescein isothiocyanate-conjugated secondary antibodies to detect injected IgG and β-galactosidase expression using the β-gal staining kit (Roche Molecular Biochemicals). Cells were viewed using a Zeiss microscope. All cells showing any trace of blue staining were scored as positive for expression.

In *Vivo Co-immunoprecipitations*—COST cells were used for high transfection efficiencies. Lysates (400 μg) prepared from pEGFP-C1 vector-, pEGFP-C1/striamin-, pEGFP-C1/N striamin-, or pEGFP-C1/C striamin-transfected cells after 48 h of transfection were incubated with anti-p53 antibody (CM-1, Novacastra Laboratories Ltd.) overnight at 4 °C. Immunocomplexes were precipitated by incubating with protein AG-Sepharose (30 min at 4 °C) and were analyzed for the presence of striamin by Western blotting with anti-GFP monoclonal antibody. Precipitation of p53 was detected by Western blotting with anti-p53 monoclonal antibody (Ab-1, Calbiochem).

### Preparation of Recombinant striamin Protein

**The ORF of striamin**—The entire cDNA sequence of striamin was determined by PCR of pEGFP-C1/striamin clone in-frame stop codon (Fig. 1). The full-length cDNA sequence thus obtained had no homology to any sequence in the DNA sequence databases. Analysis of the cDNA sequence by BLAST, PROSITE, GCG, and PSORT programs revealed no known motifs that could predict its possible function. The 5′-noncoding sequence of striamin contains C/GAAA repeats and the 3′-noncoding region contains GT repeats; however, the sequence data bases. The sequence thus obtained had no homology to any sequence in the database. The 3′-untranslated region of the gene, following which it was used for chromosomal localization by fluorescence in situ hybridization (FISH).
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Five fiber phenotypes, namely fast twitch fiber types and slow fiber type I, have been defined based on the basis of expression of the type of myosin heavy chain isoform. Strain was predominantly expressed in fast (quadriceps) versus slow fibers (soleus) (Fig. 3A). Northern analysis using RNA isolated from mouse skeletal muscles of differing fast and slow fiber content (quadriceps: 95% fast 2B, 4% fast 2X (18); extensor digitorum longus: 60% fast 2B, 28% fast 2X, 12% slow; superficial gastrocnemius: 100% fast 2B (20); diaphragm: 57% fast 2X, 34% fast 2A, 7% slow (20); and soleus: 45% fast 2A, 55% slow (21)) revealed that striamin was expressed preferentially in fast glycolytic (2B) fibers. We examined striamin expression during in vitro myogenesis of C2C12 myoblasts. These cells were cultured in differentiation medium, and RNA was isolated at various time points representing gradual formation of myotubes as observed microscopically. Interestingly, a 4-day culture that showed about 80% myotube formation had negligible expression of striamin as compared with the 1- and 2-day cultures, which had about 10–30% myotube formation (Fig. 3C). Consistent with this Northern analysis, striamin RT-PCR from C2C12 cells cultured in serum-supplemented and -deficient medium for 60–72 h exhibited its down-regulated expression in the latter (differentiated myotubes) (Fig. 3B). Cellular Localization of striamin—Northern blotting of mouse skeletal muscle cDNA is underlined up to the 5’ upstream in-frame stop codon. Expression Analyses—Northern blotting of mouse and human tissues showed a strong reactivity of the striamin probe to a 4.0 kb transcript in mouse and human skeletal muscle and heart (Fig. 2, A and B). It also reacted very weakly to transcripts of approximately 4.0 and 8.0 kb from human diaphragm. The induction of the promoter by IPTG led to the synthesis of a transfected plasmid, pEGFP-C1-striamin, encoding a GFP-striamin fusion protein. Transfected cells had distinct green fluorescence (Fig. 4A) in the cytoplasm. Interestingly, a 4-day culture that showed about 80% myotube formation had negligible expression of striamin as compared with the 1- and 2-day cultures, which had about 10–30% myotube formation (Fig. 3C). Consistent with this Northern analysis, striamin RT-PCR from C2C12 cells cultured in serum-supplemented and -deficient medium for 60–72 h exhibited its down-regulated expression in the latter (differentiated myotubes) (Fig. 2C, lanes 7 and 8).
brane (Fig. 4b), and C striamin in addition to its concentration around the nucleus was also distributed diffusely in the cytoplasm (Fig. 4c). The present data and the fact that striamin does not contain any known nuclear localization signal could suggest that the predicted high positive charge of the native protein may be responsible for its nuclear localization. Alternatively, striamin may translocate to the nucleus by interacting with some nuclear localization signal-containing protein.

Wild type (wt) p53 has a role during cell differentiation (reviewed in Ref. 22). Evidence in support of this includes the following: (i) overexpression of exogenous wt p53 or endogenous wt p53 following cell irradiation can partially restore differentiation of several tumor cells (23, 24), (ii) up-regulation of p53 mRNA occurs during C2 differentiation (25), and (iii) interference with endogenous wt p53 inhibits hematopoietic and muscle cell differentiation, which is shown to be independent of its cell cycle activity (8). In view of these reports and characteristics of striamin such as nuclear localization and down-regulation with myogenic differentiation, we asked whether striamin can interfere with p53 activity. p53−/− MEF cells were transfected with the wt p53-responsive luciferase reporter plasmid (PG-13luc), a temperature-sensitive p53 expression plasmid, pMSVp53Val135, and...
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either the pLK444 vector or pLK444/striamin. The presence of the striamin expression construct caused a significant reduction in p53 reporter gene activity in four independent experiments. This result demonstrated that striamin can inhibit the transcriptional activity of p53 (Fig. 5A). Furthermore, cotransfections of the antisense construct were seen to have a mild positive effect on p53 activity (Fig. 5A). Similar results were obtained following microinjection of pMSVp53Val135, the p53-responsive β-gal reporter, pRGCAsfos-lacZ and the various striamin expression plasmids. Injected cells were identified by coinjection of rabbit IgG that was visualized by staining with fluorescein isothiocyanate-conjugated secondary antibody. β-Gal staining was observed in 86% and 88% of cells injected with control and antisense striamin plasmid, respectively, but only in 5% of cells that were injected with striamin sense construct (Fig. 5B). These data confirmed the repression of p53 activity by striamin and were consistent with its down-regulation observed during C2C12 differentiation. To further characterize the specificity of striamin-p53 interactions, we also performed p53 reporter assays in which expression plasmids encoding N-terminal 75 (N/striamin) or C-terminal 74 (C/striamin) amino acid residues were transfected. Whereas full and C striamin were seen to repress p53 activity, transfections of N striamin were neutral (Fig. 5A).

**In Vivo and In Vitro Interactions of striamin and p53**—The effect of striamin on the transcriptional activation function of p53 prompted us to investigate a possible interaction between these two proteins. We used COS7 cells for their high amounts of wt p53 and high transfection efficiencies. p53 immunocomplexes from pEGFPC1- and pEGFPC1/striamin-transfected cells when analyzed by Western blotting with anti-GFP antibody revealed the presence of GFP-striamin (Fig. 5C). This demonstrated that the striamin interacts with p53 in vivo. Similar immunoprecipitations were also performed from cells expressing GFP-N striamin and GFP-C striamin fusion proteins. The full, N-, and C-striamin were found to interact to p53 (Fig. 5C); no coimmunoprecipitation of GFP tag was detected in these experiments.

We next performed an in vitro pull-down assay for striamin and p53. His-tagged recombinant striamin was incubated with either GST alone (negative control) or with GST-p53. Western analysis of glutathione-Sepharose-reacting complexes using an anti-His tag antibody revealed the presence of His-tagged striamin, demonstrating that striamin can physically interact with p53 (Fig. 5D). Furthermore, as in the in vivo coimmunoprecipitations, both N striamin and C striamin were found to bind to p53 (Fig. 5D).

**Chromosomal Localization**—A mouse genomic P1 clone containing the striamin gene was obtained by PCR screening of a P1 library. In FISH analysis, this clone specifically hybridized to a medium-sized chromosome, which appeared to be chromosome 12 on the basis of 4',6'-diamidino-2-phenylindole staining. To confirm the localization of striamin to mouse chromosome 12, FISH analysis was repeated using the P1 clone and chromosome 12 centromere-specific probe. The striamin P1 and chromosome 12 probes localized to the same chromosome (Fig. 6).
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6. A total of 80 metaphase cells were analyzed, out of which 71 exhibited specific labeling. Measurements of specifically hybridized chromosome 12 in 10 metaphase spreads demonstrated that striamin is located at a position that is 57% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome 12, an area that corresponds to band 12C3. This region is syntenic to human chromosome 14q21–22.

DISCUSSION

Here we report cloning and characterization of a novel gene, striamin, whose expression is restricted to the striated muscles. The expression pattern of striamin shares features in common with a few other genes, but is most similar to that of MyoD. Both are expressed in proliferating myoblasts, decline during differentiation, and yet are present in adult skeletal muscle (26). Furthermore, they appear to be preferentially expressed in fast glycolytic muscle fibers in the adult mouse (27). In adult myofibers MyoD is thought to mediate innervation and thyroid hormone effects on fiber type-specific gene expression (26) as well as repress slow isoform gene function (28). Other genes that are specific to fast glycolytic fiber include myosin heavy chain 2B (MyHC 2B) (reviewed in Ref. 29) and a muscle-specific form of the glycolytic enzyme aldolase A (M-aldA) (30). Therefore, striamin may function as a mediator of extrinsic factors on gene expression in fast glycolytic fibers, as a determinant of metabolism, or as a determinant of muscle contractile activity.

Adult skeletal muscle can undergo regeneration, repair, and growth in response to injury or various stresses (31, 32). These processes are achieved by the activation of muscle precursor or satellite cells. In normal skeletal muscle, satellite cells are mitotically quiescent, mononucleated cells that are situated between the basement membrane and the myofiber plasma membrane. Injury or stress results in the mitotic activation of the satellite cells, which proliferate and fuse to repair damaged fibers or increase the size of existing fibers. The progression from proliferating to fusion competent satellite cells is marked by a precise order of expression of myogenic regulatory factors and muscle structural proteins. This includes, in order, MyoD, myogenin, α-smooth muscle actin, and sarcomeric myosin (33). Because striamin is expressed in myoblasts in culture, it is a candidate marker for activated satellite cells and may play a role in the differentiation process in vivo.

striamin is expressed in mouse, but not in human, heart. Differences exist between rodent and human cardiac myofibers in contraction velocities and force production, which in large part reflects the ATPase activity conferred by the MyHC isoform present (34). α-MyHC, the predominant isoform in the rodent heart, confers a faster shortening velocity and low efficiency of force production. In contrast, β-MyHC predominates in the human heart, which has a slower shortening velocity and high efficiency of force production. Rodent and human hearts also differ in the relative amounts of sarcomeric actins present, cardiac and skeletal actin (35, 36), which most likely reflects a difference in force development (37). The combinations of MyHCs and sarcomeric actins in rodent versus human heart results in a rodent heart that is more similar in contractile properties to a fast-twitch skeletal muscle fiber, whereas the opposite is true for the human heart. Therefore, the expression of striamin in striated muscles and mouse heart is consistent with a role in a fast contractile phenotype.

striamin Was Found to Interact with p53 in Vitro and in Vivo—Repression of p53 activity by striamin is consistent with its down-regulation during in vitro myogenesis when significant increase in p53 activity has been reported (22). These data suggest that striamin may affect myogenesis via a direct interaction with p53. Our data suggested that both the N- and C-terminal halves of striamin protein can bind to p53; however, it is the C terminus of striamin that represses transcriptional activity of p53. This suggests that there are more than one p53 binding sites in striamin protein and vice versa. Characterization of these warrant further studies.

The myogenic differentiation program includes activation of myogenic transcription factors, intercellular fusion of myoblasts, their withdrawal from the cell cycle, and terminal differentiation to myotubes. Besides the muscle-specific family of transcription factors, myoD family, several adhesion molecules such as N-CAM, N-cadherin, very late activation antigen 4, vascular cell adhesion molecule 1 (VCAM-1), and melanin-α have been implicated in this process (38–41). Bone morphogenetic protein-12 and -13, TGF-β, and other members of the TGF-β superfamily (42, 43), ERK-6, a mitogen-activated protein kinase (44), and PAX3 (45) have been shown to interfere with or suppress in vitro myogenesis of C2C12 myoblasts. Cyclin D1 is found to be down regulated with myogenesis of C2C12, in contrast to cyclin D2, which showed transient increase, and cyclin D3, which showed 20-fold increase (7, 46). striamin does not show any structural homology to any of these proteins that have been implicated in different aspects of muscle differentiation. Of particular interest are its fast fiber specificity, nuclear localization, down-regulation with myogenic differentiation, and functional interactions with the tumor suppressor p53, which may predict it to be an important gene in the regulation of the myogenic differentiation program and warrant further studies to elucidate its role in myogenesis and the fast contractile phenotype.

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