Requirement for Down-Regulation of the CCAAT-binding Activity of the NF-Y Transcription Factor during Skeletal Muscle Differentiation

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NF-Y is composed of three subunits, NF-YA, NF-YB, and NF-YC, all required for DNA binding. All subunits are expressed in proliferating skeletal muscle cells, whereas NF-YA alone is undetectable in terminally differentiated cells *in vitro*. By immunohistochemistry, we show that the NF-YA protein is not expressed in the nuclei of skeletal and cardiac muscle cells *in vivo*. By chromatin immunoprecipitation experiments, we demonstrate herein that NF-Y does not bind to the CCAAT boxes of target promoters in differentiated muscle cells. Consistent with this, the activity of these promoters is down-regulated in differentiated muscle cells. Finally, forced expression of the NF-YA protein in cells committed to differentiate leads to an impairment in the down-regulation of cyclin A, cyclin B1, and cdk1 expression and is accompanied by a delay in myogenin expression. Thus, our results indicate that the suppression of NF-Y function is of crucial importance for the inhibition of several cell cycle genes and the induction of the early muscle-specific program in postmitotic muscle cells.

INTRODUCTION

The CCAAT-binding transcription factor NF-Y is a heteromeric protein composed of three subunits, NF-YA, NF-YB, and NF-YC, all necessary for CCAAT-binding. NF-YB and NF-YC tight association is a prerequisite for NF-YA binding and sequence-specific DNA interactions. NF-YA and NF-YC have Q-rich activation domains. Both NF-YB and NF-YC contain putative histone fold motifs (Mantovani, 1999). The CCAAT motif is present in 25% of eukaryotic promoters and NF-Y has been shown to bind >120 of these (CCAAT-containing) promoters (Mantovani, 1998). In accordance with the widespread presence of CCAAT boxes, NF-Y subunits are extremely conserved and have been identified in several eukaryotic kingdoms (Mantovani, 1999).

The NF-Y complex supports the basal transcription of a class of regulatory genes responsible for cell cycle progression, among which are cyclin A, B1, B2, cdc25B, cdc25C, and cdk1 (Zwicker *et al.*, 1995a,b; Bolognese *et al.*, 1999; Farina *et al.*, 1999; Korner *et al.*, 2001). Consistent with this, the over-

expression of a dominant negative mutant of the NF-YA subunit that inhibits DNA binding of the endogenous NF-Y results in retardation of fibroblast growth (Hu and Maity, 2000). We previously demonstrated that the cyclin B1 promoter activity is switched off during myogenic differentiation due to the loss of a functional NF-Y complex (Farina *et al.*, 1999). This evidence supports the hypothesis that the transcription of several cell cycle regulatory genes is down-regulated in differentiated muscle cells through the loss of NF-Y activity.

The differential expression of NF-YA, resulting in an alteration of NF-Y CCAAT binding activity, has been observed in several cell lines both during cell cycle progression and under specific conditions. NF-YA expression is modulated during the cell cycle, being high in G1, further increasing in S and then decreasing in the G₂/M phase (Bolognese *et al.*, 1999). Reduction of NF-YA expression has been reported in IMR-90 fibroblasts after serum deprivation and in human monocytes (Chang and Liu, 1994; Marziali *et al.*, 1997). Complete down-regulation of NF-YA protein expression occurs in terminally differentiated C2C12 muscle cells. The loss of NF-YA expression results in the loss of a functional NF-Y complex, suggesting that, although NF-Y is a ubiquitous transcription factor, differential expression of NF-YA subunit can occur

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during growth and differentiation of different cell lines (Farina *et al.*, 1999).

Permanent down-regulation of the NF-YA subunit could play a particularly important role in reducing the expression of several cell cycle control genes in differentiated muscle cells. In the present study, we show that NF-YA is not expressed in the nuclei of adult skeletal and cardiac muscle tissues, whereas NF-YB and NF-YC are still expressed. Consistent with this, by chromatin immunoprecipitation and genomic footprinting experiments, we demonstrate that *in vivo* cell cycle regulatory genes are targets for the NF-Y binding activity only in proliferating muscle cells. Interestingly, forced expression of NF-YA in cells committed to differentiate leads to a reduction in the down-regulation of cyclin B1, cyclin A, and cdk1 expression. Moreover, exogenous NF-YA protein interferes with the induction of p21, cyclin D3, and myogenin expression occurring early during muscle differentiation.

MATERIALS AND METHODS

Cell Culture

The mouse myoblast C2C12 cell line (Blau *et al.*, 1985), a clone derived from the C2C12 cell line (Yaffe and Saxel, 1977), was cultured in DMEM containing 10% fetal bovine serum (proliferating; P). Differentiation was induced by plating the cells into collagen-coated dishes and switching them for 72 h (terminally differentiated; TD) to serum-free (SF) medium: DMEM supplemented with ReduSer (Upstate Biotechnology, Lake Placid, NY) to a final concentration of 5 μ g/ml human insulin, 5 μ g/ml human (holo) transferrin, and 5 ng/ml sodium selenite. Cytosine arabinoside (50 μ M; Ara-C) was added to the SF medium to eliminate undifferentiated cells. As previously described (Tiainen *et al.*, 1996), under these conditions more than 90% of the cells become terminally differentiated (TD). For the analysis of cell cycle control and muscle-specific gene expression after overexpression of NF-YA protein (Figure 6), the cells were induced to differentiate in the absence of Ara-C for 6, 12, 24, and 48 h.

Total Cell and Tissue Lysates and Western Blot Analysis

Total cell extracts were prepared by incubating C2C12 cells in 0.15 M NaCl buffer containing 50 mM Tris-HCl, pH 8, 5 mM EDTA, 1% NP-40, 0.5% deoxycholic acid, 10 μ g/ml leupeptin, 4 μ g/ml pepstatin, 5 μ g/ml aprotinin, 50 mM NaF, 1 mM Na-orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). BDF1 wild-type adult mice were sacrificed by vertebral dislocation. Organs were dissected, immediately frozen in liquid nitrogen, and stored at -70°C until used. Purified muscle and fibroblast were obtained by treatment of the tissue with trypsin, collagenase, and mechanical disruption. Total tissue extracts were prepared by homogenization in lysis buffer (20 mM Tris-HCl, pH 8, 1% NP-40, 10% glycerol, 137 mM NaCl, 5 mM EDTA pH 8, 5 mM EGTA pH 7, 1 mM Na-orthovanadate, 50 mM NaF, 5 μ g/ml Aprotinin, 10 μ g/ml Leupeptin, 10 mM PMSF) on dry ice. Total C2C12 lysate (50 μ g) and 150 μ g of total tissue lysate were separated by SDS-PAGE (12.5%) and electroblotted onto nitrocellulose. After blocking with 5% nonfat dried milk, the filters containing tissue lysates were immunoreacted with each of the following antibodies: anti-NF-YA rabbit polyclonal antibody (0.3 μ g/ml) (Rockland, Gilbertsville, PA), anti-NF-YB rabbit polyclonal antibody (0.3 μ g/ml) (Mantovani *et al.*, 1994), anti-NF-YC rabbit polyclonal antibody (0.3 μ g/ml) (Mantovani *et al.*, 1994), anti-Hsp70 mouse monoclonal antibody (mAb) (0.25 μ g/ml) (StressGen Biotechnologies, Victoria, Canada). The filters containing C2C12 lysates were immunoreacted with 0.3 μ g/ml anti-

NF-YA rabbit polyclonal antibody (Rockland), 0.1 μ g/ml anti-cyclin B1 rabbit polyclonal antibody, 0.1 μ g/ml anti-cyclin A rabbit polyclonal antibody, 0.1 μ g/ml anti-cdk1 p34 rabbit polyclonal antibody, 1 μ g/ml anti-myogenin rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:500 anti-mouse p21 (kindly provided by C. Schneider, Laboratorio Nazionale CIB, Trieste, Italy), rabbit polyclonal antibody 0.25 μ g/ml anti-Hsp70 mouse mAb (StressGen, Biotechnologies), following the manufacturer's directions. Peroxidase activity of the appropriate secondary antibodies was visualized by enhanced chemiluminescence detection system (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom).

Immunohistochemistry

BDF1 wild-type adult mice were sacrificed by vertebral dislocation, and tissues were fixed in 100% methanol at 4°C , dehydrated, and embedded in paraffin. Five-micrometer-thick serial sections were mounted on slides, deparaffinized in xylene, and rehydrated. Sections were washed in phosphate-buffered saline (PBS), incubated 30 min. in 1 M glycine, pH 7.8, at 4°C , 10 min in 3% H_2O_2 , and incubated with 5 μ g/100 μ l anti-NF-YA rabbit polyclonal antibody (Rockland) and 2 μ g/100 μ l anti-NF-YB rabbit polyclonal antibody (Mantovani *et al.*, 1994) for 1 h at room temperature. Sections were washed four times in PBS, 1% bovine serum albumin (BSA), 0.1% Triton X-100 and incubated with a peroxidase-conjugated goat anti-rabbit antibody as secondary antibody. Sections were then washed in PBS, 0.2% BSA, 0.1% Triton X-100 and counterstained with hematoxylin. Peroxidase activity of the secondary antibodies was visualized by reaction with 3,3'-diaminobenzidine and H_2O_2 in sodium phosphate buffer.

Electromobility Shift Assays (EMSAs)

Electromobility shift assays were performed in a 25 μ l of DNA binding reaction that contained 150 μ g of murine tissues extracts, 6 fmol of labeled duplex oligonucleotide, binding buffer (20 mM Tris-HCl, pH 7.8, 60 mM KCl, 0.5 mM EDTA pH 8, 0.1 mM dithiothreitol, 3 mM MgCl_2), 1.5 μ g of poly(dI-dC), and 10 mM spermidine. The reaction was carried out on ice for 30 min and the protein-DNA complexes were subjected to native electrophoresis on 5% polyacrylamide, $0.5 \times$ TBE gels. The following oligonucleotides used as probes or as autocompetitor (300 fmol) consensus sites are underlined: B1up CCAAT, 5'-CCG CAG CCG CCA ATG GGA AGG GAG TGA; B1down CCAAT, 5'-GAA CAG GCC AAT AAG GAG GGA G; and unrelated competitor (1500 fmol): cyclin B1 (SP1) up, 5'-GGG CTG TGG CCC CGC CCC TCT C, cyclin B1 (SP1) down, 5'-GGG GAG AGG GGC GGG GCC ACA G. Production and purification of recombinant NF-YA protein were described previously (Mantovani *et al.*, 1994).

Formaldehyde Cross-linking and Chromatin Immunoprecipitations

Formaldehyde cross-linking and chromatin immunoprecipitations were performed as described previously (Fontemaggi *et al.*, 2001). Formaldehyde was added directly to proliferating and terminally differentiated C2C12 cells at a final concentration of 1% at 22°C for 10 min. The reaction was stopped by addition of glycine to a final concentration of 0.125 M. The cells were then rinsed with cold $1 \times$ PBS, incubated with $0.2 \times$ trypsin-EDTA in $1 \times$ PBS, and scraped. Cells were collected by centrifugation, washed in cold $1 \times$ PBS plus 0.5 mM PMSF, and resuspended in lysis buffer (5 mM piperazine *N,N* bis zethone sulfonic acid, pH 8, 85 mM KCl, 0.5% NP-40). Nuclei were solicated in the sonication buffer (0.1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, 0.5% deoxycholic acid) 1 time for 10 min by using microultrasonic cell disruptor. The chromatin was sheared to an average size of 500 base pairs, and immunoprecipitation was performed with protein G-agarose (KPL, Gaithersburg,

MD). The chromatin solution was precleared by adding protein G for 1 h at 4°C, aliquoted (each aliquot corresponding to 2×10^6 cells), and incubated with 2 μ g of affinity-purified rabbit polyclonal antibodies anti-NF-YB 2 μ g of affinity-purified rabbit polyclonal antibodies anti-NF-YA (Santa Cruz Biotechnology), and 2 μ g of affinity-purified rabbit polyclonal antibodies anti-E2F1 (Santa Cruz Biotechnology) overnight at 4°C with mild shaking. Before use, protein G was blocked with 1 μ g/ μ l sheared herring sperm DNA and 1 μ g/ μ l BSA for 3 h at 4°C and then incubated with chromatin and antibody for 2 h at 4°C. Immunoprecipitates were washed 10 times by adding 1 ml of immunoprecipitation buffer. The control sample supernatant (not immunoprecipitated with antibody) was collected as input sample, before washing. Immunoprecipitates were eluted from the beads with elution buffer (1% SDS, 50 mM NaHCO₃, 1.5 μ g/ml sonicated salmon sperm) at 37°C for 30 min with vigorous shaking. The samples were heated at 65°C overnight to reverse formaldehyde cross-links and ethanol precipitated. Recovered material was treated with 25 μ l of proteinase K buffer (1.25% SDS, 50 mM Tris, pH 7.5, 25 mM EDTA, pH 8), 1.5 μ l of proteinase K (Roche Diagnostics, Mannheim, Germany), 100 μ l of 1 $\times$ TE (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8), extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and ethanol precipitated. The pellets were resuspended in 30 μ l of H₂O and analyzed by using polymerase chain reaction (PCR). Total input sample was resuspended in 100 μ l of H₂O and diluted 1:100 before PCR. For PCR analysis on cyclin B2 and cyclin A, the following oligonucleotides were used: cyclin B2 up3, 5'-TGT AGA CAA GGA AAC AAC AAA GCC TGG TGG CC; cyclin B2 down2, 5'-CAG CCA CTC CGG TCT GCG ACA; cyclin A up2, 5'-CTG TAA GAT TCC CGT CGG GCC TTC G; and cyclin A down2, 5'-GTA GAG CCC AGG AGC CGC GAG.

In Vivo DNA Footprinting

The in vivo DNA footprinting was performed by using ligation-mediated PCR as described previously (Dey *et al.*, 1992). P and TD cells were treated with 0.1% dimethyl sulfate (DMS) for 2 min. After DMS treatment, cells were washed three times with cold PBS. DNA was isolated and cleaved with piperidine. Labeled PCR products were resolved on a 6% polyacrylamide-8 M urea sequencing gel. In vitro control DNA samples consisted of purified naked DNA from HeLa cells treated with 0.125% DMS for 2 min and cleaved with piperidine as described above. The following oligonucleotides were used. For cyclin B2, first primer, TM = 45°C 5'-ATATCAGGGAC-TAGAATTTG; second primer, TM = 61.4°C 5'-GACTGTAGACAAGGAAACAACAAAGCCTG; and third primer, TM = 65°C 5'-TGTAGACAAGGAAACAACAAAGCCTGGTGGCC. For cyclin A, first primer, TM = 54°C 5'-GCGGGAGGAGCGTAGAG; second primer, TM = 65.8°C 5'-GTAGAGCCCAGGAGCCGCGAG; and third primer, TM = 70°C 5'-AAGATTCCCGTCGGGCCTTCGCTCG.

DNA Transfections, Chloramphenicol Acetyltransferase (CAT), and Luciferase Assays

Stable transfections were performed as described previously (Farina *et al.*, 1999). CAT or Luc-reporter constructs carrying the cyclin B1 (Piaggio *et al.*, 1995), cdk1 (Dalton, 1992), cyclin A (Schulze *et al.*, 1995), cyclin B2 (Bolognese *et al.*, 1999), cdc25A (Vigo *et al.*, 1999), cdc25C (Manni *et al.*, 2001), and cyclin E (Botz *et al.*, 1996) were stably transfected by calcium phosphate in C2C12 cells. The obtained polyclonal cell lines were analyzed for CAT or luciferase activity. Transient transfections of p21 (kindly provided by G. Blandino, Regina Elena Cancer Institute, Rome, Italy), and myogenin (kindly provided by P.L. Puri, University of Rome, La Sapienza, Italy), promoters were performed by calcium-phosphate precipitation in proliferating C2C12 cells. Transient NF-YA overexpression was performed using the FuGENE 6 transfection reagent (catalog no. 1814443; Roche Diagnostics). Cells (1×10^6) were transfected

with 10 μ g of the eukaryotic expression vector containing the human wild-type cDNA for the NF-YA subunit under the control of the simian virus 40 promoter (NF-YA-polyII), or the vector alone (polyII), by using 40 μ l of FuGENE 6 transfection reagent. Cells were incubated with transfection medium for 16 h, and differentiation was induced after transfection by adding SF medium in the absence of Ara-C for 6, 12, 24, and 48 h. The transfection efficiency (~40%) was evaluated by scoring green fluorescent protein-positive cells in a parallel sample transfected with green fluorescent protein vector under the same experimental conditions.

Creatine Kinase Assay

Creatine kinase activity was assayed spectrophotometrically using a diagnostic kit (Sigma-Aldrich, St. Louis, MO). Briefly, 0-, 6-, 12-, 24-, and 48-h cultures of NF-YA or vector alone (polyII)-transfected cells grown in differentiation medium were washed twice with ice-cold PBS and scraped. Cells were spun at $1000 \times g$ and resuspended in PBS containing 0.1% Tween 20 and incubated on ice for 15 min. The lysates were centrifuged at $10,000 \times g$ for 15 min at 4°C, and the protein concentration in the supernatant was determined using the Bio-Rad protein assay reagent. Protein content in the samples was then adjusted to 0.4 μ g/ μ l with PBS containing 0.1% Tween 20, and, typically, 100 μ l of sample was used for creatine kinase assay according to the manufacturer's instructions. The activity of the enzyme was determined by measuring the absorbance at 510 nm and expressed as units per milligram of protein. A unit of creatine kinase activity was defined as (A_{510} units/min $\cdot$ 1000)/6.22 (extinction coefficient).

RESULTS

NF-YA Is Not Expressed in Adult Muscle Tissues

To start to investigate NF-Y expression in vivo, we performed Western blots on total protein extracts from different murine adult tissues. Figure 1A shows that the NF-YB and NF-YC subunits are expressed in all tested tissues, although to a different extent, whereas NF-YA is not detectable even upon longer exposure in skeletal muscle and heart. To confirm these observations in intact cells, we performed immunohistochemical analyses on murine skeletal and cardiac muscle tissues. The anti-NF-YA antibody did not show immunoreactivity in skeletal muscle or heart sections, whereas staining with this antibody was observed in brain. In contrast to this, an anti-NF-YB antibody showed immunoreactivity in muscle and heart as well as in brain (Figure 1B). We conclude that apparently NF-YA is not ubiquitously expressed, being undetectable in adult skeletal muscle and heart tissues, whereas the NF-YB/NF-YC heterodimer is ubiquitously expressed, being detectable in all tissues tested.

CCAAT Binding Activity of NF-Y Is Lost in Adult Muscle Tissues

To assess whether loss of the NF-YA subunit leads to loss of the CCAAT binding activity of NF-Y, we performed electrophoretic mobility shift assays with extracts from adult mouse tissues, by using an oligo containing the cyclin B1 CCAAT box (Farina *et al.*, 1999). The results demonstrate that the DNA binding activity of NF-Y is lost in skeletal and cardiac muscle extracts, whereas, as expected, it is present in the other tested tissues (Figure 2A). The lack of NF-Y binding to the CCAAT box in muscle and heart is due to the loss of the NF-YA protein as demonstrated by EMSA experiments performed in the presence of recombinant purified NF-YA pro-

Figure 1. NF-YA is not expressed in adult muscle tissues. (A) Western blot analysis was performed on total lysates from the indicated tissues. The extracts were probed with rabbit polyclonal antiserum raised against NF-YA, NF-YB, and NF-YC. To normalize protein's loading, the filter was stained with a mAb against the HSP70 protein. (B) Murine skeletal muscle, heart, and brain tissues were methanol fixed and paraffin-embedded before sectioning. Sections were incubated with polyclonal rabbit antibodies against NF-YA or NF-YB as primary antibodies and/or with peroxidase-conjugated goat anti-rabbit antibody as secondary antibody. The peroxidase activity was detected with 3,3'-diaminobenzidine before counterstaining the sections with hematoxylin.

tein. As shown in Figure 2B, the addition of 150 ng of purified NF-YA protein to skeletal muscle (lane 5) and heart (lane 8) extracts restores the binding activity of the NF-Y complex. The addition of purified NF-YA protein to brain extract (lane 2) does not substantially modify the complex mobility. Together, the results reported in Figures 1 and 2 demonstrate that of NF-YA protein cannot be detected in

terminally differentiated muscle tissues and clearly indicate that NF-YA is the limiting factor for the DNA binding activity of NF-Y complex in these tissues.

NF-Y Does Not Bind Cell Cycle-dependent Promoters in Terminally Differentiated Cells In Vivo

NF-Y supports the basal transcription of a class of regulatory genes responsible for cell cycle progression, among which are cyclin A, B1, B2, cdc25B, cdc25C, and cdk1 (Zwicker *et al.*, 1995a,b; Bolognese *et al.*, 1999; Farina *et al.*, 1999; Korner *et al.*, 2001; Manni *et al.*, 2001). To investigate the DNA-binding activity of NF-Y on cell cycle target promoters in terminally differentiated cells, we performed chromatin immunoprecipitation experiments on P and TD C2C12 skeletal muscle cells. Proliferating cells were induced to differentiate by growth factors withdrawal for 72 h. P and TD cells were treated with formaldehyde to cross-link proteins to DNA. After sonication, the cross-linked chromatin from equivalent numbers of P and TD cells was immunoprecipitated by using anti-NF-YB or anti-NF-YA antibodies. As negative controls, we included a reaction lacking primary antibody and one containing an anti-E2F1 antibody. E2F1 is a nuclear transcription factor that does not have a binding site on the cyclin B2 promoter. After immunoprecipitation, enrichment for the endogenous cyclin B2 and A promoter fragments in each sample was assessed by PCR amplification by using primers amplifying the cyclin B2 promoter region from -145 to +90 base pairs and the cyclin A promoter region from -143 to +89 base pairs, respectively. As shown in Figure 3, the anti-NF-YB and the anti-NF-YA antibodies did not immunoprecipitate the cyclin B2 (lanes 7 and 8) and cyclin A (lanes 19 and 20) promoters from differentiated chromatin. As expected, both promoters were present in the NF-YB and NF-YA immunoprecipitates in chromatin from proliferating cells (lanes 1, 2, 13, and 14). The anti-E2F1 antibody was unable to immunoprecipitate cyclin B2 promoter from both cell types (lanes 3 and 9), whereas it immunoprecipitated the cyclin A promoter in proliferating cells (lane 15). This result is in agreement with recent data demonstrating that E2F1 binds to the cyclin A promoter in proliferating glioblastoma cells (Takahashi *et al.*, 2000).

It has been described that the CCAAT displacement protein is able to displace NF-Y from several promoters containing CCAAT boxes (Nepveu, 2001). Thus, we asked whether in differentiated cells the CCAAT boxes of the cyclin B2 and cyclin A promoters are bound by proteins, other than NF-Y. To answer this question, we performed in vivo genomic footprinting analysis of both promoters. P and TD C2C12 cells were treated with the DNA-alkylating reagent DMS, and methylated G residues were identified using the ligation-mediated PCR technique (Dey *et al.*, 1992). As shown in Figure 4, in proliferating cells the CCAAT boxes of both the cyclin B2 and cyclin A promoters were protected from DMS methylation (lanes 3 and 6). In contrast, the CCAAT boxes and the flanking regions of these promoters were devoid of interactions with proteins in differentiated cells (lanes 2 and 4).

Together, these results provide further in vivo evidence for a lack of NF-Y binding activity to the CCAAT boxes of the cell cycle target promoters in skeletal muscle differentiated cells.

Figure 2. (A) CCAAT binding activity of NF-Y is lost in adult muscle tissues. EMSA were performed with B1upCCAAT 32 P-labeled oligonucleotide and 150 μ g of total extracts from the indicated tissues. The complex was specifically cross-competed by a 200-fold molar excess of unlabeled oligonucleotide (brain + autocomp.), but not by a 1000-fold molar excess of an unrelated oligonucleotide (brain + unrelated). (B) NF-YA subunit is the limiting factor for the binding of NF-Y complex to the CCAAT box. EMSAs were performed as described above, except for the adding of recombinant purified NF-YA protein (rYA) to the extracts from purified muscle, heart, and brain.

Promoter Activities of NF-Y-Target Cell Cycle Genes Are Down-Regulated in Differentiated Muscle Cells

We queried whether the lack of NF-Y binding activity to the CCAAT boxes leads to down-regulation of the activities of CCAAT-containing promoters in terminally differentiated cells. To this end, C2C12 cells were stably transfected with CAT reporter constructs carrying human cyclin B1 (Piaggio *et al.*, 1995), and human cdk1 (Dalton, 1992) promoter fragments, and luciferase reporter constructs carrying human

cyclin A (Schulze *et al.*, 1995), murine cyclin B2 (Bolognese *et al.*, 1999), human cdc25A (Vigo *et al.*, 1999), human cdc25C (Manni *et al.*, 2001), murine cyclin E promoter fragments. Except for cyclin E promoter, it has been demonstrated that NF-Y contributes to support the basal activity of these promoters in proliferating cells (Zwicker *et al.*, 1995a,b; Botz *et al.*, 1996; Huet *et al.*, 1996; Kramer *et al.*, 1997; Plet *et al.*, 1997; Liu *et al.*, 1998; Farina *et al.*, 1999; Yun *et al.*, 1999; Jung *et al.*, 2001; Manni *et al.*, 2001; Sciortino *et al.*, 2001). Figure 5 shows

Figure 3. NF-Y does not bind cell cycle-dependent promoters in terminally differentiated cells in vivo. Formaldehyde cross-linked chromatin was prepared from the same number of P and TD cells and was incubated with antibodies to NF-Y (lanes 1, 2, 7, 8, 13, 14, 19, and 20), E2F1 (lanes 3, 9, 15, and 21), or no antibody (lanes 4, 10, 16, and 22). Immunoprecipitates from each antibody were aliquoted and then analyzed by PCR with specific primers for the mouse cyclin B2 or cyclin A promoters. A sample representing 0.02% of the total input chromatin (input) was included in the PCR reactions.

Figure 4. Cyclin B2 and cyclin A promoters are devoid of sequence-specific transcription factor interactions in differentiated cells. Genomic footprinting of the coding strand of the mouse cyclin B2 and the noncoding strand of the mouse cyclin A promoters from P and TD C2C12 cells. As a control, the DMS reactivity for DNA purified from asynchronous C2C12 cells is shown (in vitro). The CCAAT boxes are indicated.

that all of promoter constructs are capable of driving transcription only in proliferating cells, whereas their activities are down-regulated in TD cells. Interestingly, the cyclin E promoter fragment that does not contain CCAAT boxes is still active in TD cells.

Forced Expression of NF-YA During the Muscle Differentiation Program Inhibits the Down-Regulation of Several Cell Cycle Gene Expression and the Induction of p21, Myogenin, and Creatine Kinase Activity

Normal differentiation of myoblasts is accompanied by a change in the expression of both cell cycle and muscle-specific genes. Initiation of muscle-specific gene expression during differentiation of muscle cells is preceded by withdrawal of the cells from the cell cycle (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990). Cell cycle arrest is a requirement for muscle differentiation, and it is accomplished by down-regulation of cyclins, with the exception of cyclin D3, which is up-regulated during differentiation and contributes to cell cycle withdrawal (Cenciarelli *et al.*, 1999). To investigate whether the presence of NF-YA could influence the gene expression profile of differentiated cells, we transiently transfected proliferating C2C12 cells with either an empty vector or a vector encoding NF-YA and then induced the cells to differentiate by withdrawing growth factors for 6, 12,

Figure 5. Promoter activities of NF-Y cell cycle target genes are down-regulated in differentiated muscle cells. CAT or Luc-reporter constructs carrying the cyclin B1, cdk1, cyclin A, cyclin B2, cdc25A, cdc25C, and cyclin E promoters were stably transfected by calcium-phosphate in C2C12 cells. The obtained polyclonal cell lines were analyzed for CAT or luciferase activity. The values in TD cells are expressed, on the y-axis, as percentages of the CAT or luciferase activity obtained from proliferating cells (100%). Results represent one typical experiment performed in triplicate.

24, and 48 h. We did not test the effect of NF-YA in terminally differentiated cells (72 h of growth factor withdrawal) because we have reported previously that in C2C12 cells transfected with NF-YA, this protein cannot be detected after terminal differentiation (Farina *et al.*, 1999). Moreover, because the rationale of this experiment was that the presence of NF-YA could retain the cells in the cell cycle, the cells were induced to differentiate in the absence of Ara-C. Indeed, in the presence of Ara-C the proliferating cells would be killed. In this experimental system, we examined the endogenous expression of several cell cycle regulatory genes by immunoblot. As shown in Figure 6A, the cells transfected with the NF-YA subunit (+) displayed an impairment in the down-regulation of cyclin B1 and cyclin A protein expression compared with cells transfected with the empty vector (–), as manifested by the presence of these proteins after 48 h of growth factor withdrawal. In cells transfected with the empty vector cdk1 expression started to be down-regulated after 24 h of growth factor withdrawal, when endogenous NF-YA is still present, thus indicating that cdk1 expression is controlled by factors different than NF-Y. However, the down-regulation of cdk1 expression is, at least in part, impaired by the presence of NF-YA after 48 h of growth factor withdrawal. Moreover, the presence of the NF-YA protein merely delayed, at least at early times, the up-regulation of the cell cycle inhibitor p21 and slightly reduced the up-regulation of cyclin D3. Consistent with this, NF-YA overexpression leads to a delay in the induction of myogenin, an early muscle differentiation marker, whereas there was no effect on MyoD expression (Sassoon, 1993; Buckingham, 1994; Tiainen *et al.*, 1996). We next examined the activity of creatine kinase, a muscle-specific enzyme marker, in cell lysates. As shown in Figure 6B, cells transfected with the empty vector exhibited an approximately eightfold increase in creatine kinase activity up to 48 h of culture in differentiation medium. NF-YA-transfected cells

Figure 6. Forced expression of NF-YA in differentiated muscle cells inhibits the down-regulation of several cell cycle gene expression and the induction of p21, myogenin, and creatine kinase activity. C2C12 cells were transiently transfected with a plasmid carrying NF-YA (+) or the empty vector (-) as indicated. After transfection, cells were grown in the absence of growth factor for 6, 12, 24, or 48 h and lysed to prepare whole cell extract (WCE) (A) and measured creatine kinase activity (B). WCEs from cells cultured in growth medium (GM) and in the absence of growth factor were subjected to Western blot analysis by using antibodies against the indicated proteins. (B) Creatine kinase activity was measured on extracts prepared from transfected and untransfected cells. Results are the mean $\pm$ S.E. of three independent experiments.

showed only an approximate twofold greater creatine kinase activity after 48 h of growth factor withdrawal. The results presented herein indicate that the down-regulation of NF-YA expression is strictly required for the correct modulation of the cell cycle and muscle-specific gene expression during differentiation of muscle cells. These results also suggest that NF-YA interferes, at least in part, with the myogenic differentiation program of C2C12 cells. To further investigate whether NF-YA directly regulates transcription of genes involved in specifying myogenic fate, we used a dominant-negative NF-YA protein, YA13m29 (Mantovani *et al.*, 1994). On transfection in mammalian cells, this vector expresses a mutant protein containing a triple amino acids substitution in the NF-YA DNA binding subdomain enabling the subunit to interact with the NF-YB-NF-YC dimer.

Figure 7. NF-YA does not directly modulate the transcriptional activity of p21 and myogenin promoters. Eighty nanograms of a vector carrying p21 promoter (A) and 2 μ g of a vector carrying myogenin promoter (B) have been transiently cotransfected in proliferating C2C12 cells together with the same amount of the empty vector or vector expressing wild-type or mutant (YA13m29) NF-YA proteins. As positive control, vector carrying p21 promoter has been cotransfected with an eukaryotic vector expressing p53 protein, and the luciferase activity of the vector carrying myogenin promoter has been measured in low-serum (1%) growth condition. As internal control of transfection efficiency, all samples have been cotransfected with the CMV β gal reporter construct. Values are the means $\pm$ standard deviations of four independent experiments.

The resulting trimer is inactive in terms of CCAAT recognition. The dominant-negative or the wild-type NF-YA proteins were cotransfected with constructs carrying p21 or myogenin promoters driving the expression of the luciferase reporter gene in proliferating C2C12 cells, and the promoter activities were determined (Figure 7, A and B). As expected for promoters that do not contain CCAAT boxes, the dominant-negative as well as the wild-type NF-YA proteins do not modulate the activities of both promoters. In contrast, the activities of p21 and myogenin promoters are induced by p53 and serum deprivation, respectively, as described previously (Edmondson *et al.*, 1992; D'Orazi *et al.*, 2002).

DISCUSSION

The results presented in this study show that the CCAAT binding activity of the NF-Y transcription factor is absent in adult skeletal and cardiac muscle tissues and that loss of this activity is due to the down-regulation of the NF-YA protein

in these tissues. The relevance of this observation resides in the fact that recent studies, in various cell types, have indicated that NF-Y could serve as a common transcription factor for an increasing number of cell cycle control genes. For instance, the cyclin B1, cyclin B2, cyclin A, cdc25B, cdc25C, and cdk1 genes all contain in their promoters NF-Y sites, which are required for their transcriptional activation at S phase (Zwicker *et al.*, 1995a,b; Bolognese *et al.*, 1999; Korner and Muller, 2001; Manni *et al.*, 2001). We have already demonstrated that the loss of a functional NF-Y complex in differentiated muscle cells leads to down-regulation of the cyclin B1 promoter (Farina *et al.*, 1999). We now extend this observation to the promoters of the other cell cycle control genes that are known to be regulated by NF-Y during the cell cycle. Moreover, the results of the *in vivo* chromatin immunoprecipitation experiments on the cyclin B2 and cyclin A promoters indicate that down-regulation of these genes in differentiated cells could be due to the absence of NF-Y on their promoters. Interestingly, the lack of NF-Y binding to cyclin B2 and cyclin A promoters in differentiated cells does not lead to the binding of other proteins to the CCAAT boxes as demonstrated by *in vivo* genomic footprinting experiments. These results provide evidence for a model in which NF-Y serves as a common transcription factor in proliferating muscle cells (myoblasts) for several key cell cycle control genes. Permanent down-regulation of NF-YA expression in differentiated cells (myotubes) leads to the inhibition of the expression of these genes. A recent analysis of a large database of 1031 human promoters indicated that the CCAAT box is present in 63% of them (Suzuki *et al.*, 2001). It will be of interest to investigate whether the transcription from other CCAAT-containing promoters, in postmitotic skeletal muscle cells, is down-regulated by the same common mechanism presently described.

In adult muscle tissues, the expression of NF-YA protein is down-regulated, whereas NF-YB and -C are still expressed. It has been demonstrated that the NF-Y histone-like dimer NF-YB/C can form complexes with H3-H4 histones for whose formation the CCAAT box is not required. Moreover, the NF-Y histone-like dimer associates with DNA during nucleosome formation in the absence of NF-YA (Caretto *et al.*, 1999). Proteins involved in histone acetylation are found associated with NF-YB (Currie, 1998; Li *et al.*, 1998). This evidence supports the hypothesis that the NF-YB/C heterodimer could serve the dual function of binding NF-YA to activate transcription of CCAAT-containing promoters and, thanks to the histone-like domains, associating DNA during nucleosome formation. Thus, skeletal muscle cells represent an excellent system to study whether the NF-YB/C dimer exerts a biological role in the absence of NF-YA.

Initiation of muscle-specific gene expression, i.e., myogenin, during differentiation of muscle cells, is preceded by withdrawal of the cells from the cell cycle. Exit from the cell cycle is accomplished by down-regulation of several cyclins and cdk1 and by up-regulation of cell cycle inhibitors, such as p21, and cyclin D3 (Perry and Rudnick, 2000). We demonstrate herein that ectopic expression of NF-YA protein could interfere with the onset of both cell cycle withdrawal and early muscle-specific program. Although NF-YA directly binds the endogenous promoters of the analyzed cell cycle regulatory genes, cyclin A and cyclin B2, as well as cyclin B1 (Sciortino *et al.*, 2001), and cdk1 (A. Gurtner, per-

sonal observation), NF-YA does not directly interfere with the muscle differentiation program. Indeed, our results indicate that NF-YA does not modulate the transcriptional activity of p21 and myogenin promoters. However, unrestricted expression of NF-YA interferes, although in an indirect way, with the differentiation process as indicated by the inhibition of creatine kinase activity occurring during differentiation in the presence of NF-YA.

Together, the above-mentioned results are consistent with a scenario in which NF-YA expression inhibits the differentiation program and keeps the myoblasts in the cell cycle. Because the induction of p21, cyclin D3, and myogenin (Molkentin and Olson, 1996; Cenciarelli *et al.*, 1999), as well as the down-regulation of the of cell cycle control genes (Perry and Rudnick, 2000), are important events in the induction of muscle differentiation, our data suggest that NF-Y exerts a biological role in the switch from proliferation to differentiation of muscle cells.

Although the presence of NF-YA in cells committed to differentiate could lead to conflicting signals, no apparent apoptosis was seen in our experimental conditions. It remains to be determined whether expression of NF-YA in the presence of differentiation stimuli for periods of time longer than those tested herein leads to apoptosis. Indeed, the level of NF-YA drops between 24 and 48 h in the transfected cells and after 72 h of growth factor withdrawal it becomes undetectable (P. Fuschi, unpublished data). This could explain why overexpression of NF-YA can only delay rather than inhibit differentiation.

Our previous results indicate that the regulation of NF-YA expression depends on a posttranscriptional level of regulation (Farina *et al.*, 1999). Based on these considerations, we tested the effect of the specific calpain I and II and cathepsin inhibitor LN-acetyl-leu-leu-norleucinal on NF-Y stability (A. Gurtner, I. Manni, and P. Fuschi, unpublished data). These preliminary experiments show that there is a cytoplasmatic increase of NF-YA protein, suggesting that the degradation of NF-YA involves a ubiquitine-proteasome pathway. The study of the regulation of NF-Y expression in adult muscle tissues, in particular of the NF-YA subunit, will be an important step toward a full understanding of cell cycle control in these cells.

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