Dynamic Transcriptional Regulatory Complexes, Including E2F4, p107, p130, and Sp1, Control Fibroblast Growth Factor Receptor 1 Gene Expression during Myogenesis*

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Developed controlled transcriptional regulation of myogenic cell proliferation and differentiation via expression of the fibroblast growth factor receptor 1 (FGFR1) gene is positively regulated by Sp1 and negatively regulated by E2F4-based transcriptional complexes. We report that p107 and p130 formed transcriptional complexes with E2F4 on the FGFR1 promoter and repressed FGFR1 gene transcription in myogenic cells. However, in Drosophila melanogaster SL2 cells, only p107 was able to repress Sp1-mediated transactivation of the FGFR1 promoter. Gel shift assays using transfected myoblast nuclear extracts showed that ectopic p107 reduced Sp1 occupancy of the proximal Sp binding site of the FGFR1 promoter, and coimmunoprecipitation studies indicated that Sp1 interacts with p107 but not with p130. Gel shift assays also demonstrated that Sp1 interacted with p107 in E2F4-p107 transcriptional complexes in myoblasts. The nature of the repressor transcriptional complex was altered in differentiated muscle fibers by the relative loss of the E2F4-p107-Sp1 transcription complex and replacement by the repressor E2F4-p130 complex. These findings demonstrate that activation and repression of FGFR1 gene transcription is governed by interplay between Sp1, p107, p130, and E2F4 in distinct transcriptional complexes during skeletal muscle development.

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1 The abbreviations used are: FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; HA, hemagglutinin; PBS, phosphate-buffered saline; BS, blocking solution; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase.
Pocket proteins regulate gene expression via different E2F-mediated mechanisms. p107 and p130 directly bind E2Fs and modulate their transcriptional activity (20, 22–24). p107 and p130 also recruit additional transcription factors to promoters containing E2F sites. For example, pRb and p130 interact directly with histone deacetylase (HDAC1) leading to local chromatin remodeling (25–27). E2F-pocket protein complexes also interact with Sp1 in the regulation of specific E2F-dependent genes. Sp1 has been reported to interact directly with p107, pRb, and E2F1–3 (28–32). However, E2F4 lacks an Sp1 binding domain and therefore does not interact directly with Sp1 (30). Nevertheless, Sp1 may contribute to E2F4-based transcriptional complexes via interaction with pocket protein co-regulators.

We demonstrated in this study that pocket protein family members p107 and p130 regulate E2F4-mediated repression of FGFR1 gene expression in skeletal muscle cells. Transient transfections in proliferating myoblasts and electromobility shift assays indicated that these proteins form functional complexes with E2F4 and repress FGFR1 gene promoter activity. We further demonstrate that in proliferating myoblasts, Sp1 interacts with p107 and masks the transcriptional repressor activity of the E2F4-p107 complex at the E2F site located in the proximal promoter region of the FGFR1 gene. These findings provide a novel mechanism for developmentally regulated expression of the FGFR1 gene during skeletal myogenesis.

**EXPERIMENTAL PROCEDURES**

*Plasmid DNA Constructs—* The plasmid m23/m42/m54FGFR1 containing mutations of three Sp1 sites in the proximal promoter region was described previously (5). Mutation of the E2F site in the above plasmid was inserted using the QuickChange site-directed mutagenesis kit (Stratagene) (6) with the following forward primer and its antisense oligonucleotide as a reverse primer 5'-GGTTCCCATGCGACTATCTATACAGGGGTATACCTGCA-3'. The base substitutions are underlined.

Mutagenesis was confirmed by DNA sequencing, and the plasmid DNA was designated as m23/m42/m54/m65FGFR1. The new truncated promoter versions of plasmid m23/m42/m54FGFR1 and m23/m42/m54/m65FGFR1 were prepared by deletion of a distal 2.2-kb SacI fragment and ligation to generate the plasmids mSp11058FGFR1 and mE2F/Sp11058FGFR1, respectively. The truncated plasmid 1058FGFR1 containing wild-type Sp1 and E2F promoter sequences was described previously (4).

*Cell Culture, DNA Transfections, and Reporter Assays—* Fetal chick myoblasts (embryonic day 13) were isolated from leg muscles and incubated in MEM supplemented with 10% FBS and 1% penicillin/streptomycin as described previously (35). Cells were cultured in FM medium (15% horse serum (Hyclone Labs), 5% FBS) in a 5% CO2 humidified incubator at 37 °C. Myoblasts were plated on collagen-coated dishes as described previously (33). Cells were plated at a density of 2.5 × 10^5 cells/6-cm dish in FM medium and transfected using Lipofectamine Plus reagent (Invitrogen) as described previously (6). For Western blots, proteins (30 μg) were resolved in 7.5% SDSPAGE and transferred onto nitrocellulose sheets as described previously (36). Cells that were blocked for 1 h at 37 °C or overnight at 4 °C in BS containing 5% nonfat dry milk in PBS with 0.05% Tween 20 at room temperature and washed three times with PBS containing 0.05% Tween 20 . After transfection, the cells were washed three times with PBS and added to the cultures for 4 h at 32 °C. The cells were harvested 48 h after transfection, and CAT assays were performed (35). For all transfection assays, four to five independent experiments were performed.

**Immunocytochemistry—* Rabbit anti-E2F4, p107, p130, Sp1, E47, Gaq, and HA primary antibodies and horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Santa Cruz Biotechnology Inc. Mouse anti-pRb antibody was obtained from BD Transduction Laboratories. Mouse anti-CMyC and β-actin antibodies were obtained from Invitrogen and Abcam, respectively. DAPI was obtained from Molecular Probes, Inc. Fluorescein-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Vector Laboratories, Inc.

Myogenic cultures were immunostained 24 h or 10 days after plating. Cells were washed three times with phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde for 5 min. Cultures were washed once and incubated for 1 h in 3% hydrogen peroxide. Cells were then blocked for 1 h at 37 °C or overnight at 4 °C in BS containing 5% nonfat dry milk in PBS with 0.05% Tween 20 . Thereafter, the cultures were incubated with rabbit polyclonal p107, p130, Sp1, E47, cMyc, or HA antibody and mouse monoclonal pRb antibody, diluted 1:1000 in BS for 1 h at room temperature, washed three times with PBS containing 0.05% Tween 20 , and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG as a secondary antibody (1:4000 dilution) for 1 h at room temperature. The cultures were washed five to six times in PBS containing 0.05% Tween 20 , and the immunocomplexes were detected using the Super Signal chemiluminescent substrate (Pierce) and x-ray film.

**Preparation of Nuclear Extracts and Electromobility Shift Assay—* Nuclear extracts were prepared as described previously (37). Complete nuclear extracts were obtained as described previously (35). Nuclear protein extracts (20 μg) were boiled for 10 min in 10× kinase buffer and allowed to cool gradually to room temperature. Approximately 200 ng of double-stranded oligonucleotide were 5’ end-labeled using T4 kinase (Promega) and γ-32P ATP (MP Biomedical). The oligonucleotides (10 μg each) were resolved in 7.5% SDS-PAGE gel. The plasmid 3284FGFR1CAT containing the full-length wild-type FGFR1 promoter was described previously (35). Myoblasts were plated at a density of 2.5 × 10^5 cells/6-cm dish in FM medium and transfected using Lipofectamine Plus reagent (Invitrogen) as described previously (6). For all transfections, 1.5 or 3 μg of 3284FGFR1CAT plasmid and 1 μg of pRSV/JGAL plasmid were kept constant. Increasing amounts of pCMVp107 and pCMVp130 were expressed as percentage activities relative to empty pCMV vector.

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other set received only 1 μg of pPacSp1 and 3 μg of pCMVp107, and the total amount of DNA transfected was adjusted to 7 μg with pRS Blue-Script DNA (Stratagene). Forty-eight hours after transfection, nuclear extracts were prepared (38), and the gel shifts were performed as described above with 3 μg of nuclear extracts.

**Immunoprecipitations**—Nuclear extracts were prepared as described for electromobility shift assays (37). Extracts from ~4 × 10^6 cells (100 μl containing 200 μg of protein) were diluted to 1 ml with chilled NET buffer (150 mM NaCl, 0.1% Nonidet P-40, and 50 mM Tris-HCl, pH 7.5) containing protease inhibitors. The nuclear lysate was precleared by incubation with 50 μl of protein A/G agarose (Santa Cruz Biotechnology) for 2 h at 4 °C and further centrifugation. The nuclear protein was incubated with 2 μg of antibody against E2F4, pRb, p107, p130, and Sp1 (Santa Cruz Biotechnology) overnight at 4 °C. After addition of 50 μl of protein A/G agarose, the suspension was incubated for another 2 h at 4 °C. The beads were pelleted by centrifugation, washed three times with cold NET buffer, and resuspended in 50 μl of 2× SDS sample buffer. After the suspension was heated to 95 °C for 10 min, 20-μl samples were resolved in denaturing SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed for the presence of specific proteins by immunodetection as described above.

**Chromatin Immunoprecipitation**—We performed chromatin immunoprecipitations using a modification of previously published methods (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40). Formaldehyde was added to a final concentration of 1% directly to the cell culture media of proliferating myoblasts and differentiated myotubes (39, 40).

Chromatin was pre-cleared with a mixture of protein A and protein G Sepharose (blocked previously with 1 mg/ml salmon sperm DNA and 1 mg/ml bovine serum albumin) at 4 °C for 4 h two times. Precleared chromatin was equally divided and was separately incubated with 4 μg of anti-E2F4, anti-p107, anti-p130, anti-Sp1, and Gaq antibodies, or no antibodies overnight at 4 °C. Immunoprecipitation, washing, and elution of immune complexes were carried out as described previously (41). Before the first wash, 20% of the supernatant from the reaction with no primary antibody for each sample (myoblasts and myotubes) was saved as input control and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Cross-links were reversed by the addition of NaCl to a final concentration of 200 mM, and RNA was removed by the addition of 10 μg of RNase A per sample followed by incubation at 65 °C for 4 to 5 h. The samples were then ethanol precipitated. The samples were reassembled in 100 μl of Tris-EDTA, pH 7.5, 25 μl of 5× proteinase K buffer (1.25% SDS, 50 mM Tris, pH 7.5, and 25 mM EDTA), and 10 μg of proteinase K (Sigma) and incubated at 45 °C for 2 h. Samples were extracted with phenol-chloroform and ethanol-precipitated. The pellets were collected by microcentrifugation, resuspended in 30 μl of water, and analyzed by PCR.

PCR mixtures contained 2 μl of immunoprecipitate or 2 μl of a 1:200 dilution of the total sample, 50 ng of each primer, 25 mM MgCl_2, 2.5 mM dNTP mix, and 2.5 units of Taq DNA polymerase in a total volume of 100 μl. After 25 cycles of amplification, the PCR products were run in 1% agarose gels and analyzed by ethidium bromide staining. The PCR primers used to amplify the −321 to +9 region were: 5’ CTGTTTTCAGTGCGCAACT3’ (forward primer, −321 to −302) and 5’ CATGGGCCCCCGTGCCCGCTG3’ (reverse primer, +9 to −13). The primers used to amplify the −1950 to −1788 region were: 5’ CCGCTGCGCAGTGCTCGTT3’ (forward primer, −1950 to −1927) and 5’ GGCGAAAGGGTTGTTATTGCCAAG 3’ (reverse primer, −1788 to −1809).

For transient chromatin immunoprecipitation assays (42), fetal chick myoblasts (embryonic day 13) were plated at a density of 2.5 × 10^6 cells/10 cm and were transfected with 1.5 μg of the 105SGFR1 wild-type promoter construct and its mutant derivatives by using Lipofectamine Plus reagent (Invitrogen) as described previously (5).

**RESULTS**

**Cellular Localization of Pocket Proteins in Chicken Muscle Cells**—We have recently shown that E2F4, present in the nuclei of both myoblasts and myotubes, acts as a negative regulator of FGFR1 gene expression (6). To identify potential E2F4 binding partners within nuclei, we analyzed the intracellular distribution of pocket proteins in undifferentiated myoblasts and differentiated myotubes by immunocytochemistry. p107, p130, and pRb proteins were localized to nuclei of both myoblasts and myotubes (Fig. 1). Immunofluorescent localization of these proteins in the nuclei of myoblasts and myotubes co-localized with 4,6-diamidino-2-phenylindole fluorescence.

Western blot analysis was carried out using fractionated cell lysates of both myoblasts and myotubes to validate the immunostaining results (Fig. 2). p107, p130, and pRb proteins were detected in the nuclear extracts from both myoblasts and myotubes. These proteins were not detected in cytoplasmic extracts. Whereas p107 was abundant in myoblasts, pRb was abundant in myotubes. Relative mobility differences of pRb proteins in extracts from myoblasts and myotubes may have been caused by differences in phosphorylation states. The relative abundance of p130 was approximately the same in myoblasts and myotubes. The p107 and p130, but Not pRb, Formed Complexes at an E2F4 Binding Site—To determine whether p107, p130, and/or pRb from chicken muscle cells interact with E2F4, we performed gel supershift assays using myoblast and myotube nuclear extracts, the E2F4 binding site of the FGFR1 promoter, and antibodies directed against p107, p130, pRb, and E2F4 (Fig. 3).

Nuclear extracts from myoblasts and myotubes formed a protein-DNA complex with the FGFR1 E2F4 binding site, which was supershifted by the E2F4 antibody. Addition of p107 and p130 antibodies in the reactions produced a supershifted protein-DNA complex, indicating the presence of these transcriptional...
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p107 and p130 were detected by Western blot analysis using cMyc and HA antibodies, respectively, directed against the expressed fusion proteins. p107 and p130 proteins repressed FGFR1 promoter activity in a dose-dependent manner (Fig. 4). Transfection of 1.5 μg of pCMVp107 reduced FGFR1 promoter activity to basal levels, whereas transfection of 1.5 μg of pCMVp130 reduced promoter activity to ~50%. Increasing amounts of pCMVp130 up to 6 μg did not further reduce promoter activity (data not shown). Repression of FGFR1 promoter activity by pCMVp107 and pCMVp130 was not caused by the presence of the strong, constitutive CMV promoter because transfection of the cloning vector pCMVTag did not reduce FGFR1 promoter activity (Fig. 4B). These results indicate that p107 is a strong repressor of FGFR1 promoter activity and that p130 functions as a repressor but is relatively less effective.

p107 Represses Sp1-mediated Activation of the FGFR1 Promoter—To assess the transcriptional effects of p107 and p130 on Sp1-mediated transactivation of FGFR1 promoter activity, the Sp1 expression construct pPacSp1 was co-transfected with pCMVp107 or pCMVp130 along with the wild-type 3284FGFR1CAT construct into D. melanogaster S2 cells. These cells have no detectable levels of endogenous Sp transcription factors (43). As shown in Fig. 5, expression of Sp1 significantly increased FGFR1 promoter activity, in agreement with our previous observations (5). Co-transfection of pCMVp107 repressed FGFR1 promoter activity to ~48% in a dose-dependent manner (Fig. 5A). In contrast, co-transfection of pCMVp130 had no effect on Sp1-mediated FGFR1 promoter activity (Fig. 5B). These results suggest that a p107-Sp1 interaction may regulate FGFR1 promoter activity in myoblasts.

Exogenous p107 Interferes with Sp1 Binding to the Proximal Region of the FGFR1 Promoter—To gain further insight into the mechanism by which p107 represses Sp1-mediated transcriptional activation of the FGFR1 gene, we first determined whether p107 interfered with Sp1 binding to FGFR1 promoter sequences. The proximal E2F4 binding site of the FGFR1 promoter located at −65 bp is near the three proximal Sp binding sites, located between −65 and −23 bp from the transcription start site (Fig. 6A). We have shown previously that the most proximal (−23 bp) Sp1 binding site conferred the strongest transcriptional activation to the FGFR1 promoter (5). Using the −23 bp Sp1 binding site sequence, we performed gel shift assays with nuclear extracts prepared from myoblasts transfected with 3 μg of pCMVp107 and pCMVp130 expression plasmids (Fig. 6B). Overexpression of p107 reduced the formation of the Sp1-DNA complex. Similar results were obtained using the consensus Sp1 binding site oligonucleotide. However, overexpression of p130 did not interfere with Sp1 binding to the −23 bp Sp1 site of the FGFR1 promoter or to the Sp1 consensus oligonucleotide. These results indicate that p107 antagonizes Sp1-mediated transcriptional activation of FGFR1 gene expression by inhibiting its occupancy of the proximal Sp binding site of the FGFR1 promoter.

Exogenous p107 Physically Interacts with Sp1 in Proliferating Myoblasts—We hypothesized that repression of Sp1-mediated transcription of the FGFR1 promoter by p107 was caused by direct protein-protein interaction. To determine whether exogenous p107 physically interacted with endogenous Sp1 independent of its binding to the FGFR1 promoter, we performed coimmunoprecipitation assays using nuclear extracts prepared from myoblasts transfected with the pCMVp107 expression plasmid. For comparison, we also used nuclear extracts from myoblasts transfected with pCMVp130 expression plasmid. The expression of each construct was examined by Western blot analysis using anti-cMyc and anti-HA antibodies.
to detect cMyc-p107 and HA-p130 fusion proteins. Detection of the fusion proteins was restricted to extracts from transfected cells (Fig. 7A). cMyc antibody coimmunoprecipitated Sp1 from the nuclear extracts prepared from myoblasts transfected with pCMVp107 (Fig. 7B). In contrast, Sp1 coimmunoprecipitation was not detected with the HA antibody from the nuclear extracts prepared from myoblasts transfected with pCMVp130 (Fig. 7C). Immunoprecipitates with nonspecific antibody (anti-E47) or without any antibody did not yield any specific, detectable interactions. These results indicate that p107 interacts with Sp1 in myoblasts, whereas p130 does not.

Sp1-p107 Interaction Is Developmentally Regulated—The interactions between endogenous Sp1 and pocket proteins in proliferating myoblasts and differentiated myotubes were examined by coimmunoprecipitation of non-transfected cells. Nuclear extracts were prepared from myoblasts and myotubes, and protein complexes were immunoprecipitated with antibodies directed against p107, p130, Sp1, and E2F4. Co-immunoprecipitated proteins were analyzed by immunoblotting. As shown in Fig. 8, Sp1 coimmunoprecipitated with p107 and vice versa in proliferating myoblasts. However, coimmunoprecipitation of Sp1 with p107 from myotube extracts was not detected. This result is consistent with previously published results demonstrating a significant decline in Sp1 abundance in myotubes relative to myoblasts (5). Sp1 coimmunoprecipitation with p130 from myoblasts and myotubes was not detected. p130 did coimmunoprecipitate with E2F4 from myoblast or myotube nuclear extracts. These results verify that Sp1 interacts with p107 and not p130 in proliferating myoblasts and that the Sp1-p107 interaction declines in differentiated myotubes.

Sp1 Is Present in the E2F4-p107 Complex Bound to the E2F Site in the FGFR1 Promoter—Having identified a specific protein-protein interaction between Sp1 and p107, we next determined whether Sp1 was present in the E2F site of the FGFR1 promoter. Electrophoretic mobility supershift assays were performed using myoblast and myotube nuclear extracts, the FGFR1 E2F4 binding site as a probe, and antibodies directed against E2F4, p130, p107, and...
Sp1 (Fig. 9). Incubation of the protein-DNA complexes with E2F4, p107, and p130 antibodies resulted in a supershifted protein-DNA complex indicating the presence of these transcription factors in the E2F4 complex in the nuclear extracts of both myoblasts and myotubes. The protein-DNA complex formed by myoblast nuclear extract and the E2F binding site
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FIG. 7. Exogenously expressed p107 interacts with endogenous Sp1. A, nuclear extracts (50 μg) of myoblasts transfected with the expression plasmids encoding cMyc-tagged p107 and HA-tagged p130 were probed with anti-cMyc and anti-HA antibodies to detect their exogenous expression levels. B, protein complexes in nuclear extracts from myoblasts transfected with the pCMVp107 expression construct were immunoprecipitated with the cMyc antibody and probed with an anti-Sp1 antibody. C, protein complexes in nuclear extracts from myoblasts transfected with the pCMVp107 expression construct were immunoprecipitated (IP) with an anti-HA antibody and Western blots were probed with an anti-Sp1 antibody. A nonspecific anti-E47 antibody was used as a control and did not immunoprecipitate Sp1. Input protein was diluted 1:3 before loading onto the gel. Sp1 communoprecipitated with cMyc-p107 but not HA-p130.

FIG. 8. Endogenous interactions between p107 and Sp1. Immunoprecipitations (IP) were performed with nuclear extracts prepared from myoblasts (MB) or myotubes (MT). A, antibodies to p107 and p130 were used to communoprecipitate p107 and p130 protein complexes. The Western blot was incubated with anti-Sp1 antibody. Sp1 communoprecipitated only with p107 in myoblast nuclear extracts. B, antibodies to Sp1 and p130 were used to communoprecipitate protein complexes from myoblasts and myotubes. The Western blot was incubated with anti-p107 antibody. p107 communoprecipitated with Sp1 in myoblast nuclear extracts. C, protein complexes from myoblasts and myotubes were communoprecipitated with E2F4 in extracts from myoblasts and myotubes. Nonspecific anti-E47 antibody was used as the control. Input protein was diluted 1:3 before loading onto the gel. Integrity of the MT nuclear extracts in A and B was verified by immunodetection of p130 and E2F4 in C.
Tripartite complex binding to the FGFR1 E2F binding site in myoblasts. Gel supershift assays with the labeled FGFR1 E2F binding site (lanes 1–6 and 9–14) or with the mutated FGFR1 E2F4 binding site oligonucleotide (lanes 7 and 8) and antibodies to E2F4, p107, p130, and Sp1 were performed using myoblast and myotube nuclear extracts. Lanes 1, 8, and 9 contain probe alone. Lanes 2–6 contain protein-DNA complexes formed with the FGFR1 E2F4 binding site oligonucleotide and myoblast nuclear extract. Lane 7 contains no protein-DNA complexes formed with the mutated FGFR1 E2F4 binding site oligonucleotide. Lanes 10–14 contain myotube nuclear extract. Antibodies added to the reactions are indicated. The arrow indicates the position of the specific E2F4 complexes, and the arrowhead indicates the position of the supershifted E2F4-based complex.

**FIG. 10.** Transient shift assays to determine Sp1-p107-E2F4 tripartite complex binding to the FGFR1 E2F binding site in *D. melanogaster* SL2 cells. As described under “Experimental Procedures,” *D. melanogaster* SL2 cells were transfected with different combinations of expression plasmids (pCMVE2F4 or pCMVP107 + pPacSp1 or pCMVP107 + pPacSp1). Forty-eight hours after transfection, nuclear extracts were prepared and used to generate complexes with the FGFR1 E2F4 binding site oligonucleotide (lanes 2–9) and a consensus Sp1 binding site oligonucleotide (lanes 10–11) as probes. Lane 1 contains probe alone. Lane 2 contains no protein-DNA complexes formed with nuclear extract prepared from non-transfected cells. Lanes 3–7 contain protein-DNA complexes formed with nuclear extracts prepared from the cells transfected with pCMVE2F4, pCMVP107, and pPacSp1 expression plasmids. Lanes 8–11 contain protein-DNA complexes formed with nuclear extracts prepared from cells transfected with pCMVP107 and pPacSp1, and not pCMVE2F4, expression plasmids. Antibodies (Ab) added to the reactions are indicated. p130 antibody was used as a control for nonspecific protein-DNA interactions. The left arrow indicates the position of the specific E2F4-DNA complexes, and the right arrowhead indicates the position of the supershifted E2F4-based complex. Formation of the Sp1-DNA complex (right arrow) was confirmed by addition of Sp1 antibody and appearance of a supershifted protein-DNA complex (right arrowhead). Expression of E2F4, p107, and Sp1 proteins formed a tripartite complex with the FGFR1 E2F binding site oligonucleotide, and this complex was not detectable in the absence of E2F4.

Analyses of the Protein-DNA complex at the E2F4 Binding Site of the FGFR1 Promoter in Vivo—The composition of the endogenous protein-DNA complexes in the proximal region of the FGFR1 promoter in both myoblasts and myotubes was analyzed by chromatin immunoprecipitation. Because FGFR1 gene expression is positively regulated in proliferating myoblasts and negatively regulated in differentiated myotubes, the occupancy of the proximal promoter region by E2F4, p107, p130, and Sp1 was determined in these two cell types (Fig. 11). In proliferating myoblasts, antibodies specific for E2F4, p107, p130, and Sp1 immunoprecipitated DNA fragments that were PCR-amplified by primers surrounding the E2F4 and Sp1 binding sites, yielding the expected product size of 330 bp. These results are consistent with the electromobility shift and supershift assays in Fig. 9. Omission of antibody and inclusion of a nonspecific antibody did not yield an immunoprecipitated DNA fragment detectable by PCR amplification. Similar to the protein-DNA complexes in myoblasts, the protein-DNA complexes in myotubes in vivo consisted of E2F4 and p130 at the E2F4 binding site. However, addition of the p107 antibody immunoprecipitated DNA from myotubes that was only weakly amplified. Furthermore, addition of the Sp1 antibody did not immunoprecipitate the FGFR1 proximal promoter DNA. Antibody specificities were demonstrated by lack of FGFR1 promoter immunoprecipitation by a nonspecific antibody. Specificity of PCR amplification of immunoprecipitated DNA was demonstrated by lack of amplified DNA using control primers for amplification of DNA spanning the –1950 to –1788-bp region of the FGFR1 promoter, which has no obvious E2F or Sp1 binding sites. These results indicate that protein-DNA transcriptional regulatory complexes consisting of E2F4, p107, p130, and Sp1 occupy the FGFR1 promoter in proliferating myoblasts. In contrast, the predominant protein-DNA complex in differentiated myotubes consists of E2F4 and p130.

To further demonstrate that in proliferating myoblasts, Sp1 interacts with the E2F4-p107 complex at the E2F4 binding site located in the proximal promoter region of the FGFR1 gene, we performed transient chromatin immunoprecipitation assays using wild-type and mutated FGFR1 promoter constructs. We showed previously that in proliferating myoblasts, three Sp1 sites (–23, –42, and –54 bp) located in the proximal promoter region of FGFR1 up-regulated transcription of this gene, and mutation of these sites abrogated promoter activity (5). In contrast, mutation of the E2F site (–65 bp) adjacent to the Sp1 binding site would not be expected to affect promoter activity (5).
sites increased promoter activity up to 3-fold, indicating that this site mediates promoter repression (6). The 2.2-kb distal promoter region containing two Sp1 sites was deleted from the wild-type and mutated FGFR1 promoter CAT constructs. After transfection of these promoter constructs into proliferating myoblasts, binding of endogenous E2F4, p107, and Sp1 was tested (Fig. 11B). Wild-type FGFR1 promoter was amplified when E2F4, p107, and Sp1 antibodies were used. No PCR product was detected with the nonspecific Goq antibody and in the absence of antibody. However, mutations of the E2F4 and Sp1 binding sites did not allow binding of E2F4, p107, and Sp1 to the FGFR1 proximal promoter region. Sp1 was abundant in myoblast nuclear extracts, and pRb was also abundant in myotube nuclear extracts. In contrast, the abundance of p107 remained unchanged in myoblast versus myotube nuclear extracts. Gel mobility shift and supershift assays with the FGFR1 E2F4 binding site oligonucleotide (Fig. 3) also differentially localized p107 and p130 to E2F4-p107 complexes. pRb did not form a protein complex with E2F4. These results indicate that a specific transition of E2F4-pocket protein complexes occurs during differentiation of chicken muscle cells and support previous findings that E2F4-p130 complexes replace E2F4-p107 complexes in fully differentiated cells (21).

E2F proteins often activate promoters, and subsequent recruitment of pocket proteins to the E2F site leads to the repression of the E2F-dependent genes such as B-myb, E2F1, cyclin A, and cyclin E (25, 44–46). Transient transfection assays in proliferating myoblasts indicated that p107 and p130 are dose-dependent corepressors of FGFR1 gene expression.

**FIG. 11.** Chromatin immunoprecipitation analyses of E2F4, p107, p130, and Sp1 binding to the FGFR1 promoter in vivo. A, immunoprecipitation of endogenous FGFR1 promoter sequences. Pre-cleared chromatin (lane 1) with no antibody incubation and before washing and elution of immune complexes was PCR-amplified using primers that flanked the FGFR1 gene at −321 bp and +9 bp. This region contains both E2F4 and Sp1 binding sites. Genomic DNA from proliferating myoblasts and differentiated myotubes was incubated with anti-E2F4 (lane 2), anti-p107 (lane 3), anti-p130 (lane 4), and anti-Sp1 (lane 5) antibodies. Amplification of genomic DNA that was precipitated in the presence of a nonspecific antibody to Goq (lane 6) or in the absence of antibody (lane 7) is shown. Cloned FGFR1 DNA as a PCR template served as a positive control (lane 8). ChIP analysis of the −1950 bp to −1788 bp region of the FGFR1 promoter devoid of potential E2F and Sp1 binding sites was performed with the above antibodies and specific primers to this region. B, transient chromatin immunoprecipitation assays of the wild-type FGFR1 gene promoter construct and its mutant derivatives. The various constructs indicating the binding site mutations are shown. Pre-cleared chromatin (lane 1) with no antibody incubation and before washing and elution of immune complexes was PCR-amplified using primers that flanked the FGFR1 proximal promoter region at −321 and +351 bp. Immunoprecipitations and PCR reactions were performed as above with antibodies specific to E2F4 (lane 2), p107 (lane 3), p130 (lane 4), Sp1 (lane 5), and Goq (lane 6, a negative control). As another negative control, PCR was performed with the sample containing no antibody (lane 7). Cloned FGFR1 DNA as a PCR template served as a positive control (lane 8). Each immunoprecipitation was done in duplicate, and two independent experiments were performed. Representative agarose gels stained with ethidium bromide are shown.
Although both p107 and p130 functioned as repressors in myoblasts, transient transfections in D. melanogaster SL2 cells indicated that p107, and not p130, repressed Sp1-mediated activation of the FGFR1 promoter. To determine the mode of repression of Sp1 activation by p107, we analyzed the protein-DNA complexes at the Sp1 binding site and showed that p107, and not p130, interfered with formation of the Sp1-DNA complex in myoblasts. Because p130 did not interact with Sp1, it is likely that p130 could not interfere with Sp1-mediated activation via direct protein-protein interaction. Furthermore, the inability of p130 to repress Sp1-mediated activation of FGFR1 promoter activity in D. melanogaster SL2 cells is probably caused by the absence of E2F-associated factors and the lack of repressor complex formation.

Repression of Sp1 activation by p107 may occur by physical interference of Sp1 binding at the promoter as a result of the proximity of the binding elements or by protein-protein interactions independent of DNA binding. The possibility of direct protein-protein interactions was assessed by coimmunoprecipitation assays. Exogenously expressed and endogenous p107 interacted with Sp1 in nuclear extracts of myoblasts. No interactions were detected in nuclear extracts of myotubes because Sp1 abundance declines during chicken myogenic differentiation (5). In contrast, p130 did not interact with Sp1 in myoblast nuclear extracts. Electromobility shift and chromatin immunoprecipitation assays in myoblasts and D. melanogaster SL2 cells demonstrated that Sp1 interacts with the transcriptional complex bound to the E2F4 binding site of the FGFR1 promoter. This interaction is mediated by specific Sp1-p107 interaction within the complex. The interactions between p107 and Sp1 in regulation of the FGFR1 promoter activity contribute to other reports of p107-Sp1 interactions in HeLa, COS, and CHOC 400 cells (28, 29).

Together, these results suggest a model for the developmentally regulated transcription of the FGFR1 gene during skeletal muscle development (Fig. 12). Sp1-mediated transcriptional activation of FGFR1 gene expression in proliferating myoblasts occurs through at least two distinct mechanisms. Sp1 activates FGFR1 promoter activity in proliferating myoblasts by directly binding to proximal and distal cis-elements (5). In addition, Sp1 interacts with p107 in the repressor p107-E2F4 complex at the E2F4 binding site in myoblasts. Because the p107-E2F4 complex is abundant in proliferating myoblasts, the repressor complex of p130-E2F4 is less abundant. During myogenic differentiation, the relative abundance of p107 declines, as does the abundance of the p107-E2F4-DNA complex relative to the p130-E2F4-DNA complex. The latter protein-DNA complex is not able to interact with Sp1 and exhibits its repressor potential. In addition, the abundance of Sp1 declines during cell differentiation, removing direct FGFR1 promoter activation and allowing repression by remaining p107-E2F4-DNA complexes. These results suggest that Sp1 masks p107-E2F4 repression of FGFR1 promoter activity in proliferating myoblasts. The results provide a model framework in which coordinated interplay between nuclear transcription factors govern the differential regulation of FGFR1 gene expression in proliferating myoblasts and differentiated muscle fibers.

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