The development of myoblasts is regulated by various growth factors as well as by intrinsic muscle-specific transcriptional factors. In this study, we analyzed the roles for STAT3 in the growth and differentiation of myoblasts in terms of cell cycle regulation and interaction with MyoD using C2C12 cells. Here we found that STAT3 inhibited myogenic differentiation induced by low serum or MyoD as efficiently as the Ras/mitogen-activated protein kinase cascade. As for this mechanism, we found that STAT3 not only promoted cell cycle progression through the induction of c-myc but also inhibited MyoD activities through direct interaction. STAT3 inhibited not only DNA binding activities of MyoD but also its transcriptional activities. However, the inhibited transcriptional activities were restored by the supplementation of p300/CBP and PCAF, suggesting that STAT3 might deprive MyoD of these transcriptional cofactors. In addition, we found that MyoD inhibited DNA binding activities of STAT3, thereby inhibiting STAT3-dependent cell growth and survival of Ba/F3 cells. These results suggest that the development of muscle cells is regulated by the coordination of cytokine signals and intrinsic transcription factors.

Skeletal muscle develops from pluripotent mesodermal stem cells through a multistep process including the commitment to myogenic precursors and the subsequent proliferation and differentiation of myoblasts. In the differentiation step, myoblasts exit from cell cycle, begin to express muscle-specific genes, and fuse with adjacent myoblasts to form multinucleated, differentiated myotubes (1, 2). Meanwhile, the maintenance and rapid regeneration after injury in adult muscle are solely dependent on a unique group of cells called satellite cells (or adult myoblasts) that are located under the basal lamina of the muscle fiber and kept quiescent in uninjured muscle. In these systems, the commitment, migration, growth, and differentiation of myoblasts are regulated by variety of growth factors such as insulin-like growth factor (IGF)-I, IGF-II, leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (3). Among these factors, IGF-I is considered to be especially important for muscle development, because mice that lacked the IGF-I receptor exhibited marked muscle hypoplasia and died soon after birth because of their inability to breathe (4). In mice lacking the HGF receptor c-met, the limb bud and diaphragm were not colonized by myogenic precursor cells, and as a consequence, skeletal muscles of the limb and diaphragm were not formed (5).

Signal transducers and activators of transcription (STAT) proteins are transcription factors that transduce pivotal biological effects of various cytokines and hormones (6). Among seven STAT family members (STAT1–4, 5A, 5B, and 6), STAT3 is activated by several cytokines, such as the IL (interleukin)-6 family of cytokines (LIF, ciliary neurotrophic factor, oncostatin M (OSM), IL-11, and cardiotrophin-1), IGF-I, HGF, epidermal growth factor, PDGF, and bFGF, and plays central roles in the regulation of growth, differentiation, and survival in various types of cells (7). For example, STAT3 promotes cell growth through the transcriptional regulation of cyclin D1 in fibroblasts (8) and of c-myc and Pim1 in hematopoietic cell lines (9, 10). In addition, STAT3 maintains embryonic stem cells in an undifferentiated state (11) and inhibits IL-6-induced neuronal differentiation in PC12 cells (12). These lines of evidence indicate that STAT3 supports cell growth and suppresses differentiation. In contrast, STAT3 also mediates cytokine-induced terminal differentiation in other cell types, e.g. IL-6-induced macrophage differentiation (13), CD40 ligand-induced B-cell differentiation (14), OSM-induced differentiation of an osteosarcoma cell line (15), and ciliary neurotrophic factor- or OSM-induced astrocyte differentiation (16, 17). Therefore, functional roles for STAT3 in cell growth and differentiation are supposed to be entirely divergent according to the cellular background and/or type of cytokine. Although STAT3 is activated by vari-
ous growth factors that regulate the growth and differentiation of myoblasts (18–20), its role in myogenesis still remains largely unknown.

The intrinsic muscle-specific transcriptional factors, such as the MyoD family and myocyte enhancer factor 2 (MEF2) family proteins, also play crucial roles in skeletal muscle development in coordination with exogenous growth factors (21). The MyoD family of basic helix-loop-helix (H-L-H) transcription factors, also referred to as myogenic regulatory factors (MRFs), includes MyoD, myogenin, Myf5, and MRF4 (for reviews, see Refs. 2 and 22). Myf5 and MyoD impose a myogenic fate on pluripotent precursor cells and induce differentiation at an earlier step, whereas myogenin and MRF4 act at a later step. These factors form heterodimers with ubiquitously expressed E-protein family members (E2A, E2B, E2-5, and HEB), bind to a consensus DNA sequence called an E-box, and regulate the expression of several muscle-specific genes and cell cycle regulatory genes. MyoD induces the expression of cyclin-dependent kinase inhibitor p21WAF1/Cip1 and Rb, which are considered to be responsible for MyoD-induced cell cycle exit (a prerequisite condition for the initiation of myogenic differentiation) (23, 24). These results suggest that MyoD would cause cell cycle arrest through its transcriptional activities. However, it was also reported that MyoD induces cell cycle arrest independently of its DNA binding activities, because MyoD carrying the mutation in the basic (DNA-binding) region inhibited cell growth as efficiently as wild-type (WT) MyoD (25, 26). With regard to this mechanism, MyoD was shown to directly interact with Rb and to inhibit its phosphorylation induced by cyclin-dependent kinases (CDKs), thereby inducing cell cycle arrest (2, 22). In addition, MyoD directly binds to MEF2 (MEF2A, C, or D) and enhances MEF2-dependent transcription (i.e. the transcription dependent on the DNA binding of MEF2) through its own transcriptional activities, and vice versa, which results in the cooperative enhancement of MEF2- and MyoD-dependent myogenic differentiation (27). Moreover, MyoD activities are positively or negatively regulated by the direct interactions with p300/CBP, PCAF, CDK4, MEK1, and Twist (2, 22, 28). Together, these results indicate that MyoD would regulate myogenic differentiation through its transcriptional activities and protein-protein interactions.

In this study, we analyzed the roles for STAT3 in the growth and differentiation of myoblasts and examined its interaction with MyoD using C2C12 (C2) cells. Here we found that STAT3 inhibits myogenic differentiation induced by low serum or MyoD as efficiently as the Ras/mitogen-activated protein kinase (MAPK) cascade. As for this mechanism, we found that STAT3 not only induces the expression of c-myc but also reciprocally antagonizes MyoD through the direct interaction. These results suggest that STAT3 would regulate the development of skeletal muscle in combination with other signaling pathways such as Ras/MAPK and phosphatidylinositol 3-kinase through cell cycle regulation and modification of MyoD activities.

**Experimental Procedures**

**Reagents and Antibodies**—The antibodies (Abs) against STAT3 (C-20X, phosho-STAT3) (B-7) and E47 (N6149) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the anti-p44/p42 and anti-phospho-p44/p42 Abs were from New England BioLabs (Beverly, MA); the anti-STAT3 Ab (S21320) was from BD Transduction Laboratories (Lexington, KY); the anti-Flag Ab (M2) was from Sigma; the anti-HA Ab (12CA5) was from Roche Molecular Biochemicals (Indianapolis, IN); the anti-MyoD Ab was from PharMingen (San Diego, CA); and the anti-G-CSF receptor (G-CSFR) Ab (MCA1544PE) was purchased from Dainippon Pharmaceutical Co. Ltd. (Tokyo, Japan). The anti-gp130 Abs was kindly provided by Dr. T. Hirano (Osaka University, Osaka, Japan).

**Plasmid Constructs and cDNA**—Various expression vectors were described previously: MyoD/ER (29), STAT3/ERT (11), MyoD/ERT (30), G-CSFR/gp130 (31), and H-Ras/G12V (32). Full-length (FL) cDNAs of MyoD and Myf5 were provided by Dr. B. Winter (University of Braunschweig, Braunschweig, Germany). Mutants of MyoD were generated by the PCR method and subcloned into the expression vector pcdNA3 (Invitrogen, De Schelp, the Netherlands). Other plasmids were gifts from other researchers: WT STAT3 and STAT3-C from Dr. J. E. Darnell, Jr. (8); Raf/ERT (33) from Dr. M. McMahon (University of California San Francisco Cancer Center, San Francisco, CA); 1-60STAT3 from Dr. T. Kitamura (University of Tokyo, Tokyo, Japan) (34); p300 from Dr. J. Boyes (The Institute of Cancer Research, London, United Kingdom); CBP from T. Kouzarides (University of Cambridge, Cambridge, United Kingdom); PCAF from Dr. Y. Nakatani (Dana-Farber Cancer Institute, Boston, MA); and cDNAs of SOCS1, CIS, and OSM from Dr. A. Yoshimura (Kyushu University, Fukuoka, Japan).

**Cell Lines and Cultures**—A myoblast cell line C2, a fibroblast cell line C3H10T1/2 (C3), and their derivative sublines were cultured in phenol red-free DMEM supplemented with 10% fetal bovine serum (Flow, North Ryde, Australia) to avoid estradiol (ED)-like effects of phenol red. Ba/F3, an IL-3-dependent cell line engineered G-CSFR/gp130, Ba/F3/GR/gp130 was cultured in RPMI supplemented with 10% fetal bovine serum and 50 ng/ml of gp130L.

**Flow Cytometry**—Surface expression of gp130 and G-CSFR/gp130 was examined by an indirect method using FACSsort (Becton Dickinson, Oxnard, CA). DNA content of cultured cells was quantitated by propidium iodide staining. Cell cycle analysis was performed with the Modfit LT2.0 program (Becton Dickinson).

**Northern Blot Analysis**—The methods for the isolation of total cellular RNA and Northern blot analysis were described previously (35).

**Communoprecipitation Analysis and Immunoblotting**—293T cells were transfected with various expression vectors by the calcium phosphate coprecipitation method. After 36 h, total cellular lysates were isolated and subjected to immunoprecipitation, gel electrophoresis, and immunoblotting as described previously (30).

**Preparation of Stable Transformants from C2, C3, and Ba/F3/G-CSFR/gp130**—We stably introduced various expression vectors into C2 cells together with the calcium phosphate coprecipitation method. After 12 h, the cells were washed, serum-deprived for 24 h, and subjected to cell lysis. After the measurement of firefly and Renilla luciferase activities, the relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the Renilla luciferase activities. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments.

**Electrophoretic Mobility Shift Assay (EMSA)**—The isolation of nuclear extracts and EMSA were performed as described previously (35). The sequences of the probes were as follows: IR, 5′-GGCTGTATTTCCC-3′; GAAAATGAGGGAGC-3′ for STAT3 (13); and E-box, 5′-CCCCCAACT-3′ for MyoD. The recognition sequences are underlined.

**GST Pull-down Assays**—The production and purification of GST-STAT3 fusion proteins were performed as reported previously (30). 35S-labeled MyoD was prepared by a TNT reticulocyte lysate system (Promega). In each binding reaction, 20 µg of GST fusion protein bound to glutathione-Sepharose beads was incubated with 50 µg of TNT reaction solution at 4 °C for 1 h. The binding complexes were separated by gel electrophoresis and subjected to autoradiography.

**GAL4 Transactivation Assays**—To construct GAL4 MyoD fusion constructs, various PCR fragments of MyoD were subcloned into the pBIND vector that contains the DNA-binding domain (amino acids 1 to 147) of GAL4 (Promega). The expression vector for GAL4-Myc-MyoD was transfected into C2 cells together with pG5-Luc, a luciferase reporter gene containing five copies of the GAL4-binding sequence (Promega), with or without the effector gene as indicated. Luciferase assays were performed as described above.
RESULTS

Effects of gp130L on the Growth and Differentiation of C2 and C3 Cells—C2 cells usually maintained in the growth medium containing 10% fetal bovine serum are known to undergo differentiation under culture with low (0.1%) serum (differentiation medium, DM). Also, MyoD/ER (a chimera consisting of full-length MyoD and the ligand-binding domain of estrogen receptor) was reported to induce differentiation in response to ED in C3 (29, 38). To examine the roles for STAT3, which can be activated by LIF, IL-6, IGF-I, HGF, PDGF, or bFGF, in myogenic differentiation under these culture systems, we introduced MyoD/ER and G-CSFR/gp130 (a chimera receptor composed of the extracellular domain of G-CSFR and the cytoplasmic domain of gp130) that can activate STAT3 into C2 and C3; these clones were designated as C2/gp130/MyoD/ER and C3/gp130/MyoD/ER, respectively. We did not detect the expression of endogenous G-CSFR on C2/gp130/MyoD/ER (data not shown) and found that G-CSFR/gp130 was expressed at a similar level to endogenous gp130 with flow cytometric analysis (Fig. 1A). Also, the equivalent level of G-CSFR/gp130 expression was detectable on C3/gp130/MyoD/ER (data not shown). When C2/gp130/MyoD/ER cells (in an ~40% confluent state) were cultured with DM or treated with ED without gp130L (in this report, we denote G-CSF as gp130 ligand (gp130L) when it acts on G-CSFR/gp130), the mRNA expression of myogenin and myosin light chain 3 was induced during culture with DM or by ED treatment. Although G-CSF by itself did not show any effects on parental C2 cells although used at a high concentration (300 ng/ml) (data not shown), it inhibited DM- or MyoD-induced myogenic differentiation through the activation of the chimeric receptor G-CSFR/gp130 in C2/gp130/MyoD/ER cells (Fig. 1B). In addition, we found that natural ligands for gp130, i.e., LIF and IL-6, could inhibit both DM- and MyoD-induced myogenic differentiation in C2/gp130/MyoD/ER cells (Fig. 1B). gp130L also significantly suppressed the ED-induced expression of myogenin in C3/gp130/MyoD/ER cells, indicating that gp130-mediated signals would inhibit both DM- and MyoD-induced myogenic differentiation in C2 and C3 cells. Next, we analyzed cell cycle profiles in these clones before and after 72-h cultures (Fig. 1C). When C2/gp130/MyoD/ER cells were cultured with DM or treated with ED for 72 h in the absence of gp130L, the proportion of proliferating cells in S-G2/M phase decreased severely in both cultures (the proportion of S-G2/M phase: 48% growth medium versus 5% DM, 12% ED). However, gp130L and LIF restored their growth significantly (gp130L, 21% DM, 28% ED; LIF, 13% DM, 20% ED). gp130L, at least in part, canceled the ED-induced growth suppression in C3/gp130/MyoD/ER cells (the change in the proportion of S-G2/M phase: from 11% to 22%) (Fig. 1C). Effects of STAT3 and Raf/MAPK on the Growth and Differentiation of C2 Cells—To clarify the mechanism by which gp130L inhibited DM- and ED-induced differentiation and restored growth in C2 cells, we explored the roles for its downstream signaling molecules. Because gp130L is known to activate STAT3 and Ras/MAPK in other cell types (9, 31), we examined whether these molecules are activated by gp130L in C2 cells. As shown in Fig. 2A, the immunoblot analyses showed that gp130L induced the phosphorylation (i.e. activation) of STAT3 and MAPK in C2/gp130/MyoD/ER cells. To determine the roles for STAT3 and Ras/MAPK in the growth and differentiation of C2 cells, we further expressed 4-hydroxytamoxifen (4-HT)-inducible forms of STAT3 and Raf (STAT3/ERT and Raf/ERT) in C2/gp130/MyoD/ER; these clones were designated as C2/MyoD/ER/STAT3/ERT and C2/MyoD/ER/Raf/ERT, respectively. As shown in Fig. 2B, both DM and ED induced the expression of myogenin in C2/MyoD/ER/STAT3/ERT cells under culture without 4-HT, whereas the expression of myogenin was reduced to an undetectable level by the 4-HT treatment (Fig. 2B). In addition, DNA content analysis showed that the 4-HT treatment significantly restored both DM- and ED-induced growth suppression in this clone (changes in the proportion of S-G2/M phase: DM, from 6% to 14%; ED, from 12% to 18%) (Fig. 2C, left panel). 4-HT-activated c-Raf also reduced the ED-induced expression of myogenin in C2/MyoD/ER/Raf/ERT cells (data not shown), which was accompanied by an increase of the proliferating cells (changes in the proportion of S-G2/M phase: DM, from 5% to 16%; ED from 12% to 19%) (Fig. 2C, right panel). Similar data were obtained from at least five clones each expressing STAT3/ERT or Raf/ERT (data not shown).
c-Myc Can Inhibit DM-induced but not MyoD-induced Differentiation in C2 Cells—Because both STAT3 and Ras/MAPK are known to mediate cell growth through the induction of c-myc (9, 39), we examined the effects of STAT3 and c-Raf on the expression of c-myc mRNA in C2 by Northern blot analysis. In the absence of 4-HT, the expression of c-myc declined under culture with DM or ED in C2/MyoD/ER/STAT3/ERT and C2/MyoD/ER/Raf/ERT cells, whereas the 4-HT treatment retained its expression in both clones for up to 72 h (Fig. 2D). This result raised the possibility that both STAT3 and c-Raf might promote cell growth and inhibit DM- and ED-induced differentiation through c-myc. To test this possibility, we stably expressed a 4-HT-inducible form of c-Myc, Myc/ERT, in C2/gp130/MyoD/ER. When these clones were cultured with 4-HT, c-Myc inhibited the DM-induced but not ED-induced expression of myogenin (a representative result is shown in Fig. 2E). These results implied that cell cycle progression driven by c-Myc is sufficient to overcome DM-induced differentiation but not MyoD-induced differentiation in C2 cells. In addition, it was speculated that both STAT3 and c-Raf would inhibit MyoD-induced differentiation independently of c-myc induction. As a matter of fact, with regard to c-Raf, its downstream molecule, MEK1, was shown recently to inhibit MyoD activities through direct interaction (28). Therefore, we analyzed the effects of STAT3 on MyoD activities in the following experiments.

STAT3 Inhibits MyoD Activities as Efficiently as c-Raf—To examine the effects of gp130L on MyoD activities, we transfected MyoD/ER or WT MyoD together with MCK-Luc (a reporter gene for MyoD) and G-CSFR/gp130 into C2 and C3 cells and performed luciferase assays. As shown in Fig. 3A, left panel, the treatment with gp130L effectively inhibited the ED-induced MyoD activity in MyoD/ER-transfected C2 cells (from 5.1-fold induction to 1.3-fold induction). gp130L also severely suppressed WT MyoD-induced MCK-Luc activity (from 5.5-fold to 1.2-fold, Fig. 3A, right panel). Similar results were obtained with C3 cells (data not shown). To further characterize the functional roles of downstream molecules of gp130 in this suppression, we examined the effects of STAT3-C (constitutively active STAT3) and H-RasG12V (oncogenic H-Ras) on MyoD activities. As shown in Fig. 3B, STAT3-C disrupted ED-induced differentiation...
MyoD activity as efficiently as H-RasG12V. 4-HT-activated STAT3/ERT and Raf/ERT also suppressed ED-induced MyoD activity as efficiently as H-RasG12V. 4-HT-activated STAT3/ERT and Raf/ERT as indicated. The transfected cells were cultured and subjected to luciferase assays. C, C2/gp130/MyoD/ER cells expressing STAT3/ERT or Raf/ERT were stimulated with 2 μM ED for 30 min with or without the 4-HT pretreatment (1 μM for 2 h). The expression of p21WAF1 was examined by Northern blot analysis.

Effects of the MyoD Family of Proteins on STAT Activities and Their Biological Significance—Next, we analyzed with luciferase assays, whether the MyoD family proteins conversely influence STAT activities in C2, C3, and NIH3T3 cells. As shown in Fig. 4A, upper panel, STAT3-C activities detected with its reporter gene, 4× APR-Luc, were severely inhibited by MyoD and myogenin but not by Myf5 in C2. In addition, gp130L-induced STAT3 activities were also inhibited by MyoD and myogenin (data not shown). Similar results were observed with C3 and NIH3T3 cells (data not shown). In contrast, none of MyoD, myogenin, or Myf5 affected 1× 6-STAT5A (constitutively active STAT5A) activities detected by 3× β-Cas-Luc (a reporter gene for STAT5) in NIH3T3 cells (Fig. 4A, lower panel). These results suggest that, among MyoD family proteins, MyoD and myogenin specifically inhibit activities of STAT3 but not of STAT5. To assess the biological significance of this suppression, we introduced MyoD/ER into a murine IL-3-dependent proB cell line Ba/F3 engineered to express G-CSFR/gp130 in which STAT3 activities were proven to be essential for gp130-mediated growth and survival (9, 31). This clone was named Ba/F3/gp130/MyoD/ER. At first, we examined the effects of ED-activated MyoD on the expression of JunB (a target molecule of STAT3) and CIS (that of STAT5) by Northern blot analysis. After deprivation of gp130L for 12 h, we stimulated Ba/F3/gp130/MyoD/ER and Ba/F3/gp130/Mock (an empty vector-transfected control clone) with gp130L or IL-3 for 30 min and investigated their induction levels. As expected, the gp130L-induced expression of Jun B was severely inhibited by the 2-h pretreatment with ED in Ba/F3/gp130/MyoD/ER but not in Ba/F3/gp130/Mock, whereas the IL-3-induced expression of CIS was hardly affected by the ED pretreatment in both clones (Fig. 4B). We also examined the effects of MyoD on the growth of these clones under culture with gp130L or IL-3. As shown in Fig. 4C, the ED treatment drastically inhibited the gp130L-dependent growth in Ba/F3/gp130/MyoD/ER but not in Ba/F3/gp130/Mock, whereas it scarcely affected the IL-3-dependent growth in both clones. Moreover, DNA content analysis revealed that the ED treatment evoked severe apoptosis in Ba/F3/gp130/MyoD/ER (the proportion of sub-G1 after the 72-h treatment, 62%) but not in Ba/F3/gp130/Mock under culture with gp130L (Fig. 4D). In contrast, the ED treatment hardly affected cell cycle profiles under culture with IL-3 in both clones. Together, these results indicate that MyoD inhibits activities of STAT3 but not of STAT5, thereby inhibiting gp130L-dependent growth and survival in Ba/F3 cells.

STAT3 and MyoD Form a Complex in Vitro and in Vivo—To elucidate the mechanism underlying the functional antagonism between STAT3 and MyoD, we examined their in vitro binding by coimmunoprecipitation methods. We transfected HA-MyoD and/or FLAG-tagged WT STAT3, together with G-CSFR/gp130, into 293T cells and isolated total cellular lysates after stimulation with gp130L. As shown in Fig. 5A, the immunoblot with the anti-FLAG Ab on the anti-HA immunoprecipitates revealed that STAT3 was coimmunoprecipitated with MyoD only when both molecules were transfected (top panel, lane 4), and vice versa (third panel, lane 4). Next, we performed in vitro binding assays using GST-STAT3. Because we could not purify the GST-STAT3 fusion protein containing the full portion because of the formation of the inclusion body, we generated several GST-STAT3 fusion proteins, each consisting of the fragment as follows: GST-STAT3 (amino acids 1–154); GST-STAT3 (303–377); GST-STAT3 (320–590); and GST-STAT3 (580–770). After verifying the quality and quantity of the fusion proteins by the Coomassie brilliant blue staining (data not shown), we analyzed their binding to the in vitro translated FL MyoD. As shown in Fig. 5B, 35S-labeled MyoD bound to GST-STAT3 (320–590), but not to GST alone, GST-STAT3 (1–154), GST-STAT3 (303–377), or GST-STAT3 (580–770). Because the DNA-binding domain of STAT3 resides around amino acids 400 to 500, it was speculated that MyoD might inhibit DNA binding activities of STAT3 through direct interaction. Next, we tried to determine which domain of MyoD interacts with STAT3 using several mutants of MyoD (Fig. 5C). As shown in Fig. 5C, ΔTAD (transactivating domain), Δ218–318, and Δ165–318 bound to GST-STAT3 (320–590) as efficiently as FL MyoD, whereas Δ1–124, Δ1–160, and Δ126–318
failed to form a complex. ΔBR and ΔH-L-H, each lacking only the basic region (BR) and H-L-H domain, showed severely reduced binding activities as well. These results indicate that both BR and H-L-H domains are necessary for MyoD to interact with STAT3. Largely consistent with this finding, mutants of MyoD lacking either BR or H-L-H domains such as BR, 1–124, 1–160, and 126–318 scarcely inhibited STAT3 activities in luciferase assays (Fig. 5D). Moreover, we found that 1–165–318 could not inhibit STAT3 activities, whereas 1218–318 was effective. This result indicates that, although the domain between H-L-H and chromatin binding domain 2 is dispensable for the binding to STAT3, it is necessary for inhibiting STAT3 activities.

**MyoD and STAT3 Reciprocally Inhibit DNA Binding Activities—Next**, we examined the effects of MyoD on DNA binding activities of STAT3 by EMSA. We transfected WT STAT3 and G-CSFR/gp130 with various amounts of MyoD (as indicated in Fig. 6A) into 293T cells and prepared nuclear extracts after 30-min stimulation with gp130L. Although the nuclear protein isolated from unstimulated 293T cells did not bind to the probe for STAT3 (lane 1), the DNA-binding complex was detected in the nuclear protein isolated from gp130L-stimulated 293T cells (lane 2). This complex was abolished by the WT competitor (lane 3), but not by the mutated competitor (lane 4), and was supershifted by the anti-STAT3 Ab (lane 5), indicating that this complex was formed from STAT3. In addition, we found that the amount of this DNA-binding complex was reduced by co-transfected MyoD in a dose-dependent manner (lanes 6–8). This result suggests that MyoD would disrupt DNA binding activities of STAT3, thereby suppressing STAT3 activities. Similarly, we examined the effects of STAT3 on DNA binding activities of MyoD by EMSA (Fig. 6B). We transfected WT
**FIG. 5.** **MyoD inhibits STAT3 activities through direct binding.**

**A** 293T cells + G-CSFR/gp130 + gp130L

- HA-MyoD
- Flag-STAT3

| IP: α-HA | IB: α-Flag | IB: α-HA | IB: α-Flag | IB: α-Flag | IB: α-Flag | IB: α-Flag |
|----------|-------------|-----------|-------------|-------------|-------------|-------------|
| (-) (-) (+) (+) | STAT3 | MyoD | (-) (-) (+) (+) | MyoD | (-) (-) (+) (+) | STAT3 |

**B**

- input
- pulled down

- GST-STAT3

- GST-STAT3

- 35S-labeled FL MyoD

**C**

- FL
- TAD CB1
- BR H-L-H
- CB2
- ABR
- ΔH-L-H
- ΔTAD
- Δ1-124
- Δ1-160
- Δ218-318
- Δ165-318
- Δ126-318

**D**

- 4x APRE-Luc
- MyoD

- FL
- ΔBR
- ΔH-L-H
- ΔTAD
- Δ1-124
- Δ1-160
- Δ218-318
- Δ165-318
- Δ126-318

| STAT3-C(-) | STAT3-C(+) |
|------------|------------|
| C          | C          |
| FL         | FL         |
| ΔBR        | ΔBR        |
| ΔH-L-H     | ΔH-L-H     |
| ΔTAD       | ΔTAD       |
| Δ1-124     | Δ1-124     |
| Δ1-160     | Δ1-160     |
| Δ218-318   | Δ218-318   |
| Δ165-318   | Δ165-318   |
| Δ126-318   | Δ126-318   |

**Reciprocal Inhibition between MyoD and STAT3**

**Fig. 5.** MyoD inhibits STAT3 activities through direct binding. A, 293T cells were transfected with HA-tagged MyoD and/or FLAG-tagged WT STAT3, together with G-CSFR/gp130, as indicated. After 36 h, the cells were stimulated with 50 ng/ml of gp130L, and total cellular lysates were prepared. The in vivo binding between STAT3 and MyoD was examined by the coimmunoprecipitation method. IB, immunoblot; IP, immunoprecipitation. B, the in vitro binding between STAT3 and MyoD was examined by GST pull-down assays. 35S-labeled FL MyoD was incubated with several types of GST-STAT3, and the binding complexes were electrophoresed and subjected to autoradiography. C, the bindings between mutants of MyoD and GST-STAT3 (320–590) were examined by GST pull-down assays. The binding reactions were performed with the in vitro translated MyoD (input), and the binding complexes were electrophoresed and subjected to autoradiography (bound). TAD, transactivating domain; CB, chromatin-binding domain. D, H-L-H C2 cells were transfected with 2 μg of 4x APRE-Luc, 2 μg of STAT3-C, and 100 ng of pRL-CMV together with 4 μg of the mutant of MyoD as indicated. After 12 h, the cells were washed, deprived of serum, cultured for 24 h, and subjected to the measurement of the firefly and the Renilla luciferase activities. The results are shown as the mean ± S.D. of triplicate experiments.
MyoD and its heterodimeric partner E47, together with various amounts of STAT3-C, into 293T cells and prepared nuclear extracts. Although the nuclear proteins isolated from Mock- or MyoD-transfected 293T cells did not bind to the probe for MyoD containing the E-box sequence (lanes 1 and 2, respectively), the DNA-binding complex was detected in the nuclear protein isolated from both MyoD- and E47-transfected 293T cells (lane 3). We confirmed that this complex was formed from the MyoD/E47 heterodimer in a sequence-specific manner using WT and mutated competitors (lanes 4 and 5), the anti-MyoD Ab (lane 6), and the anti-E47 Ab (lane 7). Furthermore, we found that this complex was reduced by STAT3-C (lanes 8 and 9), suggesting that STAT3 would inhibit DNA binding activities of MyoD.

**Fig. 6.** The mechanism of the reciprocal inhibition between STAT3 and MyoD. A, 293T cells (5 x 10⁶ cells seeded in a 10-cm dish) were transfected with G-CSFR/gp130, WT-STAT3, and HA-MyoD at the indicated amount. After 12 h, the cells were washed, deprived of serum, cultured for 24 h, and stimulated with gp130L (50 ng/ml) for 30 min. Then nuclear extracts were prepared and subjected to EMSA with the end-labeled probe for STAT3 (IR). B, DNA-binding complex. SS, supershifted band. B, 293T cells were transfected HA-MyoD, E47, and STAT3-C at the indicated amount. After 12 h, the cells were washed, deprived of serum, and cultured for 24 h, and then nuclear extracts were isolated. The nuclear extracts were subjected to EMSA with the probe for MyoD (E-box). C and D, C2 cells were transfected with pG5-Luc, pBIND (GAL4), or pBIND-FL-MyoD (GAL4-FL-MyoD fusion construct), with or without STAT3-C as indicated (C). C2 cells were also transfected with MCK-Luc, WT MyoD, STAT3-C, p300, CBP, and PCAF in the indicated combinations (D). After 12 h, the cells were washed, deprived of serum, cultured for 24 h, and subjected to luciferase assays. The results are shown as the mean ± S.D. of triplicate experiments.
Reciprocal Inhibition between MyoD and STAT3

6C). However, this activation was reduced to 12.5-fold when STAT3-C was cotransfected, indicating that STAT3-C suppresses transcriptional activities of MyoD. Because transcriptional activities of MyoD are crucially regulated by p300, CBP, and/or PCAF through the acetylation (37, 40, 41), we examined the effects of these molecules on MyoD activities suppressed by STAT3. As a result, we found that the supplement of p300, CBP, and PCAF individually restored MyoD activities suppressed by STAT3 (Fig. 6D). Because STAT3 also binds to these molecules (42, 43), this result proposed a possibility that the STAT3-MyoD binding might affect the interactions between MyoD and these transcriptional coactivators, thereby suppressing its transcriptional activities.

DISCUSSION

The biological effects of growth factors are mediated by various intracellular signaling cascades such as JAK/STAT's, Ras/MAPK, and phosphatidylinositol 3-kinase/Akt pathways. As for myogenesis, the Ras/MAPK pathway activated by IGF-I, IGF-II, HGF, or bFGF was reported to participate in cell cycle progression (44–48), although it was also shown to mediate terminal differentiation in other papers (49, 50). By contrast, IGF-I- or IGF-II-activated phosphatidylinositol 3-kinase/Akt has been shown to mediate myogenic differentiation and to support cell survival (51, 52). Although many of the growth factors that regulate the growth and differentiation of myoblasts, such as LIF, IGF-I, HGF, PDGF, and bFGF, can activate STAT3, its roles in myogenesis still remain largely unknown. Therefore, in this study, we analyzed the role for STAT3 in the growth and differentiation of myoblasts in terms of cell cycle regulation and interaction with MyoD. As a result, we found that 4-HT-activated STAT3/EVT inhibits DM- or MyoD-induced cell cycle arrest as well as Raf/EVT in C2 cells. This result suggests that STAT3 would promote cell growth in combination with the Ras/MAPK cascade in myoblasts. Regarding the mechanism underlying the STAT3-mediated cell growth, we found that STAT3 restored the expression of c-myc mRNA suppressed by DM or MyoD in C2 cells. Furthermore, we found that, in addition to the growth-supporting effects, 4-HT-activated STAT3 inhibited both DM- and MyoD-induced myogenic differentiation in C2 cells. Because 4-HT-activated Myc/EVT was able to inhibit the DM-induced but not MyoD-induced differentiation in C2 cells, it was speculated that, although STAT3-induced c-myc was sufficient to inhibit the DM (i.e., cell cycle arrest)-evoked differentiation, STAT3 would inhibit MyoD-mediated myogenic differentiation through additional mechanism(s) besides the induction of c-myc (e.g., the inhibition of MyoD activities as described here).

Although MyoD and Myf5 are expressed in proliferating myoblasts, these activities are kept silent during culture in the presence of mitogens. However, upon the deprivation of these mitogens, myoblasts withdraw from the cell cycle, and subsequently MyoD and Myf5 begin to exhibit their activities and thereby initiate the differentiation program (22). These findings imply that there is some regulatory mechanism that would control MyF activities by sensing cell cycle status in myoblasts as they respond to growth factors or external signals. As for this mechanism, it was reported that mitogen-activated CDK4 translocates into the nucleus in a complex with cyclin D1 and inhibits DNA binding activities of MyoD through direct interaction (53). In addition, activated MEK1 was found to interact with MyoD and to repress its transcriptional activities (28). Furthermore, c-Jun was proved to disrupt the interaction between MyoD and its transcriptional cofactor p300/CBP by binding to p300/CBP (22), whereas c-Jun by itself does not bind to MyoD. In addition to these reports, we showed here that gp130L-activated STAT3 inhibited MyoD activities. Regarding this mechanism, we found that STAT3 inhibited both DNA binding activities and transcriptional activities of MyoD. In addition, we found that the supplement of p300, CBP, or PCAF significantly restored its transcriptional activities suppressed by STAT3. These results raised the possibility that the amounts of these cofactors might be limited in myoblasts and that STAT3 might deprive MyoD of these transcriptional cofactors, thereby inhibiting the function of MyoD. As a matter of fact, this possibility was confirmed to be true in the case of the functional antagonism between STAT3 and Smad3/4 in a hepatocellular carcinoma cell line, HepG2 (54). However, besides this mechanism, it is also possible that, because MyoD interacts with STAT3 through its BR and H-L-H domain, necessary for the binding to its heterodimeric partner E12/E47 and its cooperative partner MEP2, STAT3 might inhibit these interactions, thereby suppressing transcriptional activities of MyoD in myoblasts.

In addition to the fact that STAT3 inhibited MyoD activities, we found that MyoD conversely suppressed STAT3 activities, thereby inhibiting STAT3-dependent growth and survival in Ba/F3 cells. As for this mechanism, we found that MyoD interacted with the DNA-binding domain of STAT3 and inhibited its DNA binding activities. Furthermore, it is also possible that MyoD might deprive STAT3 of transcriptional cofactors such as p300/CBP and PCAF as discussed above, thereby affecting the transcriptional activities of STAT3. Together, our results indicate that the balance between STAT3 activities and MyoD activities might be crucially important to determine the fate of myoblasts, i.e., growth or differentiation. However, in addition to the MyoD-STAT3 interaction, both MyoD and STAT3 have been shown to interact with various nuclear proteins and regulate their functions, and vice versa: c-Jun, p300/CBP, NeaA/SRC1a, and PIA53 for STAT3 (42, 43, 55–57); and CDK4, MEK1, p300/CBP, and PCAF for MyoD (28, 36, 39, 40, 50). Therefore, further studies to elucidate the functional cooperation among these nuclear proteins in the growth and differentiation of myoblasts would be particularly important to clarify the molecular mechanisms of myogenesis.

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