Skeletal muscle differentiation, maturation, and repair require ongoing cooperation and coordination between an intrinsic regulatory program controlled by myogenic transcription factors, including members of the MyoD and MEF2 families, and environmental cues mediated by hormones and growth factors. Insulin-like growth factors (IGFs) also play key roles in muscle development, and in the maintenance and repair of mature muscle, but their mechanisms of interaction with other muscle regulatory networks remain undefined. To evaluate the potential interplay between MyoD and IGF signaling pathways, we have studied muscle differentiation in C3H 10T1/2 fibroblasts acutely converted to myoblasts by quantitative infection with a recombinant adenovirus encoding mouse MyoD. In these cells, IGF-II gene and protein expression are induced as early events in differentiation, and the IGF-I receptor and downstream signaling molecules, including Akt, are rapidly activated. Interference with IGF-II production by a tetracycline-inhibited adenovirus expressing an IGF-II cDNA in the antisense orientation reversibly inhibited both production of muscle-specific structural proteins and myocyte fusion to form multinucleated myotubes. Similar results were achieved with a tetracycline-inhibited adenovirus expressing dominant-negative Akt. Our observations identify a robust autocrine amplification network in which MyoD enhances the later steps in muscle differentiation by induction of a locally acting growth factor.

Experimental Procedures

Materials—Fetal calf serum, newborn calf serum, horse serum, and trypsin were purchased from Invitrogen Inc. Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline were from Mediatech-Cellogrow (Herndon, VA). RIGF-I was from Gro-Pep (Adelaide, Australia). Doxycycline was from Clontech (Palo Alto, CA) and was dissolved in distilled water at a concentration of 500 μg/ml and stored at −20 °C until use. Protease inhibitor tablets were purchased from Roche Applied Sciences, okadaic acid from Alexis Biochemicals (San Diego, CA), and sodium orthovanadate from Sigma. Effectene was from Qiagen Inc. (Valencia, CA), and TransIT-LT-1 was from Mirus Corp.
(Madison, WI). The BCA protein assay kit was from Pierce, and nitrocellulose was from Omnicron (Westborough, MA). Restriction enzymes, buffers, ligases, and polymerases were purchased from Roche Applied Sciences, BD Biosciences (Clontech), and Fermentas (Hanover, MD). Reagents for enhanced chemiluminescence were from Amersham Biosciences. Several monoclonal antibodies were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA), including F5D (anti-myogenin, W. E. Wright), MP20 (ant-myosin heavy chain (MHC), D. A. Fischman), and CT3 (anti-troponin T, J. J.-C. Lin). A monoclonal antibody to MyoD was from BD Biosciences (Pharmingen, San Diego, CA). Polyclonal antibodies to Akt and phospho-Akt (Ser473) were from Cell Signaling Technology (Beverly, MA), and the polyclonal antibody to IGF-II was purchased from Abcam, Ltd. (Cambridge, UK). A monoclonal antibody to phosphotyrosine was from Santa Cruz Biotechnology (Santa Cruz, CA), as was a polyclonal antibody to the β subunit of the IGF-I receptor. Antibody conjugates were purchased from Molecular Probes (Eugene, OR); goat anti-mouse IgG2α-Alexa 488, goat anti-mouse IgG1-Alexa 594, anti-rabbit IgG-alkaline phosphatase, and anti-mouse IgG-alkaline phosphatase. The AdEasy adenoviral recombinant kit was from Q-BIO Gene (Carlsbad, CA). All other chemicals were reagent grade and were purchased from commercial suppliers.

**Cell Culture**—C3H 10T1/2 mouse embryonic fibroblasts (ATCC catalog number CCL226) were incubated on gelatin-coated tissue culture dishes in growth medium (DMEM with 10% heat-inactivated fetal bovine serum, filtered through a Gelman syringe filter (0.45 μm deoxycholate, and 1% IGEPAL CA-630). Lysates were prepared using the BCA protein assay kit. Protein samples (30 μg) were denatured by heating to 95°C in 1% SDS, 10% glycerol, 0.5% β-mercaptoethanol, 50 mM Tris-Cl, pH 6.8, 5 mM dithiothreitol, 0.1% SDS, 0.5% sodium deoxycholate, and 1% IGEPAL CA-630. Lysates were passed through a 22-gauge needle and centrifuged at 15,000 rpm at 4°C to remove insoluble material, and protein concentrations were determined using the BCA protein assay kit. Protein samples (50 μg each) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with antibodies as described previously (22). Antibodies were used at the following dilutions: anti-MHC (1:500), anti-myogenin (1:500), anti-troponin T (1:1000), anti-α-actin (1:2000), anti-phospho-Akt (Ser473) (1:1000), anti-IGF-II (1:1000), anti-IGF-I receptor β subunit (1:500), anti-phospho-tyrosine (1:1000). IGF-II was detected by immunoblotting after extraction from conditioned media. Akt enzymatic activity was assessed by immunoblotting using GSK-3β antibody followed by a stop codon and threonine 308, and serine 473 to alanines using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Each mutation was verified by restriction enzyme mapping and by DNA sequencing. The Akt1(1–60) cDNA was then subcloned via pShuttle plasmid containing a tetracycline-regulated promoter (22), as was the coding region of mouse IGF-II in the pShuttle plasmid containing a tetracycline-regulated promoter (22), as was the coding region of mouse IGF-II in the pShuttle plasmid containing a tetracycline-regulated promoter (22). As described previously (23). RNA concentration was determined spectrophotometrically at 260 nm, and its quality assessed by agarose gel electrophoresis. RNA (2.5 μg) was reverse-transcribed in a final volume of 20 μl using a RT-PCR kit (Invitrogen) with oligo(dT) primers. Each PCR reaction contained 1.0 μl of cDNA. Primer sequences were as follows: for IGF-II, 5′-CTAGTGCAGGCTAGGAG-3′ (sense) and 5′-CTCGGAAGAGGCCCGCGTGA-3′ (antisense); for IGF-I, 5′-CTCTTCATGTTGACCCACAGACAGC-3′ (antisense); for MyoD, 5′-ATGGAATGGTCATGAGCATTCC-3′ (sense), and 5′-GACGGGATCCACCCGAATCTA-3′ (antisense); for S17, 5′-ATCCCAACGAAAGCCTGCA-3′ (antisense). A linear range of product amplification was established in pilot studies for each primer pair, and the cycle number representing the approximate midpoint was used in final experiments. This varied from 20 to 25 cycles. Results were quantitated by densitometry after electrophoresis through 1% agarose gels.

**RESULTS AND DISCUSSION**

*Akt Activity Is Required for MyoD-mediated Myoblast Differentiation*—Recently published studies have demonstrated that Akt activity and protein expression are stimulated during differentiation of established muscle cell lines (17, 22, 25–28). Here we show that Akt enzymatic activity also is induced as an early event in differentiation in fibroblasts converted to myoblasts after acute infection with a recombinant adenovirus encoding mouse MyoD (Ad-MyoD) and that active Akt is needed for the later events in differentiation that culminate in myotube formation. Illustrated in Fig. 1 are results of time course experiments using 10T1/2 fibroblasts infected 1 day earlier with Ad-MyoD at an m.o.i. in which ~90% of cells express MyoD (data not shown) and subsequently incubated in DM. As seen in Fig. 1A, Ad-MyoD-infected 10T1/2 cells underwent rapid and extensive muscle differentiation, with progressive expression of myogenin and MHC, and formation of large multinucleated myotubes within 2 days. Similarly robust muscle-specific protein expression was observed by immunoblotting (Fig. 1B). Myogenin and MHC were induced in Ad-MyoD-infected cells but were not detected in fibroblasts infected with an adenovirus encoding β-galactosidase (Ad-β-Gal). Also seen in Fig. 1B is evidence of activation of Akt beginning at 16 h after addition of DM, as indicated by a progressive increase in phosphorylation on serine 473. A similarly large induction of Akt enzymatic activity was observed, as shown in Fig. 1C. In Ad-MyoD infected cells, Akt kinase activity was stimulated by ~20-fold by 1 day in DM, was increased by over 30-fold by 2 days, and was maintained at high levels for at least 3 days. Little Akt activity could be detected in fibroblasts acutely infected with Ad-β-Gal (data not shown). In contrast to these results, minimal activation of the MAP kinases, Erks 1 and 2 or p38, was seen in Ad-MyoD or Ad-β-Gal-infected cells during the same interval (data not shown).

The significance of the rise in Akt kinase activity in MyoD-
mediated muscle differentiation was tested by co-infecting 10T1/2 cells with recombinant adenoviruses expressing MyoD and a dominant-negative Akt under control of a tetracycline-regulated gene promoter (Ad-AktDN). In pilot studies, Ad-AktDN blocked IGF-induced Akt enzymatic activity (data not shown). As seen in Fig. 2, A and B, inhibition of endogenous Akt blocked MHC and troponin-T expression and myotube formation but had no effect on production of myogenin. In contrast, when expression of AktDN was impeded by the tetracycline analog, doxycycline, differentiation proceeded normally. Thus, Akt activity is required for MyoD-stimulated muscle differentiation.

IGF-II mRNA and Protein Expression Are Induced, and the IGF-I Receptor Is Activated during MyoD-mediated Muscle Differentiation—We next investigated mechanisms responsible for activation of Akt during MyoD-stimulated myoblast differentiation. We first examined components of the IGF system, as IGF signaling has been found to stimulate Akt and to enhance differentiation of established muscle cell lines and in vivo (22, 28). As shown in Fig. 3A, IGF-II gene expression, measured by semi-quantitative RT-PCR assay, was progressively induced in Ad-MyoD infected fibroblasts beginning by 8 h after incubation in DM. In contrast, little IGF-I mRNA could be detected over the same time frame, and transcripts for IGF-II and myogenin were not seen in fibroblasts infected with Ad-β-Gal and incubated in DM for the same time period. Transcripts for ribosomal protein S17 did not change in abundance.

IGF-II protein expression also was induced in Ad-MyoD-infected fibroblasts. As assessed by immunoblotting of conditioned DM, IGF-II accumulation was observed in concentrated media from cells infected with Ad-MyoD after 1 day and showed a dramatic increase by 2 days (Fig. 3B). Little IGF-II could be seen by 12 h in DM (data not shown), and none was detected in concentrated conditioned media from fibroblasts infected with Ad-β-Gal. Thus, induction of IGF-II gene and protein expression are early events in MyoD-regulated myoblast differentiation.

IGF-II activates intracellular signal transduction pathways by binding to the IGF-IR, a ligand-stimulated tyrosine protein kinase that undergoes autophosphorylation as an initial event in its activation (20). To assess phosphorylation of the IGF-IR in Ad-MyoD-infected 10T1/2 fibroblasts, lysates from cells incubated in DM were immunoprecipitated with an antibody to the β subunit of the receptor, followed by immunoblotting with an antibody to phosphotyrosine. As shown in Fig. 3C, progressively increasing tyrosine phosphorylation of the IGF-IR was detected beginning at 1 day after incubation of cells in DM. By 2 days, the extent of receptor tyrosine phosphorylation exceeded that induced in 10T1/2 cells after incubation with IGF-I for 15 min. By contrast, little receptor phosphorylation was seen after 12 h in DM in Ad-MyoD-infected fibroblasts (data not shown) or in Ad-β-Gal-infected 10T1/2 cells after up to 2 days in DM (Fig. 3C).

IGF-II Action Is Required for MyoD-stimulated Muscle Differentiation—Experiments were performed next to assess the functional significance of production of IGF-II and activation of the IGF-IR for MyoD-mediated myoblast differentiation. Fibroblasts were co-infected with Ad-MyoD and a recombinant adenovirus encoding an antisense cDNA for mouse IGF-II under control of a tetracycline-inhibited promoter (Ad-IGF-IIAS). As shown in Fig. 4A, Ad-IGF-IIAS caused a marked decline in induction of IGF-II mRNA after a 1 day incubation of cells in DM, which was reversed by doxycycline. Secretion of IGF-II also was blocked in cells expressing IGF-IIAS mRNA, and tyrosine phosphorylation of the IGF-IR was impaired, being seen only at the 2-day time point (Fig. 4, B and C). This apparent discrepancy may be explained by the partial inhibition of
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EXPERIMENTS USING 10T1/2 FIBROBLASTS INFECTED WITH AD-MYOD OR AD-β-GALACTOSIDASE (AD-β-GAL) AND INCUBATED IN DM FOR UP TO 2 DAYS. A, INDUCTION OF IGF-II AND MYOGENIN mRNA BUT NOT IGF-I OR S17 mRNA DURING INCUBATION OF AD-MYOD-INFECTED 10T1/2 FIBROBLASTS IN DM, AS ASSESSED BY SEMIQUANTITATIVE RT-PCR. B, PROGRESSIVE SECRETION OF IGF-II INTO DM BY AD-MYOD INFECTED 10T1/2 CELLS AS ASSESSED BY IMMUNOBLOTTING OF CONCENTRATED CONDITIONED MEDIUM. C, PROGRESSIVE TYROSINE PHOSPHORYLATION OF THE IGF-I RECEPTOR β SUBUNIT (IGF-IRβ) IN AD-MYOD INFECTED 10T1/2 FIBROBLASTS INCUBATED IN DM AS ASSESSED BY IMMUNOPRECIPITATION WITH ANTIBODIES TO IGF-IRβ FOLLOWED BY SEQUENTIAL IMMUNOBLOTTING WITH ANTIBODIES TO PHOSPHOTYROSINE (pTyr) AND IGF-IRβ. SIMILAR RESULTS WERE SEEN FOR A–C IN THREE INDEPENDENT EXPERIMENTS.

IGF-II mRNA BY AD-IGF-IIAS AND BY THE GREATER SENSITIVITY OF THE ASSAY FOR IGF-IR-TYROSINE PHOSPHORYLATION THAN THE ASSAY FOR IGF-II PROTEIN (WITH A DETECTION LIMIT OF ~3 nM OF IGF-II). BOTH WERE RESTORED TO NORMAL WITH DOXYCYCLINE (FIG. 4, B AND C), THUS, AD-IGF-IIAS IMPAIRED BOTH IGF-II PRODUCTION AND IGF-IR ACTIVATION.

INHIBITION OF IGF-II ALSO DIMINISHED MYOD-MEDIATED DIFFERENTIATION. AS SHOWN IN FIG. 4, D AND E, AD-IGF-IIAS REDUCED THE RISE IN MYOGENIN ACCUMULATION, BLOCKED MHC EXPRESSION, PREVENTED MYOTUBE FORMATION, AND DELAYED AKT PHOSPHORYLATION. IN CONTRAST, WHEN EXPRESSION OF IGF-IIAS mRNA WAS BLOCKED BY DOXYCYCLINE, AKT PHOSPHORYLATION WAS RESTORED AND DIFFERENTIATION PROCEEDED NORMALLY. THEREFORE, EARLY PRODUCTION OF IGF-II AND ACTIVATION OF THE IGF-IR ARE REQUIRED FOR MYOD-STIMULATED MUSCLE DIFFERENTIATION.

THE CENTRAL ROLE OF MYOD AND RELATED bHLH TRANSCRIPTION FACTORS IN MUSCLE CELL SPECIFICATION AND DIFFERENTIATION HAS BEEN KNOWN FOR OVER A DECADE (3), AND NUMEROUS STUDIES HAVE DEMONSTRATED THAT MYOD CAN READILY CONVERT A RANGE OF CELL TYPES TO MYOBLASTS (3). WE NOW FIND THAT AN ENDOGENOUSLY INITIATED SIGNALLING PATHWAY, INVOLVING INDUCTION OF IGF-II GENE AND PROTEIN EXPRESSION, AND STIMULATION OF THE IGF-IR AND AKT, ARE ADDITIONAL KEY COMPONENTS OF MYOD-MEDIATED MYOBLAST DIFFERENTIATION. IGF-II PRODUCTION, AND IGF-IR AND AKT ACTIVATION, ARE RELATIVELY EARLY EVENTS IN THE ACTIONS OF MYOD IN THIS MODEL SYSTEM, OCCURRING SOON AFTER INDUCTION OF MYOGENIN, AND THIS SIGNALLING PATHWAY APPEARS NECESSARY FOR DIFFERENTIATION TO PROCEED, AS EITHER INHIBITION OF IGF-II OR BLOCKADE OF AKT IMPAIRED EXPRESSION OF MHC AND TROPHONIN-T AND FORMATION OF MULTINUCLEATED MYOFIBERS. THUS, AT LEAST IN THE CONTEXT OF THIS MODEL, OUR RESULTS INDICATE THAT IGF-II FUNCTIONS TO INITIATE AN ESSENTIAL AUTOCRINE AMPLIFICATION CASCADE FOR MYOD-MEDIATED DIFFERENTIATION. OUR OBSERVATIONS ALSO IDENTIFY AN APPROACH THAT WILL BE USEFUL IN DEFINING THE CRITICAL SIGNALLING PATHWAYS THAT ACT DOWNSTREAM OF AKT IN MUSCLE CELLS.

THE BIOCHEMICAL MECHANISMS BY WHICH MYOD INDUCES IGF-II GENE EXPRESSION ARE UNKNOWN. PREVIOUS STUDIES HAVE ESTABLISHED THAT IGF-II GENE TRANSCRIPTION IS STIMULATED DURING DIFFERENTIATION OF ESTABLISHED MUSCLE CELL LINES (29) BUT HAVE NOT IDENTIFIED KEY DNA RESPONSE ELEMENTS OR DEFINED CRITICAL TRANSCRIPTION FACTORS. THE MOUSE IGF-II GENE IS COMPLICATED. IT CONTAINS THREE TANDEM PROMOTERS, EACH WITH UNIQUE 5′ NONCODING EXONS (30). IGF-II GENE EXPRESSION IS ALSO REGULATED BY GENOMIC IMPRINTING, AND THE GENE RESIDES ~70 KB 3′ TO THE H19 GENE WITHIN A LARGE IMPRINtED LOCUS ON MOUSE CHROMOSOME 7 (31). THE TWO GENES ARE RECIPROCALLY IMPRINTED WITH IGF-II BEING EXPRESSED FROM THE MATERNALLY DERIVED CHROMOSOME AND H19 FROM THE MATERNAL CHROMOSOME (31). INVESTIGATION INTO THE MECHANISMS OF IMPRINTING HAS Led TO IDENTIFICATION OF A KEY MATERNALLY METHYLATED GENOMIC REGION LOCATED JUST 5′ TO H19 THAT FUNCTIONS AS AN INSULATOR ELEMENT AND REGULATES RECIPROCAL EXPRESSION OF THE TWO GENES (32, 33). IT HAS BEEN POSTULATED THAT BINDING OF THE NUCLEAR ZINC FINGER PROTEIN CTCF TO THE UNMETHYLATED (MATERNAL) CHROMOSOME AT THIS SITE DIRECTS ACTIVITY OF AN ENHANCER LOCATED 3′ TO THE H19 LOCUS TO THE NEARBY H19 PROMOTER, WHILE LACK OF BINDING OF CTCF TO THE METHYLATED (PATERNAL) CHROMOSOME DIRECTS ENHANCER FUNCTION TO THE FURTHER
5' IGF-II gene (32, 33). It is now possible to determine whether this region or other nearby sites containing putative enhancer elements (34–36) are critical for MyoD-induced IGF-II gene activity in muscle.

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