Regulation of insulin-like growth factor–dependent myoblast differentiation by Foxo forkhead transcription factors

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Insulin-like growth factors promote myoblast differentiation through phosphoinositol 3-kinase and Akt signaling. Akt substrates required for myogenic differentiation are unknown. Forkhead transcription factors of the forkhead box gene, group O (Foxo) subfamily are phosphorylated in an insulin-responsive manner by phosphatidylinositol 3-kinase–dependent kinases. Phosphorylation leads to nuclear exclusion and inactivation. We show that a constitutively active Foxo1 mutant inhibits differentiation of C2C12 cells and prevents myotube differentiation induced by constitutively active Akt. In contrast, a transcriptionally inactive mutant Foxo1 partially rescues inhibition of C2C12 differentiation mediated by wortmannin, but not by rapamycin, and is able to induce aggregation-independent myogenic conversion of teratocarcinoma cells. Inhibition of Foxo expression by siRNA resulted in more efficient differentiation, associated with increased myosin expression. These observations indicate that Foxo proteins are key effectors of Akt-dependent myogenesis.

Introduction

Muscle development is a multi-step process that begins with the determination of myogenic precursors from mesodermal stem cells and concludes with differentiation of committed myoblasts (McKinsey et al., 2001). This program depends on myogenic effectors of the MyoD family (Weintraub et al., 1991) and their cooperation with myocyte-specific enhancer-binding factors, such as MEF2 (McKinsey et al., 2001). These proteins activate expression of genes required for muscle differentiation through heterodimer formation with other ubiquitous bHLH proteins and by binding to E boxes, cis-acting elements found in the promoter regions of muscle-restricted genes (Olson and Klein, 1994).

Most peptide growth factors stimulate myoblast proliferation and prevent differentiation (Coolican et al., 1997). In contrast, insulin-like growth factors (IGFs) promote myoblast differentiation in vitro (Coolican et al., 1997; Tureckova et al., 2001). In cultured myoblasts, autocrine production of IGF2 is associated with differentiation triggered by serum withdrawal (Rosenthal et al., 1991; Stewart and Rotwein, 1996; Lawlor and Rotwein, 2000a). Inactivation of IGF signaling by targeted mutagenesis of the gene encoding IGF1 receptor leads to muscle hypoplasia (Liu et al., 1993), whereas IGF1 overexpression in muscle results in enlarged myofibers (Coleman et al., 1995), suggesting that IGFs participate in myogenesis in vivo.

IGF signaling activates the phosphatidylinositol 3-kinase (PI 3-kinase) and the MAPK pathways (Kim and Accili, 2002). Although several reports indicate a requirement for PI 3-kinase activity in the activation of myogenic program (Jiang et al., 1998; Kaliman et al., 1998), the MAPK pathway does not appear to contribute to IGF-dependent myogenesis (Coolican et al., 1997; Lawlor et al., 2000; Tureckova et al., 2001; Conejo et al., 2002). Among the effectors of PI 3-kinase, the serine/threonine kinase Akt has been shown to induce transcription of muscle-specific genes (Jiang et al., 1999), thus resulting in myoblast differentiation (Lawlor et al., 2000; Lawlor and Rotwein, 2000b). However, Akt substrates required for completion of the myogenic process are unknown. Some evidence indicates that p70<sup>S6k</sup> participates in myogenesis (Cuenda and Cohen, 1999), whereas the role

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Abbreviations used in this paper: Foxo, forkhead box gene, group O; IGF, insulin-like growth factor; MyHC, myosin heavy chain; PI 3-kinase, phosphatidylinositol 3-kinase; RD, rhabdomyosarcoma-derived.
of mTOR is unclear. Analyses with the mTOR inhibitor rapamycin have yielded conflicting results, with some suggesting that rapamycin inhibits (Coolican et al., 1997; Cuenda and Cohen, 1999; Conejo et al., 2002) and others suggesting that rapamycin promotes myoblast differentiation (Erbay and Chen, 2001). Notably, other authors have reported no effect of rapamycin in this process (Canicio et al., 1998).

Forhead transcription factors of the forkhead box gene, group O (Foxo) subfamily (Kaestner et al., 2000) are phosphorylated in an insulin-responsive manner by Akt and Sgk (Brunet et al., 1999, 2001; Nakae et al., 2000). Phosphorylation leads to Foxo inhibition through nuclear exclusion. Foxo proteins mediate several transcriptional effects of insulin and IGFs, including those on hepatic glucose production (Nakae et al., 2001b, 2002), pancreatic β-cell proliferation (Kitamura et al., 2002), and adipocyte differentiation (Nakae et al., 2003).

To study the role of Foxo proteins in myoblast differentiation, we examined the effects of constitutively active and dominant-negative mutant Foxo1 on in vitro differentiation of cultured myoblasts, teratocarcinoma cells, and rhabdomyosarcoma cells. To examine pathways impinging on Foxo1 regulation of myogenesis, we also studied the ability of Foxo1 mutants to regulate myoblast differentiation in the presence of pharmacological inhibitors of the PI 3-kinase and mTOR pathways, as well as constitutively active Akt. Our data identify Foxo proteins as key effectors of Akt-dependent myogenesis.

Results and discussion
Expression and phosphorylation of Foxo isoforms during differentiation
C2C12 cells undergo growth arrest and induction of the myogenic program after incubation in serum-free medium.

There are three Foxo isoforms in mice: Foxo1, -3 and -4 (Kaestner et al., 2000). Foxo4 and Foxo1 are the most abundant transcripts in skeletal muscle (Kitamura et al., 2002). Using Western analysis, we measured expression of the three proteins in myoblasts and myotubes. The relative abundance of all three isoforms declined in myotubes compared with myoblasts, but the difference was not statistically significant (Fig. 1 A). Next, we used phospho-specific antibodies to measure phosphate content of the main phosphorylation site (S253 in Foxo1, S193 in Foxo4). In myotubes, phosphate content increased by 38 and 40% for Foxo1 and Foxo4, respectively. To measure Foxo3 phosphorylation, we used an electrophoretic gel shift assay. We detected an 85% increase in the amount of low mobility (phosphorylated) protein in myotubes (Fig. 1 B). These data indicate that differentiation is associated with increased Foxo phosphorylation. From these observations, it can be inferred that myogenic differentiation requires Foxo1 inhibition, similar to what has been observed in adipocytes (Nakae et al., 2003) and thymocytes (Leenders et al., 2000).

Foxo1 mutants modulate myoblast differentiation
Because Akt mediates IGF-dependent C2C12 differentiation (Lawlor and Rotwein, 2000b; Tureckova et al., 2001) and Foxo isoforms are Akt substrates (Brunet et al., 1999), we investigated whether they mediate myoblast differentiation. We used adenovirus-mediated gene transfer to express constitutively active (ADA) and dominant-negative (Δ256) Foxo1 mutants in myoblasts. The constitutively active mutant cannot be phosphorylated and fails to translocate in response to insulin, whereas the dominant-negative mutant lacks the transactivation domain (Nakae et al., 2000). The Δ256-Foxo1 or ADA-Foxo1 adenoviruses were expressed at high levels after transduction (Fig. 2 A, lane 2 and lane 3). After 96 h in differentiation medium, cells transduced with
the ADA mutant failed to convert to myotubes, whereas cells transfected with the Δ256 mutant were morphologically indistinguishable from control cells (Fig. 2 B). Accordingly, the ADA mutant prevented expression of myosin heavy chain (MyHC), a marker of terminally differentiated myotubes. Conversely, transduction with Δ256 caused a 30% increase in MyHC expression (Fig. 2, B and C).

Myogenin is an early differentiation marker. In myoblasts, its expression was induced between 4 and 8 h of serum withdrawal. The transient decrease in myogenin expression during differentiation has been observed by others (Langley et al., 2002), and does not appear to interfere with the cells’ ability to undergo complete differentiation into myotubes (Fig. 2 B). Cells transfected with Δ256 showed a 70% increase of myogenin levels compared with untransfected cells. In contrast, ADA-transfected cells showed a 20% decrease (Fig. 2 D). These data are consistent with the observation that the Foxo1 gain-of-function mutant impairs differentiation, whereas the dominant-negative Foxo1 increases the efficiency of differentiation.

The effect of the ADA mutant could not be accounted for by nonspecific inhibition of Akt function because phosphorylation of Gsk3, another Akt substrate, was unaffected in cells expressing the ADA-Foxo1 mutant (Fig. 2 E).
We obtained additional evidence that Foxo inhibition is required for differentiation using siRNA to decrease Foxo mRNA levels. We designed two siRNAs, one directed against a Foxo1-specific sequence, and one against a pan-Foxo–specific sequence. Transfection of the pan-Foxo siRNA resulted in 70–90% decreases in mRNA levels encoding all three isoforms (Fig. 3 A), and was accompanied by a 30% increase in MyHC expression (Fig. 3 C). In contrast, transfection of the Foxo1-specific siRNA inhibited expression of Foxo1 (Fig. 3 B), but not of Foxo3 and -4, and induced a lesser increase in MyHC expression (Fig. 3 C).

**ADA Foxo1 prevents differentiation induced by Myr-Akt**

Akt stimulates myoblast differentiation (Coolican et al., 1997; Lawlor and Rotwein, 2000b). Transduction with a constitutively active Myr-Akt promoted differentiation and increased MyHC expression twofold. However, cotransduction of Myr-Akt and ADA-Foxo1 prevented differentiation induced by Myr-Akt and decreased MyHC expression to the same levels seen in cells transduced with ADA-Foxo1 alone (Fig. 4 B). These data suggest that Akt-induced myogenesis is mediated by Foxo.

**Δ256-Foxo1 fails to reverse rapamycin inhibition of differentiation**

Among the PI 3-kinase effectors, some authors have shown that p70s6k is required for differentiation (Coolican et al., 1997; Cuenda and Cohen, 1999; Conejo et al., 2002). Inhibition of mTOR by rapamycin resulted in an approximate 70% decrease of MyHC levels and blocked myogenic differentiation. The inhibition of MyHC expression by rapamycin was unaffected by Δ256-Foxo1 (Fig. 4 C; P < 0.001), suggesting that the mTOR pathway does not act through Foxo to promote differentiation.

**Δ256-Foxo1 induces differentiation of teratocarcinoma, but not of rhabdomyosarcoma cells**

P19 teratocarcinoma cells are able to undergo myogenic conversion when cultured with DMSO. When P19 cells were transduced with the Δ256-Foxo1, differentiation oc-
curred in the absence of DMSO, and MyHC expression increased to levels similar to DMSO-treated cells (Fig. 4 D, lanes 1–4 and bar graph). In contrast, transduction with the ADA mutant inhibited DMSO-induced differentiation and reduced MyHC expression by ~70%. Thus, a loss-of-function Foxo1 mutation enhances myoblast differentiation of P19 cells, whereas a gain-of-function mutation impairs it.

Rhabdomyosarcoma-derived (RD) cells lack functional IGF signaling (Merlino and Helman, 1999). Most rhabdomyosarcomas carry chromosomal translocations in which the DNA-binding domain of Foxo1 is fused to the transactivation domain of Pax3 or Pax8 (Wang et al., 1998; Merlino and Helman, 1999). We tested whether Foxo1 inhibition would restore differentiation by transducing cells with Δ256-Foxo1 and by culturing them in differentiation medium. However, we failed to detect morphological changes or increases in MyHC expression (Fig. 3 D, lane 5 and lane 6). Thus, Foxo1 inhibition alone cannot rescue the transformed phenotype of RD cells.

Conclusions
Our data provide evidence for a key role of Foxo transcription factors as mediators of IGF-dependent myoblast differentiation. We provide two lines of evidence to support our conclusions. Foxo1 gain-of-function is associated with impaired myoblast differentiation, whereas loss-of-function is able to partly restore inhibition of differentiation by wortmannin. Furthermore, constitutively active Foxo1 inhibited, whereas dominant-negative mutant Foxo1 caused a slight, but significant increase in the expression of differentiation markers. Consistent with the notion that Foxo is regulated by Akt, we also found that the ability of constitutively active Akt to induce differentiation (Rommel et al., 2001) can be blocked by the phosphorylation-defective Foxo1 mutant. These data identify Foxo1 as a key component of the Akt pathway in differentiation. This pathway requires activation of the cell cycle inhibitor p21 (Lawlor and Rotwein, 2000b), a Foxo1 target in differentiating adipocytes (Nakae et al., 2003). Although we have not examined p21 expression in C2C12, it is likely to be an important Foxo target in myoblasts as well.

The inability of the dominant-negative Foxo1 to completely reverse the inhibition of differentiation caused by wortmannin suggests that multiple effectors of differentiation act downstream of PI 3-kinase. Similarly, the dominant-negative Foxo1 failed to reverse rapamycin inhibition of myoblast differentiation, indicating that Foxo1 phosphorylation by mTOR is not required for this process. We suggest that signals regulating myoblast differentiation diverge downstream of PI 3-kinase, with both Akt and mTOR playing a role in the process.

Foxo1 inhibition restored myogenic differentiation of teratocarcinoma-derived cells, but not of rhabdomyosarcoma-derived cells. RD cells express myogenic factors, such as
MyoD and myogenin, but these factors are transcriptionally inactive (Tapscott et al., 1993). It has also been shown that RDs are defective in their PI 3-kinase→Akt signaling (Xu et al., 2002), and that constitutively active forms of PI 3-kinase and Akt fail to restore differentiation. Now, we extend those data by showing that inhibition of Foxo1 function is equally unable to restore the differentiated phenotype, suggesting that the defect in RD cells is distal to Foxo1.

After the submission of this manuscript, it has been reported that Foxo1 promotes differentiation of primary myoblasts (Bois and Grosveld, 2003). These conclusions are at odds with our data, and appear to be due to substantial differences in the experimental system, such as the dissociation of Foxo1 phosphorylation from nuclear localization, the lack of Akt regulation of Foxo1, and the failure of dominant-negative Akt to affect differentiation.

In conclusion, we show that Foxo1 plays in important role in regulating skeletal muscle differentiation through the PI3K→Akt pathway. Further work will identify Foxo1 targets involved in this process.

Materials and methods
Reagents
We obtained C5C12, P19, and RD cells from the American Type Culture Collection, anti-Foxo1 antibodies from Santa Cruz Biotechnology, Inc., anti-PAK antibody 12CA5 from Boehringer, anti-MyHC (clone MF-20) and anti-myogenin antibodies (clone F5D) from the Hybridoma Bank (University of Iowa, Iowa City, IA), and anti-phospho-Gsk3 and anti-Gsk3 antisera from Upstate Biotechnology, C5C12, P19 (Wilton and Skerjane, 1999), and RD cells (Merlino and Helman, 1999) were maintained and differentiated as described previously (Astolfi et al., 2001). Foxo1 and Akt adenoviral vectors have been described previously (Nakae et al., 2001b). Western blotting was performed according to standard procedures.

Design and transfection of siRNAs
We used the target finder and design tool (Ambion) to identify target siRNAs. The Foxo1-specific sequence was 5′-AAAAATGCTTCCAGATTGTCTG-3′. The pan-Foxo sequence was 5′-AAGGATAAGGGCGACAAGGACC-3′. SiRNA synthesis and transfection with siPORT™ reagents was performed according to the manufacturer’s instructions (Ambion). Foxo expression was measured by real-time RT-PCR using a LightCycler PCR instrument (Roche) and LightCycler SYBR Green I reaction mix (Roche). Each reaction was performed in triplicate under standard reaction conditions. β-Actin was used as a control of amplification efficiency.

Immunofluorescence
Cells were cultured on microscope slides, differentiated, and fixed in 4% PFA. After incubation with anti-MyHC IgG2b at 1:500, detection was performed using DAPC-conjugated secondary antibody (Nakae et al., 2003).

Statistical analysis
Statistical analysis was performed using t test for paired data.

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