Functional inactivation of the IGF-I receptor delays differentiation of skeletal muscle cells

Z-Q Cheng¹, S Adi¹, N-Y Wu¹, D Hsiao¹, E J Woo¹, E H Filvaroff², T A Gustafson³ and S M Rosenthal¹

¹Department of Pediatrics, University of California, San Francisco, California 94143, USA
²Department of Growth and Development, University of California, San Francisco, California 94143, USA
³Section of Signal Transduction, Metabolex, Inc., Hayward, California 94545, USA

(Requests for offprints should be addressed to S M Rosenthal, Division of Pediatric Endocrinology, University of California, San Francisco, California 94143–0434, USA; Email: smr@itsa.ucsf.edu)

Abstract

Skeletal myoblasts are inherently programmed to leave the cell cycle and begin the differentiation process following removal of exogenous growth factors. Serum withdrawal results in a marked induction of IGF production which is essential for skeletal muscle differentiation in vitro. However, the potential role of the tyrosine kinase IGF-I receptor (thought to be the principal mediator of both IGF-I and II signaling in skeletal muscle) in the decision of myoblasts to begin differentiation following serum withdrawal is unknown. To explore the role of the IGF-I receptor in this decision by skeletal myoblasts, we functionally inactivated endogenous IGF-I receptors in mouse C2C12 cells using a dominant negative, kinase-inactive IGF-I receptor in which the ATP-binding site lysine (K) at residue 1003 has been mutated to alanine (A). Cell lines with the greatest degree of mutant IGF-I receptor expression (A/K cells) demonstrated functional inactivation of endogenous IGF-I receptors as determined by their impaired ability to phosphorylate the principal substrate of the IGF-I receptor, IRS-1, in response to treatment with IGF-I. In addition, the proliferative response of myoblasts to IGF-I was completely abolished in A/K cells. Following withdrawal of exogenous growth factors, A/K cells demonstrated a marked delay in the induction of the gene expression of myogenin, a skeletal muscle-specific transcription factor essential for differentiation, and a subsequent delay in the induction of muscle creatine kinase activity. Delayed differentiation in A/K cells was associated with prolonged phosphorylation of the cell cycle regulatory retinoblastoma (Rb) protein; it is the un- (or hypo-) phosphorylated form of Rb which is known to promote differentiation in skeletal myoblasts. Thus, the IGF-I receptor regulates the timing of myoblast differentiation induced by serum withdrawal. The delayed differentiation of skeletal myoblasts with functionally inactive IGF-I receptors may result, at least in part, from delayed induction of myogenin gene expression and prolonged phosphorylation of the Rb protein.

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Introduction

Skeletal myoblasts are inherently programmed to leave the cell cycle and begin the differentiation process following removal of exogenous growth factors (Molkentin & Olson 1996). Serum withdrawal results in a marked induction of insulin-like growth factor (IGF) peptide production several days before attainment of the mature muscle phenotype (Tollefsen et al. 1989a, b, Florini et al. 1991, Rosenthal et al. 1991, Rosen et al. 1993), and IGF antisense oligodeoxynucleotides or cRNAs inhibit the differentiation process (Florini et al. 1991, Montarras et al. 1993).

While autocrine/paracrine production of IGF peptides plays a critical role in muscle differentiation, the potential role of the IGF-I receptor in the decision of skeletal myoblasts to begin differentiation following serum withdrawal is unknown. The tyrosine kinase IGF-I receptor is thought to be the principal mediator of both IGF-I and II signaling in skeletal muscle and in a wide variety of tissues (Ewton et al. 1987, LeRoith et al. 1995). IGF-I receptors are abundant in murine skeletal myoblasts (Beguinot et al. 1985, Tollefsen et al. 1989a, Rosenthal et al. 1991), including C2C12 cells (Palmer et al. 1997), during initiation of the differentiation process which occurs following serum withdrawal.

To explore the role of the IGF-I receptor in the ability of skeletal myoblasts to begin differentiation following serum withdrawal, we functionally inactivated the IGF-I receptor by a dominant negative strategy using a...
kinase-inactive IGF-I receptor. The dominant negative strategy for functional inactivation of endogenous IGF-I receptors is based on knowledge that the IGF-I receptor is a heterotetrameric complex resulting from dimerization of two proreceptor monomers, each of which is processed into α and β subunits (LeRoith et al. 1995). Ligand binding is thought to induce a conformational change in the IGF-I receptor which ultimately leads to its activation (LeRoith et al. 1995). Activation of the IGF-I receptor, itself a tyrosine kinase, results principally from intramolecular trans-autophosphorylation of one β-subunit by the other β-subunit within the receptor complex (Frattali & Pessin 1993). Previous studies have shown that overexpressed human IGF-I receptors form hybrids with endogenous rodent IGF-I receptor halves (Prager et al. 1992, Kato et al. 1993, Li et al. 1994, Prager et al. 1994, Webster et al. 1994, Burgaud et al. 1995, Kulik et al. 1997) and that hybrid receptors containing a kinase-inactive receptor half will bind ligand normally but will be functionally inactive since both receptor halves must have normal kinase activity for signaling to occur (Treadway et al. 1991, Frattali & Pessin 1993).

A dominant negative strategy to assess endogenous IGF-I receptor function has been used in a variety of cell types. Functional inactivation of endogenous IGF-I receptors with kinase-deficient IGF-I receptors has been reported in NIH-3T3 (Kato et al. 1993) and mouse wild-type fibroblasts (Li et al. 1994), rat-1 fibroblasts (Prager et al. 1994, Kulik et al. 1997), rat GC pituitary cells (Prager et al. 1992, Webster et al. 1994), and rat C6 glioblastoma cells (Burgaud et al. 1995). In the present study, we find that functional inactivation of endogenous IGF-I receptors in skeletal myoblasts with a kinase-inactive, dominant negative IGF-I receptor abolishes IGF-I induced proliferation and markedly delays the onset of differentiation normally induced by serum withdrawal.

Materials and Methods

Materials

The following chemicals were purchased: 

<table>
<thead>
<tr>
<th>Substance</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>25 Ci/mmol from Amersham Corp. (Arlington Heights, IL, USA)</td>
</tr>
<tr>
<td>α-32P-deoxy-CTP</td>
<td>3000 Ci/mmol from DuPont-New England Nuclear (Boston, MA, USA)</td>
</tr>
<tr>
<td>IRS-1 antibody</td>
<td>Rabbit polyclonal IgG from Upstate Biotechnology, Inc. (Lake Placid, NY, USA)</td>
</tr>
<tr>
<td>Phosphotyrosine antibody</td>
<td>Coupled to horseradish peroxidase from Transduction Laboratories (Lexington, KY, USA)</td>
</tr>
<tr>
<td>monoclonal antibody</td>
<td>G3-245 to the human retinoblastoma (Rb) gene product from PharMingen (San Diego, CA, USA)</td>
</tr>
<tr>
<td>FBS</td>
<td>Serum from the Cell Culture Facility (University of California, San Francisco, CA, USA)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Gift from Ciba-Geigy Corp. (Summit, NJ, USA)</td>
</tr>
<tr>
<td>Myogenin</td>
<td>cDNA from Dr. W. W. Wright (University of Texas Southwestern Medical Center, Dallas, TX, USA)</td>
</tr>
</tbody>
</table>

IGF-I receptor function has been used in a variety of cell types. Functional inactivation of endogenous IGF-I receptors with kinase-deficient IGF-I receptors has been reported in NIH-3T3 (Kato et al. 1993) and mouse wild-type fibroblasts (Li et al. 1994), rat-1 fibroblasts (Prager et al. 1994, Kulik et al. 1997), rat GC pituitary cells (Prager et al. 1992, Webster et al. 1994), and rat C6 glioblastoma cells (Burgaud et al. 1995). In the present study, we find that functional inactivation of endogenous IGF-I receptors in skeletal myoblasts with a kinase-inactive, dominant negative IGF-I receptor abolishes IGF-I induced proliferation and markedly delays the onset of differentiation normally induced by serum withdrawal.

Hormone binding assays

Binding of 125I-IGF-I to cell monolayers in six-well plates was carried out as previously described (Rosenthal et al. 1991). Nonspecific binding was determined in the presence of 3 × 10⁻⁸ M unlabeled IGF-I. Binding determinations were carried out in triplicate for each concentration of unlabeled ligand. Binding in each well was normalized to total protein content (Lowry et al. 1951).

Cross-linking studies

Covalent cross-linking of 125I-IGF-I to cell monolayers was carried out with disuccinimidyl suberate as previously described (Rosenthal et al. 1994). Cells were subsequently

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solubilized and analyzed by SDS-PAGE on 5–12% gradient gels as previously described (Rosenthal et al. 1994). Samples in each lane were normalized for cellular protein. Gels were stained, destained and dried, and autoradiography was carried out.

**Immunoprecipitation and immunoblotting analysis**

For IRS-1 phosphorylation studies, cells were switched from DMEM–20% FBS to DMEM–1% BSA for 16 h. Cells were then placed in fresh DMEM–1% BSA in the absence or presence of IGF-I (100 ng/ml) for 1 min at 37 °C. Cells were rinsed twice with ice-cold PBS and lysed in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4; 1% nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 1 µg/ml each of aprotinin, leupeptin and pepstatin; 1 mM NaVO₄; and 1 mM NaF). Lysates normalized for total protein were pre-cleared with protein A-agarose beads which were subsequently washed with ice-cold RIPA buffer and placed in 2 x Laemmli sample buffer. Samples were boiled for 5 min and analyzed by SDS-PAGE on 5–12% gradient gels. After electrotransfer and membrane blocking as previously described (Silverman et al. 1995), membranes were treated with an antiphosphotyrosine antibody coupled to horseradish peroxidase at a 1:1000 dilution in blocking buffer overnight at 4 °C. Membranes were subsequently washed three times in blocking buffer, and immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham ECL kit RPN 2108). Autoradiography was subsequently carried out. To measure total IRS-1 protein, blots were stripped and immunoreactive bands were washed with ice-cold RIPA buffer and placed in 2 x Laemmli sample buffer. Samples were boiled for 5 min and analyzed by SDS-PAGE on 5–12% gradient gels. After electrotransfer and membrane blocking as previously described (Silverman et al. 1995), membranes were treated with an antiphosphotyrosine antibody coupled to horseradish peroxidase at a 1:1000 dilution in blocking buffer overnight at 4 °C. Membranes were subsequently washed three times in blocking buffer, and immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham ECL kit RPN 2108). Autoradiography was subsequently carried out. To measure total IRS-1 protein, blots were stripped and treated with the same anti-IRS-1 antibody used for immunoprecipitation.

For Rb protein phosphorylation studies, cells were lysed in sample buffer, boiled and analyzed by SDS–7.5% PAGE as previously described (Rosenthal & Cheng 1995). After electrotransfer, nitrocellulose blots were incubated with an anti-Rb protein antibody that recognizes both phosphorylated and hypophosphorylated forms of Rb. When analyzed by SDS–7.5% PAGE, phosphorylated forms of Rb migrate above an un- (or hypo-) phosphorylated form of slightly greater than 105 kDa (Gu et al. 1993, Rosenthal & Cheng 1995). Immunoreactive bands were visualized as previously described (Rosenthal & Cheng 1995).

**mRNA analysis**

Total RNA was isolated by extraction in guanidinium isothiocyanate (Sambrook et al. 1989b). RNA was quantitated by spectrophotometric determination at 260 nM. Twenty-five micrograms of RNA per sample were denatured in formaldehyde, subjected to electrophoresis in 1% agarose gels and transferred to nitrocellulose. Myogenin cDNA was labeled using random primers to a specific activity of 10⁹ c.p.m./µg. Nitrocellulose membranes were pre-hybridized, hybridized and washed as described (Hartmann et al. 1990). Autoradiography was carried out, and mRNA abundance was determined by scanning densitometry.

**Muscle creatine kinase (MCK) assay**

At the indicated times, cells in DMEM–2% horse serum were washed with PBS, solubilized in 50 mM glycylglycine, 1% nonidet P-40, and homogenized in a 1 ml Dounce homogenizer (Olson et al. 1983). Muscle creatine kinase (MCK) assays were performed by reacting 0.1 ml lysate with phosphocreatine and ADP-gluthione (Sigma kit procedure no. 520, Sigma Chemical Co., St Louis, MO, USA). After 30 min, the reaction was quenched with p-hydroxymercuroibenzoate. Color reaction was induced by the addition of a-naphthol and 0.05% diacetyl. Spectrophotometric readings were taken at 520 nM, and MCK units were determined from a calibration curve generated by creatine standards. MCK values were normalized to total protein content. All samples were assayed in triplicate.

**Cell number**

At the indicated times, cell number was determined with a hemocytometer after dissociation with 0.25% trypsin and 0.5% EDTA at 37 °C. Determinations were carried out in triplicate for each condition. Cell viability was determined by exclusion of trypan blue (Resnicoff et al. 1995).

**Statistical analysis and densitometry analysis**

Cell proliferation rates, MCK and Rb data were analyzed by analysis of variance, with subsequent comparisons to control values made by unpaired t-test. For densitometric scanning of myogenin mRNA Northern blots and IRS-1 and Rb Western blots, analysis was performed on a G4 Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

**Results**

**Cross-linking and binding studies**

Six randomly selected control clones transfected with the insertless pECE vector and the neomycin resistance plasmid (pSV2 neo) and ten randomly selected clones expressing both the mutant kinase-inactive IGF-I receptor and the gene for neomycin resistance were screened by IGF-I
Functional inactivation of endogenous IGF-I receptors

To determine whether overexpression of this kinase-inactive IGF-I receptor resulted in functional inactivation of endogenous IGF-I receptors in skeletal myoblasts, we examined the ability of the IGF-I receptor to phospho-

ylate the principal substrate of the IGF-I receptor, IRS-1 (LeRoith et al. 1995), in response to treatment with IGF-I. A band of approximately 180 kDa corresponding to phosphorylated IRS-1 was seen in control cells treated with IGF-I (100 ng/ml for 1 min) but not in untreated control cells, as expected (Fig. 2). Phosphorylated IRS-1 was not detected in A/K-10 cells before IGF-I treatment. After IGF-I treatment, phosphorylated IRS-1 expressed as a percentage of total IRS-1 was reduced by approximately 80% in A/K-10 vs control cells (Fig. 2). Phosphorylated IRS-1 was similarly reduced in IGF-I-treated A/K-1 cells (data not shown). To confirm functional inactivation of endogenous IGF-I receptors, we examined the ability of IGF-I to induce a proliferative response in myoblasts. Cells growing in 20% FBS-supplemented medium were switched into serum-free medium with 1% BSA in the absence or presence of 20 ng/ml IGF-I for 24 h. This concentration of IGF-I for 24 h has been previously shown to stimulate a doubling in number of murine skeletal myoblasts (Ewton & Florini 1995). IGF-I treatment resulted in a significant proliferative response in control cells; however, no proliferative effect of IGF-I was seen in either A/K-1 or A/K-10 cells (Fig. 3).

Thus, overexpression of kinase-inactive IGF-I receptors resulted in functional inactivation of endogenous IGF-I receptors as determined both by impaired IGF-I-induced IRS-1 phosphorylation in A/K-1 and A/K-10 cells and lack of a mitogenic response to IGF-I. These cells were therefore used to explore the role of the IGF-I receptor in the ability of myoblasts to begin differentiation following serum withdrawal.

Functional inactivation of the IGF-I receptor delays myoblast differentiation

To evaluate the consequences of functional inactivation of endogenous IGF-I receptors on the ability of myoblasts to undergo spontaneous differentiation following serum withdrawal, we determined the ability of the IGF-I receptor to phospho-

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withdrawal, myoblasts were switched from medium supplemented with 20% FBS to medium supplemented with 2% horse serum, and early and late markers of muscle cell differentiation were assessed. This reduction in serum is well known to initiate the differentiation process (Molkentin & Olson 1996). The gene expression of myogenin, a member of the MyoD family of skeletal muscle-specific transcription factors which plays an essential role in muscle differentiation (Hasty et al. 1993, Nabeshima et al. 1993), was minimally detectable in control myoblasts in high serum and increased 10- to 20-fold after 24–48 h in low serum (1.8 kb mRNA) (Fig. 4), as expected. In contrast, the onset of myogenin mRNA induction was delayed in A/K-1 cells, with levels that were minimally detectable at 24 h and which did not increase significantly until 48 h in low serum (Fig. 4). A similar delay in the induction of myogenin mRNA was seen in A/K-10 cells (data not shown). In addition, expression of MCK activity, a later marker of skeletal muscle cell differentiation, was also delayed in A/K cells. While MCK activity increased significantly over time 0 values in control cells after 2 days in low serum, a significant increase in MCK activity did not occur in A/K cells until 6 days in low serum (Fig. 5). Of note, the percentage of viable cells was 95% in both control and A/K cells for up to 6 days in low serum-supplemented medium (data not shown).

Functional inactivation of the IGF-I receptor prolongs Rb phosphorylation following serum withdrawal

To explore a potential mechanism by which functional inactivation of endogenous IGF-I receptors delays skeletal muscle cell differentiation, we examined the phosphorylation state of the ubiquitous nuclear Rb protein. Phosphorylated Rb inhibits myoblast differentiation while un- (or hypo-) phosphorylated Rb promotes the differentiation process (Gu et al. 1993). We therefore hypothesized that delayed differentiation in A/K cells would be associated with prolonged Rb phosphorylation. When analyzed by SDS–7.5% PAGE and immunoblotting, phosphorylated forms of Rb migrate above an un- (or hypo–) phosphorylated form of slightly greater than 105 kDa (Gu et al. 1993, Rosenthal & Cheng 1995). Rb in proliferating myoblasts (maintained in serum supplemented with 20% FBS, time 0) was present predominantly in the phosphorylated form in control as well as in A/K-1 cells (Fig. 6A). Control cells switched into medium supplemented with 2% horse serum demonstrated a progressive decrease in phosphorylated Rb and an increase in hypophosphorylated
Rb, such that by 24 h, virtually all of the Rb was present only in the hypophosphorylated form, typical of differentiating cells (Gu et al. 1993, Rosenthal & Cheng 1995) (Fig. 6A, B). In contrast, the A/K mutant cells demonstrated prolonged Rb phosphorylation (Fig. 6A). At 24 h, the ratio of phosphorylated to un- (or hypo-) phosphorylated Rb was significantly greater in A/K vs control cells (1.10 ± 0.46 vs 0.37 ± 0.21 (mean ± s.d.), P<0.05) (Fig. 6B). Ultimately, Rb was present in a predominantly hypophosphorylated form in A/K cells, but only after 48–72 h in 2% horse serum-supplemented medium (data not shown).

Discussion

In the present study, we found that functional inactivation of endogenous IGF-I receptors in skeletal myoblasts with a kinase–inactive, dominant negative IGF-I receptor abolished IGF-I-induced proliferation and resulted in a delay in the differentiation process normally induced by withdrawal of exogenous growth factors. In contrast, when wild-type human IGF-I receptors were overexpressed in bovine or rodent skeletal muscle cells, an IGF-I-induced proliferative response was augmented in comparison to control cells, and differentiation occurred more rapidly than in control cells following serum withdrawal (Quinn et al. 1994, Quinn & Haugk 1996). Previous in vivo studies demonstrated that targeted disruption of the IGF-I receptor in mice resulted in skeletal muscle hypoplasia, but those cells that were present had undergone differentiation (Liu et al. 1993). A potential limitation of the in vivo knockout study, however, is that the IGF-I receptor was absent in all tissues (Liu et al. 1993), raising the possibility that the muscle phenotype was due, at least in part, to absent IGF-I receptors in the surrounding cell types which affect skeletal muscle biology, e.g. nerve cells and endothelial cells. We therefore developed a cell autonomous model in order to examine the consequences of IGF-I receptor inactivation on the ability of skeletal myoblasts to differentiate normally.

To explore potential mechanisms by which functionally inactivated IGF-I receptors delay differentiation of skeletal myoblasts, we examined myogenin gene expression and the phosphorylation state of the cell cycle regulatory retinoblastoma protein. Myoblasts expressing a dominant negative IGF-I receptor demonstrated a marked delay in the induction of myogenin expression and MCK activity. This delay in myogenin expression likely contributes to the delay in later markers of differentiation, such as MCK activity, since myogenin is essential for skeletal muscle differentiation (Hasty et al. 1993, Nabeshima et al. 1993). In addition, we found that myoblasts expressing a dominant negative IGF-I receptor demonstrated prolonged Rb phosphorylation in comparison to control cells. The delayed induction of myogenin gene expression and MCK activity in cells with functionally inactive IGF-I receptors may be a consequence of prolonged Rb phosphorylation, since myoblast differentiation is thought to require Rb dephosphorylation (Gu et al. 1993). In addition, the unphosphorylated but not the phosphorylated form of Rb can interact directly with members of the MyoD family of skeletal muscle-specific transcription factors, including myogenin (Gu et al. 1993). This interaction may be required for terminal myoblast differentiation because Saos-2 osteosarcoma cells, which express a nonfunctional Rb protein, can be converted to skeletal muscle by cotransfection with Rb and myogenin expression constructs but cannot be so converted with a myogenin construct alone (Gu et al. 1993). Thus, prolonged Rb phosphorylation itself could also contribute to delayed differentiation in A/K myoblasts with functionally inactive IGF-I receptors.

Since IGF-I receptors can form hybrids with insulin receptors (Bailyes et al. 1997), it is possible that the delayed differentiation we observed in myoblasts expressing a dominant negative IGF-I receptor is a consequence of functional inactivation of endogenous insulin receptors as
well as of endogenous IGF-I receptors. The contribution of insulin receptors in the present study, however, is likely to be minimal in view of studies demonstrating that insulin binding and insulin receptor mRNA are virtually non-detectable in the closely related C2 myoblasts, but increase only after myoblasts have differentiated into myocytes (Brunetti et al. 1989).

In view of recent studies which demonstrate a role for the IGF-I receptor in cell survival (Resnichoff et al. 1995, Kulik et al. 1997, Hongo et al. 1998), it is noteworthy that the percentage of viable cells was 95% in both control and A/K myoblasts expressing the dominant negative IGF-I receptor for up to 6 days in low serum-supplemented medium. The consequences of IGF-I receptor inactivation on cell survival appear to vary with cell type and experimental conditions. In rat-1 fibroblasts, a similar IGF-I receptor A/K mutant sensitized cells to apoptosis (Kulik et al. 1997), while expression of a closely related IGF-I receptor ATP-binding site mutant in rat C6 glioblastoma cells did not cause cell death (Burgaud et al. 1995). The lack of cell death in our studies with C2C12 myoblasts may reflect the fact that these experiments were carried out in monolayers. Numerous studies with compromised IGF-I receptors have demonstrated that decreased cell survival occurred in anchorage-independent or in in vivo conditions, yet apoptosis did not occur when the same cells were studied in monolayer (Resnichoff et al. 1995, Hongo et al. 1998).

In summary, while the IGF-I receptor is not essential for skeletal muscle differentiation, the IGF-I receptor significantly influences the timing of myoblast differentiation: overexpression of the IGF-I receptor accelerates myogenic differentiation (Quinn et al. 1994, Quinn & Haugk 1996), while functional inactivation of the IGF-I receptor results in delayed muscle differentiation. The delayed differentiation of skeletal myoblasts with functionally inactive IGF-I receptors may result, at least in part, from prolonged phosphorylation of the Rb protein and delayed induction of myogenin gene expression.

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