Role of the p66Shc Isoform in Insulin-like Growth Factor I Receptor Signaling through MEK/Erk and Regulation of Actin Cytoskeleton in Rat Myoblasts*

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To investigate the role of Shc in IGF action and signaling in skeletal muscle cells, Shc protein levels were reduced in rat L6 myoblasts by stably overexpressing a Shc cDNA fragment in antisense orientation (L6/Shca). L6/Shcas myoblasts showed marked reduction of the p66Shc protein isoform and no change in p52Shc or p46Shc proteins compared with control myoblasts transfected with the empty vector (L6/Neo). When compared with control, L6/Shcas myoblasts demonstrated 3-fold increase in Erk-1/2 phosphorylation under basal conditions and blunted Erk-1/2 stimulation by insulin-like growth factor I (IGF-I), in the absence of changes in total Erk-1/2 protein levels. Increased basal Erk-1/2 activation was paralleled by a greater proportion of phosphorylated Erk-1/2 in the nucleus of L6/Shcas myoblasts in the absence of IGF-I stimulation. The reduction of p66Shc in L6/Shcas myoblasts resulted in marked phenotypic abnormalities, such as rounded cell shape and clustering in islets or finger-like structures, and was associated with impaired DNA synthesis in response to IGF-I and lack of terminal differentiation into myotubes. In addition, L6/Shcas myoblasts were characterized by complete disruption of actin filaments and cell cytoskeleton. Treatment of L6/Shcas myoblasts with the MEK inhibitor PD98059 reduced the abnormal increase in Erk-1/2 activation to control levels and restored the actin cytoskeleton, re-establishing the normal cell morphology. Thus, the p66Shc isoform exerts an inhibitory effect on the mitogen-activated protein kinase signaling pathway in rodent myoblasts, which is necessary for maintenance of IGF responsiveness of the MEK/Erk pathway and normal cell phenotype.

Skeletal muscle represents an important site of action for the IGFs,¹ because specific high affinity IGF-I receptors are expressed in this tissue (1, 2), and both IGF-I and IGF-II stimulate growth and differentiation of skeletal muscle cells (3). Muscle cells also synthesize and secrete IGF-I, II, and IGF-binding proteins, providing a cell model for integrated autocrine and paracrine control of mitogenic and metabolic actions by the IGFs. IGF signaling in skeletal muscle involves activation of specific cell surface receptors, containing a tyrosine kinase domain within their cytoplasmic portion and undergoing autophosphorylation on specific tyrosine residues upon ligand binding. Receptor autophosphorylation triggers tyrosine phosphorylation of intracellular substrate proteins, including IRS-1, IRS-2, Crk-II, and the Shc proteins (4, 5).

The Shc proteins are widely expressed signaling mediators that are tyrosine-phosphorylated by multiple receptor or receptor-associated tyrosine kinases and are capable of stimulating multiple signaling pathways, including IRS-1, IRS-2, Crk-II, and the Shc proteins (4, 5).

¹ The abbreviations used are: IGF, insulin-like growth factor; Erk, extracellular signal-regulated protein kinase; IRS, insulin receptor substrate; PTB, phosphotyrosine binding; SH2, Src homology 2; CH1, -2, collagen homology 1 and 2; MEK, mitogen-activated and extracellular signal-regulated protein kinase kinase; PD98059, an inhibitor of activation of MEK by Raf; MEM, minimum essential medium; BCS, bovine calf serum; BSA, bovine serum albumin; PY99, phosphotyrosine antibody; Akt, protein kinase B; MAP, mitogen-activated protein; FITC, fluorescein isothiocyanate; Grb2, growth factor receptor binding protein-2; SOS, son of sevenless; EGF, epidermal growth factor; TBS, Tris-buffered saline.
oxidative stress, apoptosis, and life span (12, 13). However, the intracellular signaling pathways mediating the unique actions of p66Shc remain to be established.

The mitogenic and survival signals evoked by the IGFs have been extensively studied in multiple cell types, including skeletal muscle cells. Activation of the MEK/Erk pathway in response to the IGFs appears to mediate myoblast growth and differentiation (14, 15). IGF-I stimulation of DNA synthesis is blocked by the MEK inhibitor PD98059 (16), and myotubes do not differentiate when the MEK/Erk pathway is similarly blocked by PD98059 (17). Furthermore, suppression of IGF-I-stimulated cell proliferation in dexamethasone-treated L6 myoblasts is associated with increased Shc and decreased IRS-1 tyrosine phosphorylation (18, 19), suggesting that under conditions of glucocorticoid excess the mitogenic response of muscle cells to the IGFs can be modulated by altering the activity of the Shc/Erk pathway. The specific contribution of the p66Shc isoform to IGF-I action on skeletal muscle cells has not been investigated.

In this study, we show that selective reduction of p66Shc in L6 skeletal muscle cells results in up-regulation of the MEK/Erk pathway, leading to increased Erk-1/2 phosphorylation and nuclear localization in the absence of IGF-I stimulation. This is associated with a dramatic perturbation of the actin cytoskeleton, leading to abnormal cell shape, growth, and differentiation of the L6 myoblasts. The abnormalities in both signaling reactions and organization of the actin cytoskeleton are corrected by the MEK inhibitor PD98059, suggesting that in skeletal muscle cells the p66Shc isoform may physiologically exert an inhibitory role on MEK/Erk, which is necessary for full responsiveness of this signaling pathway to the IGFs and maintenance of normal cell morphology.

EXPERIMENTAL PROCEDURES

Cell Culture—L6 rat skeletal muscle myoblasts were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% donor calf bovine serum (BCS) (both from Invitrogen), 2 mM l-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and non-essential amino acids in a 5% CO2 atmosphere at 37 °C. Myoblasts were differentiated into myotubes with 2% horse serum (Invitrogen), 2 mM triiodothyronine (Sigma), and 20 nM insulin. For IGF-I studies, cells were incubated in complete medium containing 0.5% bovine serum albumin (BSA) without calf bovine serum for 16 h, and then stimulated with 100 nM IGF-I (GRO PEP, Adelaide, Australia) for the indicated times or left untreated. To block the MEK/Erk signaling pathway, cells were incubated with 20 μM PD98059 (Calbiochem, Merck KGaA) for the indicated times.

Antibodies—A monoclonal phospho-tyrosine antibody (PY99) and polyclonal IGF-I R β-subunit (C-20) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal MEK-1/2, phospho-MEK-1/2 (Ser-217/Tyr-221), Akt, phospho-Akt (Ser-473), and phospho-p42/44 MAP kinase (Erk-1/2) (Thr-202/Tyr-204) antibodies were from Cell Signaling Technology (Beverly, MA). Polyclonal and monoclonal Shc antibodies were from BD Transduction Laboratories (Lexington, KY). A monoclonal MAP kinase (Erk-1/2) antibody was from Zymed Laboratories (San Francisco, CA). A monoclonal vinculin antibody was from Sigma-Aldrich.

Transfection Studies—To generate L6 myoblasts stably expressing reduced levels of the p66Shc isoform, a 287-bp fragment of the cDNA encoding the p66/p46Shc proteins (from nucleotide 55 to nucleotide 342), indicated as as2, was generated by PCR amplification using the pMShc plasmid (kindly provided by Dr. J. Schlessinger, New York, NY) as a template. A 326-nucleotide cDNA fragment corresponding to the NH2-terminal 109 amino acids unique to p66Shc, indicated as as2, was generated by PCR amplification using the rat p66Shc cDNA (kindly provided by Dr. J. E. Pessin, New York, NY). To generate stable transfectants, the cDNAs of interest were cloned in antisense orientation into the mammalian expression vector pCR3.1 (Invitrogen), containing a G418 resistance gene, under control of the cytomegalovirus promoter. The as1- and as2-containing plasmids were transfected into L6 myoblasts by liposome-mediated gene transfer using LipofectAMINE™ (Invitrogen), and stable transfectants, indicated as L6/Shcas1 and L6/Shcas2, were selected by their ability to grow in neomycin-containing medium. Multiple stable clones of L6 cells harboring Shc were obtained within 4 weeks. Plasmid integration into the cell genome was confirmed by PCR amplification of genomic DNA with forward and reverse oligonucleotide primers corresponding to the pCR3.1 (5'-TAA TAC GAC TCA CTA TAG GG-3') and Shc (5'-CTG CAG TGG CCC TGT CCA TCC-3') sequences, respectively. The amount of Shc protein expressed in transfected L6 myoblasts was determined by immunoblotting total cell lysates with Shc antibodies, as previously described (18).

Immunoprecipitation and Immunoblotting—For immunoprecipitation studies, L6 skeletal muscle cells were washed twice with Ca2+/Mg2+-free PBS and then scraped in ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM MgCl2, 1% Nonidet P-40, 50 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 mM sodium orthovanadate, and 1% Nonidet P-40. Cell lysates were centrifuged at 12,000 × g for 10 min, and the resulting supernatant was collected and assayed for protein concentration using the Bradford dye binding assay kit with BSA as a standard. Equal amounts of cellular proteins (500 μg) were subjected to immunoprecipitation with the indicated antibodies overnight at 4 °C. The resulting immune complexes were adsorbed onto 70 μl of protein A-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C, washed three times with lysis buffer, and then eluted with Laemmli buffer for 1 h. The immunoprecipitates were resolved by electrophoresis on 7% or 10% SDS-polyacrylamide gels, as appropriate, directly or following immunoblotting with the specific antibodies, as indicated. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences) using a transfer buffer containing 192 mM glycine, 20% (v/v) methanol, and 0.02% SDS. To reduce nonspecific binding, the membranes were incubated in TNA buffer (10 mM Tris-HCl, pH 7.8, 0.9% NaCl, 0.01% sodium azide) supplemented with 5% BSA and 0.05% Nonidet P-40 for 2 h at 37 °C, or in phosphate-buffered saline (PBS) supplemented with 3% nonfat dry milk for 2 h at room temperature, as appropriate, and then incubated overnight at 4 °C with the indicated antibody. The proteins were then visualized by enhanced chemiluminescence (ECL) using horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG (Amersham Biosciences) and quantified by densitometric analysis using OptiLab® image analysis software (Grafter SA, Mirande, France).

Immunofluorescence Analyses—To visualize the actin cytoskeleton, L6 cells were grown on coverslips in complete medium for the indicated times, then fixed with 4% paraformaldehyde and permeabilized at −20 °C with 100% methanol. Fixed cells were incubated with fluorescein isothiocyanate-conjugated (FITC) phallodin (purchased from Sigma-Aldrich) for 30 min and subsequently washed with PBS. Coverslips were mounted on glass slides with Gel mount (Biomeda, Foster City, CA). Fluorescent signals were visualized using a laser-scanning spectral confocal microscope (Leica DMLB microscope with a Sensicam 12 Bitled charge-coupled device camera, Bensheim, Germany), and all images were captured at the same magnification.

To examine vinculin-containing focal adhesions, L6 cells grown on glass coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Incubation with primary antibody against vinculin (1:400) was conducted at room temperature for 16 h, followed by incubation with secondary Alexa Fluor anti-mouse antibody (1:500, Molecular Probes, Eugene, OR) for 1 h and washing three times with PBS. Cells were finally stained with TO-PRO-3 (1:10,000, Molecular Probes) to visualize nuclei, and coverslips were mounted using Gel mount (Biomeda). Images were acquired on a laser-scanning spectral confocal microscope (Leica Microsystems, Heerbrugg, Switzerland), and all images were taken at the same magnification.

To study the intracellular localization of Erk-1/2, L6 cells were grown on glass slides, arrested at 50% confluence in serum-free medium for 24 h, and then incubated in the absence or presence of 100 nM IGF-I for 30 min. To assess total Erk-1/2 control and treated cells were fixed with methanol/acetic acid (70:30, v/v) for 10 min at −20 °C. After a 10-min rehydration with multiple PBS washes at 25 °C, the cells were blocked with PBS/10% BCS for 45 min and then incubated with the monoclonal MAP kinase antibody in PBS/10% BCS (1:100) for 60 min at room temperature. Coverslips were visualized by FITC-conjugated anti-mouse IgG in PBS/10% BCS (1:100) for 60 min at 25 °C in the dark. To evaluate the localization of phospho-Erk-1/2, control and IGF-I-treated cells were fixed with 3% paraformaldehyde for 20 min and permeabilized with 100% methanol for 5 min at −20 °C. After a 10-min rehydration with multiple PBS washes at

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RESULTS

Generation of L6 Skeletal Muscle Myoblasts with Reduced p66Shc Protein Levels—To determine the specific contribution of the p66Shc isoform to IGF-1 action and signaling in skeletal muscle cells, the cellular levels of this IGF-1 receptor substrate were selectively reduced by expressing specific Shc antisense RNA sequences. For this purpose, undifferentiated L6 skeletal muscle cells were stably transfected with two independent Shc antisense sequences, designated as1 and as2. as1 represents a 287-nucleotide cDNA fragment corresponding to both the p46/p52Shc and p66Shc mRNA transcripts, whereas as2 represents a 326-nucleotide cDNA fragment specific to the NH2-terminal 109 amino acids unique to the p66 Shc mRNA transcript (Fig. 1A). Independent clones of L6 cells showing decreased expression of p66Shc were identified by immunoblotting with anti-Shc antibodies and selected for further analyses.

Both as1 and as2 induced a marked and selective reduction in p66Shc protein levels in multiple clones of L6 myoblasts (Fig. 1, B and C). as1 decreased the p66Shc protein ~90% compared with untransfected wild-type L6 myoblasts (p < 0.05) or L6/Neo myoblasts transfected with the empty pCR3.1 vector. By contrast, the protein levels of the other Shc isoforms, i.e. p52Shc and p46Shc, were not significantly different in L6/Shc1, L6/Neo, and untransfected L6 myoblasts (Fig. 1B). A 65% reduction in p66Shc was obtained following overexpression of the as2
cDNA construct (p < 0.05), which also did not affect p52Shc or p46Shc protein levels (Fig. 1C). Therefore, L6 skeletal muscle cell lines with marked reduction of p66Shc were established by stable transfection of as1 or as2. The selective inhibition of p66Shc expression in the absence of changes in expression levels of the other Shc isoforms may be potentially explained by different sensitivity to antisense-mediated inhibition of protein translation of the two mRNA transcripts encoding the Shc proteins (8–10). Therefore, both as1 and as2 interfere with the mRNA transcript encoding p66Shc, whereas the mRNA transcript encoding p46/52Shc may be resistant to interference by as1 (Fig. 1A).

**Erk-1/2 Signaling in L6/Shc Myoblasts**—The Shc proteins regulate cellular responses through activation of the Grb2/Sos/Ras signaling cascade, leading to phosphorylation and activation of the MAP kinase family members Erk-1 and Erk-2. To verify whether selective reduction of the p66Shc protein levels could modify this signaling pathway, we analyzed basal and IGF-I-induced Erk-1/2 phosphorylation in L6/Shc1 and L6/Shc2 myoblasts with decreased p66Shc protein levels. Activation of Erk-1 and Erk-2 was evaluated by immunoblotting with phospho-Erk-1/2 (Thr-202/Tyr-204) antibodies in two independent clones of L6/Shc1 myoblasts (C6 and D28) and two independent clones of L6/Shc2 myoblasts (E15 and E21). In the L6/Shc1 clones, basal Erk-1 and Erk-2 phosphorylation was increased 411 and 360% of control, respectively (p < 0.05) (Fig. 2A). By contrast, basal phosphorylation of Erk-1 and Erk-2 was similar in L6 myoblasts transfected with the empty vector (L6/Neo, clones N1 and N5) and in untransfected wild-type L6 myoblasts (respectively, p = 0.32 and p = 0.97), indicating that plasmid transfection and clonal selection in neomycin-containing medium did not affect the level of Erk phosphorylation. No change in total Erk-1 and Erk-2 protein content was evident in wild-type L6, L6/Neo, and L6/Shc1 myoblasts (Fig. 2A). Increased phosphorylation of Erk isoforms in the basal state was also evident in two independent clones of L6/Shc2 myoblasts compared with control (Fig. 2B; p < 0.05), although this change was of lower magnitude (i.e. 280 and 200% increases of Erk-1 and Erk-2 phosphorylation, respectively, in clones E15 and E21 versus control) as compared with that seen in L6/Shc1 myoblasts. Total protein content of Erk-1 and Erk-2 was slightly and not significantly increased in L6/Shc2 compared with control myoblasts (Fig. 2B). Therefore, stable overexpression of either as1 or as2 in L6 myoblasts results in decreased p66Shc content and increased phosphorylation of Erk-1 and Erk-2 not due to changes in total Erk protein content. L6/Shc1 myoblasts were used in subsequent studies, because they showed greater decrease in p66Shc protein levels and more prominent increase in Erk-1/2 phosphorylation as compared with L6/Shc2 myoblasts.

To assess the responsiveness of Erk-1/2 phosphorylation to IGF-I stimulation, control and L6/Shc2 myoblasts were treated with 100 nM IGF-I for various times and subjected to immunoblotting with phospho-Erk-1/2 antibodies. In control L6/Neo cells, IGF-I markedly increased phosphorylation of both Erk isoforms, which was evident after 3 min of stimulation, peaked at 5 min, and remained sustained up to 30 min (Fig. 2C). By contrast, Erk-1/2 phosphorylation was elevated in the basal state in L6/Shc2 myoblasts and showed no further increase during IGF-I stimulation (Fig. 2C).

Activation of Erk-1/2 following phosphorylation on Thr-202/Tyr-204 results in translocation of the phosphorylated kinases from the cytoplasm, where they are normally retained presumably via a cytoplasmic anchoring complex, to the nucleus (20). To investigate the intracellular localization of activated Erk-1/2 in myoblasts with reduced p66Shc levels, L6/Shc2 cells were studied by immunofluorescence using antibodies to total or phosphorylated Erk-1/2 and compared with control. In control L6 myoblasts, Erk-1/2 appeared uniformly distributed in the cell cytoplasm under basal conditions. IGF-I stimulation induced an increase in the Erk-1/2 signal in the cell nucleus and perinuclear region (Fig. 3A). Increased nuclear fluorescence in IGF-I-treated cells was also observed using antibodies to phosphorylated Erk-1/2 (Fig. 3B), indicating IGF-I-dependent nuclear translocation of the phosphorylated form of Erk-1/2. As compared with control cells, L6/Shc2 myoblasts showed a greater amount of Erk-1/2 in their nucleus already in the basal state, which did not augment upon IGF-I stimulation (Fig. 3A). The nuclear Erk-1/2 in the unstimulated L6/Shc2 myoblasts was constitutively phosphorylated, as demonstrated by immunofluorescence with phospho-Erk-1/2 antibodies (Fig. 3B). Thus, L6/Shc2 myoblasts show constitutive activation and nuclear translocation of Erk-1/2 proteins in the absence of IGF-I stimulation.

IGF-I exerts a dual effect on proliferation and differentiation of skeletal muscle cells, which relies upon an intact Erk-1/2 responsiveness to this growth factor (16, 17, 21). Because Erk activation was altered in L6/Shc2 compared with control L6 myoblasts, IGF-I effects on DNA synthesis and cell differentiation were next determined. In untransfected L6 and L6/Neo myoblasts IGF-I stimulation resulted in a 3-fold increase in DNA synthesis, measured by determining the rates of [3H]thymidine incorporation into DNA (p < 0.05 versus basal) (Fig. 4A). By contrast, in L6/Shc2 myoblasts the IGF-I effect on DNA synthesis was modest and statistically not significant (Fig. 4A). Differentiation into myotubes, which is largely mediated by IGFs secreted in an autocrine manner (3), was also impaired in the L6/Shc2 myoblasts. Both wild-type L6 and L6/Neo myoblasts became elongated and fused to form the characteristic multinucleated myotubes when grown in the differentiation medium (Fig. 4B). By contrast, L6/Shc2 myoblasts showed elongation and some degree of alignment, but did not develop into myotubes (Fig. 4B).

**IGF-I Receptor Signaling in L6/Shc Myoblasts**—The constitutive activation of Erk in the absence of IGF-I stimulation in myoblasts with reduced p66Shc levels could potentially result from increased activity of steps in the IGF-I signaling cascade upstream of Erk. To explore this possibility, tyrosine phosphorylation and total protein levels of the IGF-I receptor were measured in control and L6/Shc2 myoblasts. As shown in Fig. 5A, IGF-I receptor protein levels were similar in wild-type L6, L6/Neo, and L6/Shc2 myoblasts. Tyrosine phosphorylation of the IGF-I receptor was very low under basal conditions and was increased severalfold following incubation with 100 nM IGF-I for 10 min in wild-type L6, L6/Neo, and L6/Shc2 myoblasts (Fig. 5A). Tyrosine phosphorylation of p52Shc, the predominant Shc isoform undergoing tyrosine phosphorylation in response to IGF-I stimulation in L6 cells (18), was also low in the absence of IGF-I in wild-type L6, L6/Neo, and L6/Shc2 myoblasts, and was increased to a similar extent in all cell lines after stimulation with IGF-I (Fig. 5B). Tyrosine phosphorylation of p66Shc was induced by IGF-I in wild-type L6 and L6/Neo myoblasts but not, as expected, in the L6 myoblasts expressing low levels of this Shc isoform following transfection of the p66Shc antisense (Fig. 5B). These results indicate that the constitutive activation of Erk in myoblasts with reduced p66Shc is not the consequence of increased tyrosine phosphorylation of the IGF-I receptor or Shc proteins.

Erk-1/2 phosphorylation and activation in response to growth factor stimulation is mediated by the serine kinase MEK. To further investigate the mechanisms responsible for the increased activity of Erk in the L6/Shc2 myoblasts, the...
activation of MEK was measured by immunoblotting with specific phospho-MEK (Ser-217/Ser-221) antibodies. In wild-type L6 and L6/Neo myoblasts, MEK phosphorylation was low in the basal state and increased 7-fold after IGF-I stimulation (Fig. 5C). In contrast, MEK phosphorylation was already augmented in the basal state in L6/Shc as myoblasts (5-fold versus control, \( p < 0.05 \)) and showed no significant changes when cells were stimulated with IGF-I. These findings parallel the changes in Erk-1/2 phosphorylation observed in L6/Shc as myoblasts (Fig. 2, A–C). The increased MEK phosphorylation was not explained by changes in MBK protein levels, which were similar in wild-type L6, L6/Neo, and L6/Shc as myoblasts (Fig. 5C). Thus, constitutive MEK activation in the absence of IGF-I stimulation appears to account for the increased basal phosphorylation of Erk-1/2 in L6/Shc as myoblasts.

To investigate another signaling pathway that is largely independent of the MEK/Erk pathway, the activation state of Akt was also assessed. As shown in Fig. 5D, the level of Akt phosphorylation on Ser-473 was similarly low in control and L6/Shc as myoblasts and was increased markedly following stimulation with IGF-I. Therefore, while MEK/Erk kinases are de-regulated in the L6/Shc as myoblasts, the Akt pathway is not altered and shows normal responsiveness to IGF-I.
A representative of three experiments is shown. Basal and IGF-I-stimulated wild-type L6 and L6/Shc isoform myoblasts were grown on glass slides, arrested at 50% confluence in serum-free medium for 24 h, and then incubated in the absence or presence of 100 nM IGF-I for 30 min. Cells were fixed and incubated with Erk-1/2 or phospho-Erk-1/2 antibodies, respectively. As described under “Experimental Procedures,” total Erk-1/2 detected by immunostaining with Erk-1/2 antibodies, phospho-Erk-1/2 detected by immunostaining with phospho-Erk-1/2 antibodies, and selective reduction of p66Shc were established. These cells showed: (i) specific reduction of p66Shc in the absence of significant changes in the other Shc isoforms, (ii) marked de-regulation of the MEK/Erk signaling pathway, with increased basal phosphorylation and blunted response of these kinases to IGF-I stimulation, and (iii) altered actin cytoskeleton and cell shape.

Furthermore, correcting the overactivity of Erk results in restoration of the actin cytoskeleton, suggesting a key function of the Erk signaling pathway in the regulation of this cellular component in skeletal muscle cells.

Although all three Shc isoforms can be tyrosine-phosphorylated upon growth factor stimulation, p46/52Shc are coupled to growth and survival signals, whereas p66Shc also undergoes serine phosphorylation and mediates pro-apoptotic responses to oxidative stress (12). Specifically, the p66Shc has been shown to regulate intracellular oxidant levels and hydrogen peroxide-mediated forkhead inactivation (23), effects that are probably relevant to the reported ability of p66Shc to control lifespan in mammals (12) and are unique to this Shc isoform. Additional evidence for different promoter regulation by methylation and histone deacetylation (10) and subcellular localization (24) strengthen the concept of the biological diversity of the Shc isoforms. Consistent with their biological diversity, p66Shc and p46/52Shc have been shown to exert opposite effects on the MEK/Erk signaling pathway. Overexpression of p46/52Shc enhanced EGF-induced Erk activation, whereas p66Shc overexpression had no effect (9) or inhibited (25) this response. The latter study suggested that p66Shc might function to provide for a feedback inactivation of the Erk signaling pathway in contrast to the other Shc isoforms. Also in the present study, p66Shc found to exert an inhibitory effect on Erk, because reduced expression levels of p66Shc were associated with per-
sistent Erk activation. The opposite effects of p46/p52 Shc and p66Shc on Erk activation are of pathophysiological significance, since human breast cancer tissues with high p46/p52 Shc to p66Shc expression ratios show increased proliferative activity and are associated with poor prognosis (26).

The mechanism underlying the inhibitory effect of p66Shc on the Erk-signaling pathway is not fully understood. In L6/Shc as myoblasts, Erk was constitutively activated and poorly responsive to IGF-I stimulation, and so was MEK, the signaling molecule upstream of Erk. By contrast, tyrosine phosphorylation of the IGF-I receptor and the p46/p52Shc isoforms and Akt phosphorylation were normal, indicating that the increased Erk phosphorylation in myoblasts with reduced p66Shc was not the consequence of enhanced IGF-I receptor signaling and that other signaling pathways did not exhibit constitutive activation. According to Okada et al. (25), p66Shc is serine-phosphorylated in an MEK-dependent manner, and the phosphorylated p66Shc binds to Grb2 but is not capable of associating with receptor tyrosine kinases. Because p66Shc and p46/p52Shc compete for binding to a limited pool of Grb2 molecules, increased cellular abundance of p66Shc may result in removal of Grb2-Sos complexes from the receptor at the cell membrane and consequent inhibition of Ras activation (25). Conversely, one could envision that reduction of p66Shc protein levels, which was achieved in this study, may allow more p46/p52Shc binding to Grb2 in the proximity of the tyrosine kinase receptor, leading to sustained Ras and Erk activation.

L6 myoblasts with reduced p66Shc levels showed rounded shape and altered growth properties when compared with control myoblasts (Fig. 4A) and were characterized by complete disruption of the actin stress fibers, focal adhesions, and cell cytoskeleton (Fig. 6). Importantly, inhibition of Erk restored the myoblast phenotype in L6/Shc as cells, suggesting a role for the MEK/Erk pathway in the regulation of actin polymerization and focal contacts. The Shc proteins have been implicated in the control of actin cytoskeleton and cell motility in multiple

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**Fig. 4. Abnormal growth and differentiation properties of L6 myoblasts with reduced p66Shc levels.**

A. basal (white bars) and IGF-I-stimulated (black bars) [3H]thymidine incorporation into DNA in wild-type L6, L6/Neo (clones N1 and N5), and L6/Shc as (clones C6 and D28) myoblasts. Cells were grown in complete medium to ~50% confluence and then incubated in serum-free medium for 16 h in the absence or presence of 100 nM IGF-I, as described under “Experimental Procedures.” Data represent the quantitation of basal and IGF-I-stimulated DNA synthesis from five independent experiments. *, p < 0.05 versus basal.

B. phenotypic characteristics of wild-type L6, L6/Neo (clone N5), and L6/Shc as (clone C6) cells before (top) and after (bottom) differentiation into myotubes, carried out as described under “Experimental Procedures.” Magnification, ×200.
cell types. Fibroblasts obtained from mice with targeted disruption of the Shc gene show aberrant rounded morphology and a disorganized actin cytoskeleton (27), and down-regulation of p46/52 Shc isoforms results in reduced EGF-dependent motility in MCF-7 breast cancer cells (28). Conversely, overexpression of p46/52 Shc was found to promote some extent of organization of actin cytoskeleton and focal adhesions, associated with a random type of cell motility, in a glioblastoma-derived cell line (29), and to improve motility in hepatocyte growth factor-stimulated melanoma cells (30). Even though one study demonstrated that p46/p52 Shc can translocate to the cytoskeleton and directly bind to F-actin in PC-12 cells (31), suggesting direct regulation of actin polymerization by the Shc proteins, current evidence indicates that Shc promotes cytoskeletal rearrangement in response to growth factor stimulation through the Erk pathway (27, 32). In addition, the p46/52 Shc isoforms bind to and are tyrosine-phosphorylated by integrin-activated tyrosine kinases such as FAK and Src (33, 34) and, similarly, promote Erk activation. Thus, growth factor- and integrin-triggered signals converge on the Shc/Erk pathway and dynamically regulate actin fiber assembly, together with signals transduced through the FAK-p130Cas complex. Consistent with this model, regulation of actin cytoskeleton was observed following overexpression of activated MEK, implying the Ras/Raf/MEK/Erk pathway in the p46/p52 Shc-dependent effects (29). The link between Erk and the cytoskeleton is demonstrated by the finding that activated Erk can directly phosphorylate and activate myosin light chain kinase, leading to phosphorylation of myosin light chains and subsequent promotion of the cytoskeletal contraction necessary for cell movement (35).

If MAP kinase promotes actin organization, why did constitutive activation of Erk-1 and Erk-2 cause actin fiber disassembly in the L6/Shc myoblasts? First, persistent up-regulation of Erk activity may be inappropriate for maintenance of normal organization of the actin cytoskeleton. Consistent with this concept, up-regulation of MEK activity in rat kidney cells, obtained by expressing a temperature-sensitive v-Src mutant

![Figure 5](image-url)
for at least 24 h, or by stably expressing v-Src, has been recently shown to cause disruption of the actin cytoskeleton and loss of focal contacts (22). This effect was due to MEK-dependent inhibition of the Rho-ROCK-LIM kinase pathway (22), which promotes actin stress fiber stabilization and actomyosin-based cell contractility (36, 37). A similar mechanism, i.e. ROCK inhibition by sustained MEK/Erk signaling, has been described in Ras-transformed fibroblasts (38, 39). The phosphatidylinositol 3-kinase/Akt pathway was reported to be not involved in the v-Src- or Ras-induced disruption of the actin cytoskeleton (22, 38), and this is consistent with the finding of normal Akt activation in L6/Shc1 myoblasts in this study (Fig. 5D). The ability of constitutive Erk activation to cause changes in cell morphology and rearrangement of actin cytoskeleton has been recently demonstrated also in a macrophage cell line following overexpression of annexin 1 (40). Second, persistent MEK activation may induce altered subcellular localization of Erk kinases. To regulate the adhesion/cytoskeletal network, activated Erk has to be translocated to newly forming focal adhesions, because MEK inhibition suppresses this peripheral targeting of Erk and integrin-dependent focal adhesion assembly (41). In this study, we found that the L6 myoblasts with reduced p66Shc had constitutive IGF-I-independent translocation of activated Erk proteins in the nucleus. It is possible, therefore, that constitutive nuclear targeting of Erk-1 and Erk-2 in the L6/Shc1 myoblasts may diverge these kinases from critical sites in the cytoskeletal network, leading to impaired Erk regulation of the actin cytoskeleton.

Myoblasts with increased Erk activity and disruption of actin stress fibers showed impaired DNA synthesis in response to IGF-I stimulation and incomplete differentiation into myotubes. Changes in actin filament-associated proteins and loss of actin stress fibers can contribute to aberrant growth control, because this may alter both growth factor- and integrin-mediated entry into the S phase of the cell cycle (42–44). In addition, focal adhesion-associated proteins may play an important role in skeletal muscle differentiation, as treatment with cytochalasin D, a selective disruptor of actin filaments, reportedly inhibits differentiation of myoblasts into myotubes (45). Alternatively, impaired growth and differentiation responses of L6/Shc1 myoblasts could be due to abnormal regulation of the

**Fig. 6.** Actin cytoskeleton and focal adhesions in L6/Shc1 myoblasts and the effects of PD98059 treatment. Control L6/Neo and L6/Shc1 myoblasts were incubated with 20 μM PD98059 for 72 h or left untreated. A, effects of PD98059 on the actin cytoskeleton in control L6/Neo (clone N5) and L6/Shc1 (clone C6) myoblasts. Cells were stained with FITC-phalloidin, as described under “Experimental Procedures.” Scale bars, 10 μm. B, effects of PD98059 on focal adhesions in control L6/Neo (clone N5) and L6/Shc1 (clone C6) myoblasts. Cells were stained with vinculin antibodies (green) and TO-PRO-3 (blue) to visualize focal adhesions and nuclei, respectively, as described under “Experimental Procedures.” Scale bars, 40 μm.

**Fig. 7.** Effects of PD98059 on Erk and MEK phosphorylation in L6/Shc1 myoblasts. Wild-type L6, L6/Neo, and L6/Shc1 myoblasts were incubated with 20 μM PD98059 for 72 h or left untreated. A, effects of PD98059 on MEK-1/2 phosphorylation in wild-type L6, L6/Neo (clone N5), and L6/Shc1 (clones C6 and D28) myoblasts. Cell lysates were analyzed by immunoblotting with phospho-MEK-1/2 (Ser-217/Ser-221) or MEK antibodies to assess MEK phosphorylation and total protein content, respectively. Data shown are representative of three independent experiments. B, effects of PD98059 on Erk-1/2 phosphorylation in wild-type L6, L6/Neo (clone N5), and L6/Shc1 (clones C6 and D28) myoblasts. Cell lysates were analyzed by immunoblotting with phospho-p42/p44 MAP kinase (Thr-202/Tyr-204) or MAP kinase antibodies to study Erk-1/2 phosphorylation and total protein content, respectively. Data shown are representative of three independent experiments.
MEK/Erk signaling pathway. It is well recognized that IGF-I stimulates mitogenesis in L6 myoblasts via Erk (14) and that Erk inhibition is associated with enhanced differentiation into myotubes (14). Furthermore, Erk phosphorylation is initially increased and then decreased in response to IGF-I, this biphasic and opposite response being required for the stimulatory effects of IGF-I on myoblasts proliferation and differentiation, respectively (21). In L6/Shoc5 myoblasts, the phosphorylation levels of both MEK and Erk were persistently elevated and unresponsive to IGF-I (Figs. 2 and 5). Constitutively active MEK/Erk has been shown to block S-phase entry in fibroblasts and other cell types (40, 46), and this may explain the lack of IGF-I responsiveness of MEK/Erk. Loss of p66Shc in L6 skeletal muscle myoblasts, which is necessary for a physiologically relevant, inhibitory signaling effect on Erk inhibition is associated with enhanced differentiation into myotubes (40, 46), and this may explain the lack of mitogenic response of the L6/Shoc5 myoblasts to IGF-I, even in the presence of normal IGF-I signaling through Akt (Fig. 5D).

In addition, the activation levels of MEK and Erk were persistently higher in the L6/Shoc5 myoblasts compared with control myoblasts examined at various times during differentiation. The inappropriateness of this high MEK/Erk signaling activity may have similarly led to impaired terminal differentiation activity into myotubes.

In conclusion, this study shows that the p66Shc isoform exerts a physiologically relevant, inhibitory signaling effect on the Erk pathway in skeletal muscle myoblasts, which is necessary for coordinated actin cytoskeleton polymerization and normal IGF-I responsiveness of MEK/Erk. Loss of p66Shc in L6 myoblasts results in an altered cell phenotype resembling that of transformed cells, with rounded shape, disruption of actin fibers, growth factor-insensitive DNA synthesis, and inability to undergo complete differentiation. In future studies, it will be important to assess whether variations in p66Shc expression levels may contribute to phenotype changes in other cell types, including cancer cells.

REFERENCES

1. Beguinot, F., Kahn, C. R., Moses, A. C., and Smith, R. J. (1985) J. Biol. Chem. 260, 15892–15898
2. Shimizu, M., Webster, C., Morgan, D. O., Blau, H. M., and Roth, R. A. (1986) Am. J. Physiol. 251, E611–E615
3. Florini, J. R., Ewton, D. Z., and Coolican, S. A. (1996) Endocrinology 327, 2299–2306
4. Okada, S., Rao, A. W., Ceresa, B. P., Blaikie, P., Margolis, B., and Pessin, J. E. (1997) J. Biol. Chem. 272, 28042–28049
5. LeRoith, D., Werner, H., Beinert-Johnson, D., and Roberts, C. T., Jr. (1995) Endocr. Rev. 16, 143–163
6. Egan, S. E., Giddings, W. B., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) Nature 363, 45–51
7. Pronk, G. J., de Vries-Smits, A. M., Buday, L., Downward, J., Maassen, J. A., Medema, R. H., and Bos, J. L. (1994) Mol. Cell. Biol. 14, 1575–1581
8. Pelczi, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallio, F., Bernardi, I., Nisini, L., Grignani, F., Pawson, T., and Pelczi, P. G. (1992) Cell 70, 93–104
9. Migliaccio, E., Mele, S., Salcini, A. E., Pelczi, G., Lai, K. M., Superti-Furga, G., Pawson, T., Di Fiore, P. P., Lanfrancone, L., and Pelczi, P. G. (1997) EMBO J. 16, 706–716
10. Ventura, A., Lunt, L., Pacini, S., Baldari, C. T., and Pelczi, P. G. (2002) J. Biol. Chem. 277, 22370–22376
11. Kao, A. W., Waters, S. B., Okada, S., and Pessin, J. E. (1997) Endocrinology 138, 2474–2480
12. Migliaccio, E., Giorgio, M., Mele, S., Pelczi, G., Reboli, P., Pandolfi, P. P., Lanfrancone, L., and Pelczi, P. G. (1999) Nature 402, 309–313
13. Napoli, C., Martín-Padura, I., de Nigris, F., Giorgio, M., Mansuetu, G., Somma, P., Condorelli, M., Sica, G., De Rosa, G., and Pelczi, P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2112–2116
14. Denecke, J., Calera, M. R., Farmer, R. S., and Pilch, P. F. (1996) Mol. Cell. Biol. 16, 5964–5973
15. Bennett, A. M., and Tonks, N. K. (1997) Science 278, 1288–1291
16. Giorgino, F., and Smith, R. J. (1995) J. Clin. Invest. 96, 1473–1483
17. Giorgino, F., Pedrini, M. T., Matera, L., and Smith, R. J. (1997) J. Biol. Chem. 272, 7455–7463
18. Lenormand, P., Sardet, C., Pages, G., L'Allemainn, G., Brunet, A., and Pouyssegur, J. (1993) J. Cell Biol. 122, 1078–1088
19. Adi, S., Bin-Abbas, B., Wu, N. Y., and Rosenthal, S. M. (2002) Endocrinology 143, 511–516
20. Pawlak, G., and Helfman, D. M. (2002) J. Biol. Chem. 277, 26927–26933
21. Nemoto, S., and Finkel, T. (2002) Science 295, 2306–2307
22. Ventura, A., Macarana, M., Raker, V. A., and Pelczi, P. G. (2004) J. Biol. Chem. 279, 2299–2306
23. Lai, K. M., and Pawson, T. (2000) Genes Dev. 14, 1132–1145
24. Yamada, K. M. (1999) J. Cell Biol. 146, 389–403
25. Pelczi, G., Giordano, S., Zhen, Z., Salcini, A. E., Lanfrancone, L., Bardelli, A., Papaioannou, V., and Pelczi, P. G. (1995) Oncogene 10, 1631–1638
26. Thomas, D., Patterson, S. D., and Brashad, R. A. (1996) J. Biol. Chem. 270, 29924–29931
27. Giancotti, F. G., and Ruoslahti, E. (1999) Science 285, 1028–1032
28. Schlafpefer, D. D., and Hunter, T. (1997) J. Biol. Chem. 272, 13189–13195
29. Schlafpefer, D. D., Hauck, C. R., and Sieg, D. J. (1999) Prog. Biophys. Mol. Biol. 71, 435–478
30. Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Lanerolle, P., and Cheresh, D. A. (1997) J. Cell Biol. 137, 481–492
31. Yang, N., Higuchi, O., Ohishi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998) Nature 393, 509–512
32. Yonemura, S., Fujihara, S., and Kishida, Y. (2000) EMBO J. 19, 2911–2923
33. Ben-Ze’ev, A., and Bershadsky, A. D. (1997) Adv. Mol. Cell. Biol. 24, 125–163
34. Olsen, M. F., Ashworth, A., and Hall, A. (1995) Science 268, 1270–1272
35. Roswarski, K., and Assoian, R. K. (2003) Mol. Cell. Biol. 23, 4283–4294
36. Lee, K. H., Lee, S. H., Kim, D., Ghee, S., Kim, C., Chung, C. H., Kwon, H., and Kang, M. S. (1999) Exp. Cell Res. 252, 401–415
37. Rescan, C., Contant, A., Talari, H., Theer, N., Glaise, D., Gaguen-Guilhouzou, C., and Baffet, G. (2001) Mol. Cell. Biol. 12, 725–738

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