NIK and IKKα signal myogenesis

NUCLEAR FACTOR-κB-INDUCING KINASE AND IκB-KINASE-α SIGNAL SKELETAL MUSCLE CELL DIFFERENTIATION

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Abbreviations: IGFs, insulin-like growth factors; PI 3-kinase: phosphatidylinositol 3-kinase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; IκBα, inhibitor NF-κB; IKKs, IκB kinases; NIK, NF-κB inducing kinase; MHC, myosin heavy chain; GFP, green fluorescent protein; GST, glutathione S-transferase.
SUMMARY

Nuclear factor-kappaB (NF-κB)-inducing kinase (NIK), IkappaB kinases (IKK) α and β, and IkappaBα are common elements that signal NF-κB activation in response to diverse stimuli. In the current study we analysed the role of this pathway during insulin-like growth factor II (IGF-II)-induced myoblast differentiation. L6E9 myoblasts differentiated with IGF-II showed an induction of NF-κB-DNA binding activity that correlated in time with the activation of IKK-α, IKK-β and NIK. Moreover, the activation of IKK-α, IKK-β and NIK by IGF-II was dependent on phosphatidylinositol (PI) 3-kinase, a key regulator of myogenesis. Adenoviral transduction with the IkBα (serine32,36alanine) mutant severely impaired both IGF-II-dependent NF-κB activation and myoblast differentiation, indicating that phosphorylation of IkBα at serine 32 and serine 36 is an essential myogenic step. Adenoviral transfer of wild-type or kinase-deficient forms of IKKα or IKKβ revealed that IKKα is required for IGF-II-dependent myoblast differentiation whereas IKKβ is not essential for this process. Finally, overexpression of kinase-proficient wild-type NIK showed that the activation of NIK is sufficient to generate signals that trigger myogenin expression and multinucleated myotube formation in the absence of IGF-II.
INTRODUCTION

The IGFs are the only known growth factors that are crucial to myogenesis (1). IGF-I and IGF-II switch on the myogenic program through the IGF-I receptor (2), activating the expression of myogenic transcription factors, cell cycle arrest, muscle-specific protein expression and cell fusion to form multinucleated myotubes (3,4). Phosphatidylinositol 3-kinase (PI 3-kinase) is an essential second messenger for myogenesis (5-9). We have recently described a myogenic signalling cascade initiated by IGF-II that leads to biochemical and morphological skeletal muscle cell differentiation and which involves PI 3-kinase activation, NF-κB activation and iNOS expression and activation (10). In this report, we further analyse the role of the NF-κB activating signalling cascade in myogenesis. NF-κB transcription factors are key mediators of inflammatory responses, immune system functioning, transformation, oncogenesis and antiapoptotic signalling (11-13). NF-κB exists in the cytoplasm in an inactive form by virtue of its association with inhibitory proteins termed IκB (11-15). NF-κB translocation to the nucleus and activation is most frequently achieved through the signal-induced proteolytic degradation of IκB in the cytoplasm. Two kinases, IKKα and IKKβ, which are contained in a high-molecular-weight multiprotein complex, show inducible IκB kinase activity and play a key role in NF-κB activation by a variety of stimuli (16-19). Despite their high sequence similarity, IKKα and IKKβ have different regulatory and functional roles. In mice lacking IKKβ, the activation of NF-κB by cytokines is abolished and mouse embryos die on day 12 to 13 of gestation due to massive liver apoptosis (20). In contrast, IKKα is dispensable for proinflammatory responses but plays an essential role in embryonic development. Mice lacking IKKα exhibit defective proliferation and differentiation of epidermal keratinocytes and defective limb and skeletal patterning (21,22). IKKα and IKKβ are themselves phosphorylated and activated by one or more upstream kinases, like NIK, which is a member of the mitogen-activating protein (MAP) kinase kinase kinase (MAPKKK) family (23-25).

We report here that IκBα phosphorylation at Ser 32 and S36 is required for both IGF-II-dependent NF-κB activation and differentiation in L6E9 myoblasts. We show that IKKα is involved in IGF-II-dependent multinucleated myotube formation and muscle-specific gene onset, whereas
IKKβ is not essential for these processes. Our data suggest that NIK activation triggers myogenin expression and multinucleated myotube formation in the absence of IGF-II.
EXPERIMENTAL PROCEDURES

Materials. IGF-II was kindly given by Eli Lilly (Indianapolis, IN). L6E9 rat skeletal muscle cell line was kindly provided by Dr. B. Nadal-Ginard (Harvard University). 293 cells, low passage, were from Microbix (Ontario, Canada). PI 3-kinase inhibitor LY294002 was from BioMol Research Laboratories (Plymouth, USA). The NF-κB probe for electrophoretic mobility shift assay was kindly given by Dr. Jean-François Peyron (Inserm U364, Nice, France). The cDNAs encoding FlagIKKα, FlagIKKα(K44A), FlagIKKβ and FlagIKKβ(K44A) were provided by Dr. D. Goeddel (Tularik, Inc.). The cDNA encoding glutathione S-transferase (GST)-IkBα(1-54) was provided by Dr. M. Karin (University of California, San Diego). Recombinant adenoviral vectors expressing IkBα(S32,36A), kinase-proficient Flag-tagged NIK, LacZ and green fluorescent protein (GFP) were provided by Dr. Yibin Wang (University of Maryland, Baltimore). Polyclonal antibody C38320 against caveolin-3 was from Transduction Laboratories (KY, USA). Mouse monoclonal antibody MF 20, which stains all sarcomeric myosin heavy chain isoforms and mouse monoclonal anti-rat myogenin antibody (F5D) were from Developmental Studies Hybridoma Bank (MD, USA). Polyclonal antibodies against IκBα (C-15), IKKα, IKKβ and NIK were from Santa Cruz Biotech. (CA, USA). Anti-β-actin (clone AC-15) and anti-FLAG M2 monoclonal antibodies were from Sigma.

Cell culture. Rat L6E9 myoblasts were cultured as described previously (5). Subconfluent myoblasts were differentiated by serum depletion in DMEM plus antibiotics with or without IGF-II (40 nM) in the absence or presence of other compounds, as indicated for each experiment. Cells were photographed after staining the nuclei with MAYER's hemalum solution for microscopy (Merck, Darmstadt, Germany) and cell fusion was quantified by counting nuclei in myotubes from a total of at least 1000 nuclei from 10-20 randomly selected microscope fields for each condition. For adenoviral transduction, subconfluent L6E9 myoblasts were transduced at a multiplicity of 50-100 particles/cell and then cultured for additional 36 h before inducing differentiation with or without IGF-II. To compare the impact of IKKα and IKKβ on myoblast differentiation, experimental
conditions were selected to ensure similar levels of expression of IKKα and IKKβ constructs (data not shown).

**Construction of recombinant adenovirus.** Recombinant adenovirus expressing Flag-tagged versions of either wild type or dominant-negative mutant K44A of human IKKα and IKKβ were generated by homologous recombination as described by Graham and Prevec (38). cDNAs were cloned into the shuttle plasmid pAdl1/RSV and co-transfected with pJM17 into 293 cells to achieve homologous recombination. Individual plaques were isolated and checked for recombinant protein expression after infection of 293 cells. Recombinant adenovirus were further amplified in 293 cells, purified by cesium chloride gradient centrifugation, dialyzed against 1 mM MgCl₂, 10 mM Tris pH 7.4, 10% glycerol and stored at -80°C (39). Viral stocks were titrated by infecting 293 cells with serial dilutions of the preparation and observing the cytopathic effect (CPE) on the cells 48 h after infection. An infectious titer was given assuming that a multiplicity of infection (MOI) of 10 is required to cause a complete CPE at 48 h. In addition, the OD₂₆₀ of the preparation was measured to estimate the particle titer (1OD₂₆₀ unit = 10¹² particles/ml).

**Electrophoresis and immunoblotting of membranes.** Cells were lysed for 30 min at 4°C in 50 mM Tris pH 7.5, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 15 mM Na₄P₂O₇, 20 mM NaF, 0.1% PMSF, 0.1% aprotinin, supplemented with 1% Nonidet-P40. Cell extracts were centrifuged at 10,000xg for 15 min at 4°C and 50 µg of the solubilized proteins was loaded. SDS-PAGE and immunoblot analysis were performed as described previously (5).

**Immunoprecipitation and kinase assays.** Cells were washed in PBS and resuspended at 10⁶ cells/10 µl in hypotonic solution (10 mM Hepes pH 7.8, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 15 mM Na₄P₂O₇, 20 mM NaF, 0.1% PMSF and 0.1% aprotinin). After 10 min at 4°C, NP40 was added to 1% and cells were centrifuged in a microfuge at 10,000 x g for 15 min. The supernatant, containing the cytoplasmic fraction, was recovered.

For immunoprecipitation, antibodies were preadsorbed on protein-G-Sepharose at 4°C for 1 h and washed twice in hypotonic solution/1% NP40, before being incubated with the protein extracts for 2 h at 4°C. The immunopellets were washed 6 times in the same buffer and once in kinase buffer.
(20 mM Hepes, 10 mM MgCl2, 100 µM Na3VO4, 20 mM β-glycerophosphate, 2 mM DTT, 50 mM NaCl pH 7.5). Kinase reactions were carried out for 30 min at 30°C using 5 µCi of [γ-32P]-ATP and GST-IkBα(1-54) as substrate (except for NIK kinase assays, in which autophosphorylation of immunoprecipitated NIK was analysed). The reaction products were analysed on 10% polyacrylamide gels and revealed by autoradiography.

**Electrophoretic Mobility Shift Assay** NF-κB-DNA binding activity was analysed in total cellular extracts made in Totex lysis buffer (20 mM Hepes, 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1% PMSF, 0.1% aprotinin) (10). Supernatants from a 15 min 10,000 x g centrifugation were collected. The NF-κB probe was a synthetic double-stranded oligonucleotide containing the NF-κB binding site of the IL-2 gene promoter (5’-GATCCAAGGGACTTTCCATG-3’). Samples (70 µg) were incubated for 10 min at 4°C with 30 ng of polyIdC and 5 µl of 5x NF buffer (50 mM Tris pH 7.5, 500 mM NaCl, 5 mM DTT, 5 mM EDTA, 20% glycerol, 0.4 mg/ml salmon sperm DNA) in a final volume of 25 µl. The end-labeled probe was added for a further incubation of 25 min at 25°C. The specificity of the bands detected was verified by adding 10-100 fold excess of competing unlabeled NF-κB probe. NF-κB unrelated oligonucleotide probe controls did not show any specific binding activity (data not shown).

**Immunofluorescence.** Cells grown on coverslips were fixed for 20 min with 3% paraformaldehyde in PBS, washed three times in PBS and then treated as follows: (a) 10 min in PBS containing 50 mM NH₄Cl, (b) 10 min in PBS containing 20 mM glycine and (c) 30 min in PBS containing 10% fetal bovine serum. Subsequently, coverslips were incubated with primary antibodies (polyclonal anti-NIK antibodies, 1 µg/ml; monoclonal anti-myogenin antibody, undiluted culture supernatant) for 1 h at room temperature. After washing in PBS, coverslips were incubated with fluorochrom conjugated (Oregon Green or Texas Red) antibodies for 45 min. Cells were washed three times in PBS and then mounted in immunofluore medium (ICN Biomedicals Inc., Aurora, OH). Images were obtained using a Leica TCS 4D laser confocal fluorescence microscope with a 40 x objective.
RESULTS

IGF-II-induced skeletal muscle cell differentiation involves NF-κB signalling cascade activation.

IGF-II induces NF-κB DNA binding activity as an early event during L6E9 myoblast differentiation (10). Most extracellular stimuli that activate the NF-κB pathway induce phosphorylation of the NF-κB repressor IκBα at Ser-32 and Ser-36 as a requisite for its degradation (19). However, alternative pathways for NF-κB activation have been described (26,27). To analyse the mechanism required by IGF-II for NF-κB activation during differentiation, L6E9 myoblasts were transduced with an adenovirus expressing an IκBα mutant with Ser-32 and Ser-36 replaced by alanine residues (adv/IκBαAA). This mutant inhibits NF-κB in pathways involving Ser phosphorylation and subsequent proteasome degradation of IκBα. L6E9 myoblasts differentiated for 24 h with IGF-II exhibited an induction of NF-κB-DNA binding activity (Fig. 1, A), which was blocked in myoblasts overexpressing IκBαAA (Fig. 1, B). Myoblasts infected with an adenovirus expressing green fluorescent protein (adv/GFP) were used as control (Fig. 1, B). Next, we analysed the impact of IκBα phosphorylation on IGF-II-dependent myoblast differentiation. After 4 days of IGF-II treatment, the expression of muscle-specific proteins such as myosin heavy chain (MHC) and caveolin-3, was highly decreased in myoblasts overexpressing IκBαAA mutant (50±12%, n=3) compared to control non-transduced cells or cells transduced with adv/GFP, while the expression of the non-muscle specific protein β-actin was similar in all conditions (Fig. 1, C). Moreover, cells overexpressing IκBαAA mutant did not fuse into myotubes whereas control cells (adv/GFP) showed 82% of the nuclei in myotubes from a total of 1661 nuclei randomly counted (Fig. 1, D). These data suggest that IGF-II requires NF-κB activation through a mechanism that involves IκBα phosphorylation to trigger skeletal muscle cell differentiation.
IGF-II induces PI 3-kinase-dependent IKKα and IKKβ activation during myoblast differentiation.

Data presented above indicating that IκBα phosphorylation was required by IGF-II to induce myoblast differentiation, led us to analyse the activity and expression of IKKα and IKKβ during this process. L6E9 myoblasts exhibited a peak of IKKα activity after 24 h in IGF-II-containing differentiation medium (Fig. 2 A, upper panel). The kinetics of IKKα activation was consistent with that of IGF-II-dependent NF-κB DNA-binding activation, which we have previously shown to be dependent on PI 3-kinase activity (10). To analyse whether IGF-II-dependent IKKα activation involved PI 3-kinase, L6E9 myoblast differentiation was induced with or without IGF-II in the absence or presence of the PI 3-kinase inhibitor, LY294002. PI 3-kinase inhibitor (20 μM) blocked the ability of IKKα to phosphorylate GST-IκBα(1-54) in response to IGF-II (Fig. 2 B, upper panel). Neither IGF-II nor LY294002 altered IKKα protein expression (Fig. 2 C, upper panel), however, the 24 h delay between the start of the IGF-II treatment and the activation of IKKα seemed to indicate that IKKα was not direct target of the IGF-induced phosphorylation cascade. This appears to be the case as IKKα activation by IGF-II was blocked in the presence of cycloheximide (CH) (5 μg/ml), indicating that it depends on de novo protein synthesis (Fig. 2 D, upper panel).

Phosphorylation of GST-IκBα (1-54) substrate by IKKβ was also induced 24 h after triggering differentiation with IGF-II (Fig. 2 A, lower panel). IKKβ activation by IGF-II was blocked by PI 3-kinase inhibitor LY294002 (Fig. 2 B, lower panel). Neither IGF-II nor LY294002 treatments modified the level of IKKβ expression in differentiating myoblasts (Fig. 2 C, middle panel), indicating that changes in activity are caused by activation of the kinase rather than variations in IKKβ protein content. As for IKKα, the activation of IKKβ by IGF-II was blocked by cycloheximide (Fig. 2 D, lower panel).

IGF-II requires IKKα but not IKKβ to induce skeletal muscle cell differentiation.

To determine whether the activation of IKKs by IGF-II is functionally linked to myogenic differentiation, we generated replication-deficient adenoviral vectors expressing Flag-tagged wild type or dominant negative forms of IKKα and IKKβ (adv/FlagIKKα, adv/FlagIKKα(K44A),
adv/FlagIKKβ, adv/FlagIKKβ(K44A), respectively). Subconfluent L6E9 myoblasts were transduced with the different recombinant adenoviruses using adv/GFP as a control and, 36 h later, differentiation was induced by placing the cells in an IGF-II-containing medium. Expression and kinase activity of the transduced proteins were evaluated 24 h after inducing differentiation with IGF-II. Cells transduced with adv/FlagIKKα exhibited GST-IκBα(1-54) phosphorylation activity on immunoprecipitates with an anti-Flag monoclonal antibody while no specific IκBα phosphorylation activity was detected in immunoprecipitates from cells transduced either with adv/GFP or adv/FlagIKKα(K44A) (Fig. 3 A, upper). In these conditions, similar levels of wild type and dominant negative forms of IKKα were expressed, as verified by immunoblotting using anti-Flag antibody (Fig. 3 A, lower). After 4 days of differentiation in the presence of IGF-II, cells overexpressing FlagIKKα(K44A) remained highly undifferentiated, as measured by caveolin-3 and MHC expression, compared to cells transduced with either adv/GFP or adv/FlagIKKα. The expression of the non-muscle specific protein β-actin was not altered by FlagIKK(K44A) overexpression (Fig. 3 B).

To analyse the role of IKKβ activity in myoblast differentiation, cells were transduced with adv/FlagIKKβ or adv/FlagIKKβ(K44A). Both proteins were expressed to similar levels as detected on anti-Flag immunoblots (Fig. 3 C, lower) and adv/FlagIKKβ(K44A) transduced cells exhibited no IκBα kinase activity on anti-Flag immunoprecipitates (Fig. 3 C, upper). In contrast to IKKα, IKKβ does not seem to play an essential role in myoblast differentiation as cells transduced with adv/FlagIKKβ(K44A) expressed skeletal muscle-specific proteins as efficiently as non-transduced cells or cells transduced with adv/GFP or adv/FlagIKKβ (Fig. 3 D). At the morphological level, overexpression of FlagIKKβ(K44A) did not alter the ability of myoblast to fuse in response to IGF-II (78% of nuclei into myotubes from a total of 1488 nuclei randomly counted: 78%, t= 1488) compared to non-transduced control cells (78%, t= 1616), cells transduced with adv/GFP (82%, t=1661) or cells transduced with adv/FlagIKKβ (80%, t=1011) (Fig 4, arrows show large accumulations of nuclei into myotubes). Conversely, when FlagIKKα(K44A) was overexpressed, only thin, spindle-like myotubes were formed compared to the large myotubes observed in non-transduced cells, cells transduced with adv/GFP or cells transduced with adv/FlagIKKα (Fig. 4). Indeed, after 4 days in IGF-II-differentiating medium, only 25% of the nuclei (t=1367) in cells overexpressing FlagIKKα(K44A) were in myotubes with more
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than 10 nuclei. Under the same culture conditions, 77% of the nuclei from cells overexpressing FlagIKKα (t=2987) were in myotubes with more than 10 nuclei. Taken together, these results suggest that IKKα plays a relevant role in IGF-II-dependent morphological and biochemical differentiation of skeletal muscle cells while IKKβ is not essential to this process.

NIK is activated in differentiating myoblasts.

NIK is a common mediator in the NF-κB signalling cascades and IKKα has been reported to be a better substrate than IKKβ for phosphorylation by NIK (24). The endogenous kinase activity and protein expression of NIK in differentiating L6E9 myoblasts was studied. Induction of NIK autophosphorylation activity was detected after 24 h in IGF-II-containing differentiation medium (Fig. 5, A). As observed for NF-κB DNA-binding activation (10) and IKKα and IKKβ activities (Fig. 2, B), the activation of NIK was totally blocked by LY294002 indicating that IGF-II requires PI 3-kinase to activate NIK in differentiating myoblasts (Fig. 5, A). No changes were detected in NIK protein expression in response to IGF-II or LY294002 (Fig. 5, B). As for IKKα and IKKβ, NIK activation by IGF-II was blocked by cycloheximide indicating that it required de novo protein synthesis (Fig. 5, C).

Interestingly, LY294002 inhibited NIK activation only when it was present together with IGF-II during the whole 24 h-treatment. In contrast, when LY294002 was added during the last 6 h or the last 1 h of IGF-II incubation (at 18 h or at 23 h of differentiation, respectively), no inhibition of NIK activity was observed (Fig. 5, D). These results suggest that PI 3-kinase is most probably involved in the de novo synthesis of the factor/s required for NIK activation rather than being a direct upstream element of NIK activation cascade.

To test whether the activation of NIK by IGF-II was a key event during differentiation, we transduced L6E9 myoblasts with recombinant adenovirus expressing kinase-proficient wild type Flag-tagged NIK that exhibited a high degree of basal autophosphorylation activity on anti-Flag or anti-NIK immunoprecipitates (Fig. 6, A; the lower band that is detected with anti-NIK antibodies in cells transduced with adv/FlagNIK is probably due to cross-activation of endogenous NIK by the overexpressed kinase). After 2 days in the absence of IGF-II (DMEM), myoblasts infected with control adv/GFP fused poorly (12% of a total of 1713 nuclei counted at random were found inside multinucleated myotubes) (Fig. 6, B). In contrast, transduction with adv/FlagNIK promoted
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myoblast fusion (48% of nuclei inside myotubes of a total of 1789 nuclei counted at random) (Fig. 6, B). Co-immunofluorescence assays showed that NIK-overexpressing cells highly expressed myogenin in their nuclei (Fig. 6 C). The number of nuclei expressing myogenin was 6 fold higher in cells overexpressing NIK (adv/NIK) than in control cells (adv/lacZ) (15 randomly selected fields were analysed from each one of 2 independent experiments with each condition performed in triplicate).
DISCUSSION

We describe a pathway by which IGF-II modulates skeletal muscle cell differentiation through activation of the IKK complex. The activation of the NF-κB cascade (NIK, IKKα and IKKβ activation, IκB degradation, NF-κB DNA-binding activation) is detected 24 h after placing subconfluent myoblasts in IGF-II-containing differentiation medium. These are early events during the differentiation program induced by IGF-II but they differ greatly in their kinetics compared with the rapid activation of this cascade that can be detected within minutes of exposure of cells to cytokines or other stimuli. These observations indicate that IKK might not be a direct target of the IGF-induced phosphorylation cascade, an assumption reinforced by the fact that IGF-mediated IKK and NIK activation required de novo protein synthesis. The nature of the factor/s induced by IGF-II to trigger the NF-κB cascade activation during myogenesis remains undefined. Among the possibilities to be considered, IGF-II could induce the secretion of an autocrine factor by differentiating myoblasts. In this context, we did not detect expression of TNFα—a classical activator of NF-κB—by RT-PCR studies in myoblasts induced to differentiate by IGF-II (data not shown). Another possibility is that the newly synthesized protein is a differentiation-induced kinase that may be a target for IGF-II itself or for an alternative factor generated in response to IGF-II.

The involvement of NF-κB in myogenic signalling has been described in rat, human and chick embryonic myoblasts (10,28,29) although differentiation of C2C12 skeletal muscle cells in a 2% horse serum-containing medium seems to occur through a signalling cascade in which NF-κB plays a negative regulatory role (30). We have previously established that PI 3-kinase, NF-κB and iNOS are elements of a common myogenic cascade in which IGF-II induces, through a PI 3-kinase-dependent pathway, a decrease in IκB-α protein content that correlates with a decrease in the amount of IκB-α associated with p65 NF-κB, NF-κB DNA-binding activation and NO production (10). PI 3-kinase is a key mediator of myogenesis (5-9) and the role of PI 3-kinase in NF-κB cascade activation during myogenesis is reinforced by data presented here showing that both NIK and IKK activation by IGF-II in differentiating myoblasts are blocked by inhibiting PI 3-kinase. PI 3-kinase is known to be directly involved in the activation of NF-κB in processes like anti-apoptotic PDGF signalling (31) and TNF-mediated immune and inflammatory responses (32). However, in
view of our results, PI 3-kinase does not seem to directly activate NIK phosphorylation (LY294002 inhibited NIK activation only when it was present together with IGF-II during the whole 24 h treatment but not during the last 6 h or the last 1 h). These data suggest that PI 3-kinase is most probably involved in the de novo synthesis of protein/s required for NIK activation rather than being an upstream element in the activation of NIK. In contrast, our results do not rule out a biphasic role for IGF-II: first, in initiating the de novo synthesis of a NIK-activating factor and, second, in promoting NIK activation by the newly synthesized protein that -in view of the results presented in figure 5 D-could only occur through a PI 3-kinase-independent pathway.

Although the stimuli that activate IKKβ and the substrates that mediate its biological activity are known, the stimuli and the relevant substrates for IKKα are less characterized. IKKα and IKKβ appear to exert different and non-interchangeable physiological roles. Gene targeting experiments revealed that although not involved in the activation of IKK by proinflammatory stimuli, IKKα is involved in morphogenesis (20-22). In this context, our results show that while IGF-II induced both IKKα and IKKβ activities early during the differentiation program, the overexpression of a kinase-deficient mutant of IKKβ did not alter the expression of muscle-specific proteins nor the formation of multinucleate myotubes. The differentiation process was, however, blocked by a kinase-deficient mutant of IKKα, suggesting that endogenous IKKβ cannot substitute IKKα in the myogenic signalling. Interestingly, the skeletal muscle poorly expresses IKKβ while it is one of the tissues with higher expression levels of IKKα (33).

Proliferation precedes differentiation in IGF-stimulated myogenesis and the opposing early and late effects of IGF during myogenesis are reflected in the phosphorylation state of the cell cycle regulatory retinoblastoma protein in skeletal myoblasts (34,35). Before exiting from the cell cycle, IGFs induce a last round of proliferation in which NF-κB is required to increase cyclin D1 expression and pRb hyperphosphorylation (33,35,37). Then, a decrease in NF-κB activity followed by a decrease in cyclin D1 levels seems to be required to allow the exit from the cell cycle that precedes differentiation (3,36). A causative relation between NF-κB down-regulation and myogenesis was initially proposed, without considering that the NF-κB activity detected after 24 h in differentiation medium -even if it was lower than that observed during proliferation- could exert a myogenic role (36). Indeed, this appears to be the case as, consistent with data reported here, NF-κB activity was shown to be required by human, mouse and chicken myoblasts to fuse (10,31,32).
We show here that IGF-II-dependent differentiation triggers a delayed induction of the NF-κB-activating cascade which requires PI 3-kinase activity and \textit{de novo} synthesis of still-undefined factors. Our data suggest that the activation of NIK and IKKα and the subsequent phosphorylation of IκBα at Ser-32 and Ser-36 are key events in skeletal muscle differentiation induced by IGF-II.
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FIGURE LEGENDS

Figure 1. IGF-II-induced skeletal muscle cell differentiation requires IκBα phosphorylation at Ser-32 and Ser-36 for NF-κB activation. (A) Total cell extracts from L6E9 myoblasts differentiated with or without 40 nM IGF-II for 24 h were incubated with a $^{32}$P-labeled NF-κB probe corresponding to the κB site in the IL-2 gene promoter and analysed by EMSA. The specificity of the bands was verified by adding 50 fold excess of competing unlabeled κB probe (cold probe) or unrelated oligonucleotide probes (data not shown). (B) Total cell extracts from L6E9 myoblasts transduced with adv/IκBα AA or with adv/GFP and differentiated with IGF-II 40 nM for 24 h were incubated with a $^{32}$P-labeled NF-κB probe and analysed by EMSA. (C) Expression of IκB-α, myosin heavy chain (MHC) and caveolin-3 (cav-3) was analysed by immunoblot in total cell lysates from non-transduced myoblasts (nt), or myoblasts transduced with adv/GFP or adv/IκBα AA, differentiated for 4 days with IGF-II. β-actin content was analysed as a control of relative amounts of proteins in each sample. (D) L6E9 myoblasts non-transduced or transduced with adv/GFP or adv/IκBα AA were grown to confluence and then allowed to differentiate in the presence of IGF-II for 4 days. Morphological differentiation was assessed by myotube formation. Images shown are representative of 15-25 microscope fields taken at random. The scale is the same for all panels. All infections were performed in duplicate and the results shown are representative of three independent experiments.

Figure 2. Endogenous IKK activation during IGF-II-induced myoblast differentiation. (A) Cell extracts (50 µg) from L6E9 myoblasts differentiated with IGF-II (40 nM) were immunoprecipitated (IP) with anti-IKKα (upper panel) or anti-IKKβ (lower panel) antibodies (10 µg) preadsorbed on protein G-Sepharose beads and kinase assay was performed as described in Methods, using GST-IκBα(1-54) as a substrate. Non-specific kinase activity was analysed using a non-immune antibody (data not shown). (B) Kinase assay was performed as described in A, from cells differentiated for 24 h in the absence or presence of IGF-II (40 nM), with or without LY294002 (20 µM). (C) IKK expression was analysed by immunoblot (IB) in cell lysates obtained from myoblasts differentiated for 24 h in the absence or presence of IGF-II with or without LY294002. The relative amount of proteins in each sample was checked by expression of the non-muscle-specific protein β-actin. (D) Subconfluent L6E9 myoblasts were differentiated for
24 h in DMEM containing or not IGF-II (40 nM) with or without cycloheximide (CH, 5 µg/ml). Kinase assay was performed as described above. Results shown are representative of 3-4 independent experiments.

**Figure 3. IGF-II requires IKKα but not IKKβ activation to induce skeletal muscle cell differentiation.** Subconfluent L6E9 myoblasts were transduced either with adv/GFP, adv/FlagIKKα or adv/FlagIKKα(K44A) (A and B) or with adv/GFP, adv/FlagIKKβ or adv/FlagIKKβ(K44A) (C and D). After 36 h, cells were induced to differentiate with IGF-II.

(A and C, upper) Kinase activity of the transduced proteins was measured in anti-Flag immunoprecipitates after 24 h in IGF-II differentiation medium, by in vitro phosphorylation of GST-IκBα (1-54) fusion protein. Non-specific (ns) IκBα phosphorylation activity was analysed using a non-immune antibody. (A and C, lower) Expression of transduced proteins was checked by immunoblotting using anti-Flag antibody. (B and D) Muscle-specific protein expression in total cell lysates from undifferentiated myoblasts (mb) or differentiated for 4 days with IGF-II, 36 h after being transduced either with adv/GFP, adv/FlagIKKα, adv/FlagIKKα(K44A), adv/FlagIKKβ or adv/FlagIKKβ(K44A) or left untransduced (nt). Relative amount of proteins in each sample were checked by expression of the muscle-specific protein β-actin. All infections were done in duplicate and the results shown are representative of three independent experiments.

**Figure 4. Overexpression of dominant negative FlagIKKα(K44A) in L6E9 myoblasts impairs IGF-II-induced myotube formation.** Subconfluent myoblasts transduced either with adv/GFP, adv/FlagIKKα, adv/FlagIKKα(K44A), adv/FlagIKKβ or adv/FlagIKKβ(K44A) were differentiated with IGF-II for 4 days. Cells were photographed after nuclear staining and myotube formation was quantified. Proliferating myoblasts are also shown. Images are representative of 25 microscope fields taken at random from each of at least 5 independent experiments. The scale is the same for all panels.

**Figure 5. NIK is activated in IGF-II-dependent differentiation.** (A) Cell extracts (50 µg) from L6E9 myoblasts differentiated for 24 h in DMEM in absence or in presence of IGF-II (40 nM), with or without LY294002 (20 µM), were immunoprecipitated with anti-NIK antibody (5 µg) preadsorbed on protein G-Sepharose beads. Autophosphorylation assay was performed as
NIK and IKKα signal myogenesis

described in Methods and non-specific (ns) NIK activity was tested using a non-immune antibody. (B) NIK expression was analysed by Western blot in cell lysates obtained at 24 h of differentiation and relative amounts of proteins in each sample were checked by expression of β-actin. (C) Subconfluent L6E9 myoblasts were differentiated for 24 h in DMEM containing or not IGF-II (40 nM), with or without cycloheximide (CH, 5 µg/ml). Kinase assay was performed as described above. (D) Subconfluent L6E9 myoblasts were differentiated for 24 h with IGF-II (40 nM) with or without LY294002 (20 mM), added either for the whole 24 h or during the last 6 h or the last 1 h of IGF-II treatment. Kinase assay was performed at t=24 h, as described above. Data shown are representative of three independent experiments.

Figure 6. NIK overexpression induces myoblast differentiation in the absence of IGF-II. (A) 293 cells were transduced with adv/FlagNIK. Non-transduced cells were used as control (nt). Cell extracts obtained 24 h after infection were immunoprecipitated with antibodies against NIK or Flag preadsorbed on protein G-Sepharose beads. Autophosphorylation assay was performed as described in Methods. Non-specific (ns) NIK autophosphorylation activity was tested using non-immune antibodies. (B) Subconfluent L6E9 myoblasts were transduced with adv/FlagNIK or control adv/GFP. 36 h after infection, cells were allowed to differentiate for 2 days in the absence of IGF-II (DMEM). Morphological differentiation was assessed by myotube formation and cells were photographed after nuclear staining. Images shown are representative from 25 microscope fields taken at random from each one of 3 independent experiments. The scale is the same for all panels. (C) Myogenin expression in L6E9 cells transduced with adv/FlagNIK or control adv/lacZ. Cells were grown to subconfluence on glass coverslips and maintained for 2 days in serum-free medium in the absence of exogenous IGF-II (DMEM). For immunofluorescence detection, cells were fixed and simultaneously probed for myogenin and NIK, as described in Methods. Cells showing myogenin nuclear staining were counted and averaged from a minimum of 15 randomly selected fields. All infections were done in triplicate and the results shown are representative of two independent experiments.
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Figure 1. Canicio et al, 2001

A. NF-κB

B. adv/GFP adv/IKBAA NT

C. IκBα MHC Cav-3 βactin

D. non-transduced adv/GFP adv/IκBαAA
Figure 2. Canicio et al, 2001

A.

IP:
- anti-IKKα
- anti-IKKβ

IGF-II (h): 0, 4, 8, 12, 24

32P-IκB-α

B.

IP:
- anti-IKKα
- anti-IKKβ

DMEM  IGF-II  IGF-II + LY294002

32P-IκB-α

C.

IB:
- IKKα
- IKKβ
- β-actin

D.

IP:
- anti-IKKα
- anti-IKKβ

DMEM  IGF-II  IGF-II + CH

32P-IκB-α
Figure 3. Canicio et al, 2001

A. 
IP: Flag
32P-IκB-α
IB: Flag

B. 
MHC
Caveolin 3
β-actin

C. 
IP: Flag
32P-IκB-α
IB: Flag

D. 
MHC
Caveolin 3
β-actin
Figure 4. Canicio et al, 2001

Myoblasts
adv/GFP
adv/FlagIKKβ
adv/FlagIKKβ(K44A)
adv/FlagIKKα
adv/FlagIKKα(K44A)
Figure 5. Canicio et al, 2001

A. IP: anti-NIK

B. NIK

C. IP: anti-NIK

D. NIK activity at t=24 h

LY294002 treatment (h):

| Treatment (h) | 24 | 6  | 1  | ns |
|---------------|----|----|----|----|
| IGF-II        | +  | +  | +  | ns |
| IGF-II + LY294002 | +  | +  | +  | ns |

$^{32}$P-NIK
Figure 6. Canicio et al, 2001

A.  

| IP | Flag | NIK | ns |
|----|------|-----|-----|
| adv/Flag-NIK nt | nt | nt | adv/Flag-NIK |

32 P-FlagNIK

B.  

DMEM adv/GFP

DMEM adv/FlagNIK

C.  

anti-NIK anti-myogenin

adv/lacZ

adv/NIK
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