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Differential signaling through NFκB does not ameliorate skeletal myoblast apoptosis during differentiation

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Abstract During 23A2 skeletal myoblast differentiation, roughly 30% of the population undergoes apoptosis. Further, constitutive signaling by G12V:H-Ras or Raf:CAAX abrogates this apoptosis. In this study, we demonstrate an increase in NFκB activity in myoblasts that have survived and are expressing muscle-specific genes. NFκB activity is also elevated in myoblasts expressing constitutively active G12V:H-Ras but not Raf:CAAX. Expression of a dominant negative IκB (IκB-SR) sufficient to eliminate this elevated level of NFκB activity, in either the 23A2 myoblasts or their G12V:H-Ras-expressing counterparts, however, does not affect survival. Furthermore, expression of a constitutively active IκB kinase in 23A2 myoblasts does not protect these cells from the apoptosis associated with differentiation. Since signaling by IκB kinase can abrogate differentiation, this result demonstrates that abrogated differentiation and abrogated apoptosis are separable phenotypes.

Key words: NFκB; Myoblast; Apoptosis; Ras

1. Introduction

Apoptosis associated with skeletal myoblast differentiation has been documented in cultures of primary myoblasts [1,2], in established muscle cell lines [3,4] and in an established muscle satellite cell line [5]. As is the case with any important physiological process [6,7], inappropriate myoblast apoptosis contributes pathologically to muscle degeneration [8–10]. Thus, an understanding of the molecules that coordinately regulate skeletal myoblast differentiation and apoptosis is critical to the design of effective therapies.

Constitutive signaling by Ras [11–13], or its downstream effector kinase Raf [14–17], is sufficient to abrogate skeletal myoblast differentiation. We have reported that either constitutive signaling by Ras [4] or Raf [17] can also abrogate the apoptosis associated with 23A2 myoblast differentiation. The signaling molecules downstream of either Ras or Raf responsible for these phenotypes, however, remain elusive [4,14,17–19]. A candidate for this signaling molecule, known to be activated by either Ras [20,21] or Raf [22,23] signaling in fibroblasts, is the transcription factor NFκB. Like constitutive signaling by either Ras or Raf, constitutive signaling by NFκB is sufficient to inhibit skeletal myoblast differentiation [10,24,25]. Constitutive NFκB activity, however, is not required for the Ras-induced differentiation-defective phenotype [25]. Since signaling through NFκB has been shown to play an important role in survival in other systems [26–30], we investigated the contribution of NFκB signaling to the survival of 23A2 myoblasts during differentiation and to the abrogated apoptotic phenotype of myoblasts expressing constitutively active G12V:H-Ras or Raf:CAAX.

2. Materials and methods

2.1. Cells and cell culture

All cells were cultured on gelatin-coated plates and maintained in growth medium (GM), which consists of basal modified Eagle's medium (BME), 10% fetal bovine serum (FBS), and a 1% combination of 10 000 I.U./ml penicillin and 10 000 μg ml^{-1} streptomycin (1% P/S). Differentiation was induced by switching cells from GM to differentiation medium (DM), which consists of BME, 1% P/S and 0% FBS. Cells were incubated at 37°C in 5% CO_2. The growth and differentiation properties of 23A2 myoblasts and the 23A2 derivative expressing G12V:H-Ras (A2:H-Ras myoblasts) have been reported previously [4,18].

2.2. Transient transfection and luciferase assay

Equal cell numbers were plated and the next day transfected using Lipofectamine Plus per manufacturer's instructions (Gibco BRL). Luciferase activity was determined using the DLR Dual Luciferase Kit per manufacturer's instructions (Promega). Experiments were performed within the linear range of the assay and a background (no lysate) measurement was taken and subtracted for each experiment. The activity of *Photinus pyralis* luciferase encoded by the reporter plasmid was normalized in each transfection to the activity of *Renilla reniformus* luciferase. Transfected G12V:H-Ras is expressed in the 23A2 myoblasts at levels comparable to c-Ras. The activity of *Renilla* luciferase in these A2:G12V:H-Ras myoblasts is similar (10% variation at most) to that assayed from lysates of identically transfected 23A2 myoblasts.

2.3. DNA binding assay

Nuclear extracts were prepared using the NE-PER™ nuclear and cytoplasmic extraction reagent kit (Pierce) per manufacturer's instructions. Protein concentrations were determined using Coomassie Protein Assay Reagent (Pierce) and samples were stored at −80°C. DNA-binding activity was assessed using the EZ-Detect™ NFκB(p65) Transcription Factor Kit (Pierce) per manufacturer's instructions. Briefly, to a streptavidin-coated well loaded with biotinylated NFκB consensus duplex, 7 μg of each nuclear extract (<10 μl) is added along with 50 μl of binding buffer containing poly dI-dC. Where indicated, wild-type (WT) NFκB competitor duplex or mutant (M) NFκB competitor duplex (40 pmol) was added prior to the addition of extract. After 60 min at room temperature with mild agitation, wells were washed three times followed by incubation with 100 μl of NFκB(p65) primary antibody. After 60 min at room temperature with no agitation, wells were washed three times followed by incubation with 100 μl of HRP-conjugated secondary antibody. After 60 min at room temperature with no agitation, wells were washed four times. To each well, 100 μl of

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equal parts luminol/enhancer solution and stable peroxide solution was added and chemiluminescence was measured using a luminometer. Baseline chemiluminescence, determined by excluding extract, was subtracted from each value obtained.

2.4. Cytosolic nucleosome enzyme-linked immunosorbent assays (ELISAs)

DNA fragmentation and nuclear membrane disruption, as monitored by the presence of cytosolic nucleosomes, was used as a marker for apoptosis. Cytosolic nucleosomes were measured using the Cell Death Detection ELISA Plus Kit (Roche Diagnostics) per manufacturer's instructions. Transfected cells were switched to fresh GM or DM for various times. Attached cells were rinsed in phosphate-buffered saline and then lysed by incubation in 300 μl cell lysis buffer from the kit on a rocker for 30 min at room temperature. Samples were diluted four-fold and 20 μl of each sample was transferred to a 96-well, flat-bottomed, streptavidin-coated microtiter plate. Seventy-two microliters of 1× incubation buffer from the kit, and 4 μl each of biotin-conjugated anti-histone antibody and peroxidase-conjugated anti-DNA antibody was added to the lysate in the microtiter plate and incubated at 4°C overnight. Following three washes with 300 μl of incubation buffer, the presence of cytosolic nucleosomes was measured by adding 100 μl of the substrate, ABTS. This was incubated in the microtiter plates for 5–20 min at room temperature prior to assessment of activity using a spectrophotometer at 405 nm. Experiments were performed within the linear range of the assay. Attached cells from parallel plates were counted and absorbance was normalized to cell number. A background (no lysate) measurement was taken and subtracted for each experiment.

2.5. Cell morphology assay

Adherent cells round up during the apoptotic process allowing apoptosis to be monitored via a cell morphology-based assay [31]. Cultures transiently transfected with plasmid encoding β-galactosidase and test vector were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as described [31]. The transfection efficiency of 23A2 myoblasts and A2:G12V:H-Ras myoblasts varied by at most 6–7%. Percentage of apoptotic cells for cultures in DM for 3 or 9 h was determined as follows: (round blue cells divided by the total number of blue cells)×100. At least 400 cells were scored from duplicate plates for each condition in a blind manner. After culture in DM for 24 h, the majority of apoptotic 23A2 myoblasts had detached from the plate [4]. For this reason, total cell numbers were also determined for both attached and corresponding detached cells from parallel plates. Similar to the calculation performed following TUNEL analysis [4], the total percentage of apoptotic cells for each plate after 24 h in DM was calculated as follows:

{[total attached cells×percent round/blue cells]+[total detached cells×transfection efficiency]} divided by [total attached cells×percent blue cells]+{[total detached cells×transfection efficiency]}.

3. Results and discussion

3.1. NFκB activity is elevated in differentiating 23A2 myoblasts and in myoblasts expressing constitutively active G12V:H-Ras but not Raf:CAAX

Although apoptosis is initiated concurrent with differentiation, apoptosis occurs within 8–12 h in DM, while differentiation is completed only after 48 h in DM [4]. Others have reported an increase in NFκB DNA-binding activity after 24 h in DM that is critical to the differentiation of rat L6E9 skeletal myoblasts [32,33]. These reports, however, did not examine NFκB transcriptional activity and also did not examine the temporal activation of NFκB. We have analyzed the transcriptional activation of NFκB in murine 23A2 skeletal myoblasts using a luciferase reporter construct containing five tandem κB sites (pNFκB-Luc). Apoptosis in these myoblasts is easily measured after 3 h in DM and is essentially complete after 8–12 h in DM [4]. We, therefore, analyzed the transcriptional activity of NFκB in 23A2 after 3, 9 and 24 h in DM. We found that the NFκB activity remained constant when 23A2 myoblasts were switched from GM to DM for either 3 or 9 h, but that this activity increased roughly two-fold after 24 h in DM (Fig. 1A). At first, this increase in NFκB signaling during the differentiation process would appear to contradict the finding that constitutive NFκB activity inhibits differentiation [10,24,25]. However, a similar situation has been reported for the IFN-inducible protein 202 (p202). Expression of p202 increases during skeletal myoblast differentiation, yet constitutive expression of p202 inhibits differentiation [34]. In both cases, it is likely that while NFκB and p202 activity each play an important role in the differentiation process, the temporally inappropriate activity of either actually abrogates differentiation. The role of NFκB signaling in myoblast survival, however, has not been examined.

Since activated Ras has previously been shown to signal through NFκB [20,21], we examined the NFκB activity in A2:G12V:H-Ras myoblasts, which do not differentiate or undergo apoptosis in DM [4]. We detected a constitutively elevated NFκB signal in the A2:G12V:H-Ras myoblasts that is 3.5-fold greater than that observed in 23A2 myoblasts in either GM or DM for 3 or 9 h (Fig. 1A). We next assessed the ability of transiently expressed G12V:H-Ras or Raf:CAAX to activate NFκB signaling. Expression of G12V:H-Ras, sufficient to abrogate differentiation and the associated apoptosis of 23A2 myoblasts [17], also constitutively activated NFκB signaling roughly 3.5-fold (Fig. 1B). Although Raf signaling activates NFκB in other systems [22,23], expression of Raf:CAAX, sufficient to abrogate differentiation and the associated apoptosis of 23A2 myoblasts [17], did not result in the constitutive activation of NFκB (Fig. 1B).

Classical increases in NFκB-dependent transcription are mediated through nuclear translocation and subsequent specific DNA binding [35]. We, therefore, assessed the specific DNA binding of nuclear NFκB during differentiation. As previously reported using L6E9 rat myoblasts [32], the specific DNA binding of p65-containing nuclear NFκB from 23A2 myoblasts cultured in DM for 24 h is increased when compared to that from 23A2 myoblasts cultured in GM (Fig. 1C). Signaling by Ras, however, does not increase the nuclear translocation and subsequent specific DNA binding of NFκB [20]. Ras-induced increases in NFκB-dependent transcription are mediated through the functional activation of the p65 subunit of nuclear NFκB [20]. Consistent with this previous report using NIH 3T3 fibroblasts, the specific DNA binding of p65-containing nuclear NFκB from A2:G12V:H-Ras myoblasts, or 23A2 myoblasts transfected with G12V:H-Ras or Raf:CAAX, was comparable to similarly cultured parental 23A2 myoblasts (Fig. 1C).

3.2. Constitutive signaling through NFκB is not sufficient to enhance myoblast survival during differentiation

Since constitutive signaling by NFκB is sufficient to inhibit skeletal myoblast differentiation [10,24], we assessed the ability of NFκB signaling to abrogate the apoptosis associated with differentiation. NFκB is kept inactive in the cytoplasm through association with IκB. The IκB kinase (IKK) complex is composed of one regulatory subunit and two catalytic subunits (IKK-1 and IKK-2). IKK-2 is responsible for the specific phosphorylation of IκB in vivo. This phosphorylation signals the degradation of IκB, allowing NFκB to translocate to the nucleus and regulate transcription [35]. To investigate the possibility that increased NFκB signaling might be suffi-

cient to decrease the apoptosis associated with differentiation, we utilized a constitutively active mutant of IKK-2 (S177E, S181E:IKK-2) [36]. Since the luciferase reporter assay allows detection of NFκB activity only in transfected cells, we utilized this assay to determine the amount of the plasmid encoding activated IKK-2 (pS177E, S181E:IKK-2) needed to increase the level of NFκB activity in 23A2 myoblasts, prior to the induction of differentiation, to at least the level found in 23A2 myoblasts that have survived the apoptosis associated with differentiation. We determined that transfection of 50 ng of the pS177E, S181E:IKK-2 increased the NFκB transcriptional activity to levels greater than that found in 23A2 myoblasts that have survived the apoptosis associated with differentiation (Fig. 1A) to levels comparable to that found in A2:G12V:H-Ras myoblasts (Fig. 2A). The assay to detect p65-containing NFκB DNA-binding activity assesses the entire culture. We reasoned, however, that we might be able to detect an increase in p65-containing NFκB DNA-binding activity following transfection of pS177E, S181E:IKK-2, since our transfection efficiency is routinely around 50% ([17] and personal observations). Nuclear extracts from 23A2 myoblasts transfected with 50 ng of the pS177E, S181E:IKK-2 possessed increased p65-containing NFκB DNA-binding activity relative to nuclear extracts from mock transfected 23A2 myoblasts (Fig. 2B).

We next assessed the effect of this IKK-2-mediated increase in NFκB activity on the apoptosis associated with 23A2 myoblast differentiation. To do this, we first performed an ELISA, which measures cytosolic nucleosomes, as an indicator of DNA fragmentation in the entire culture. With a transfection efficiency of 50%, we have previously been able to use this assay to detect a decrease in DNA fragmentation as a consequence of transient transfection of activated G12V:H-Ras or Raf:CAAX [17]. Transient transfection of pS177E, S181E:IKK-2, sufficient to increase NFκB activity in 23A2 myoblasts comparable to that observed in G12V:H-Ras myoblasts (Fig. 2A), did not reduce the DNA fragmentation that occurs when a culture of myoblasts is induced to differentiate (Fig. 2C). We next employed a morphology-based assay to assess the percentage of apoptotic cells [31]. To assess only transfected cells, an expression vector encoding β-galactosi-

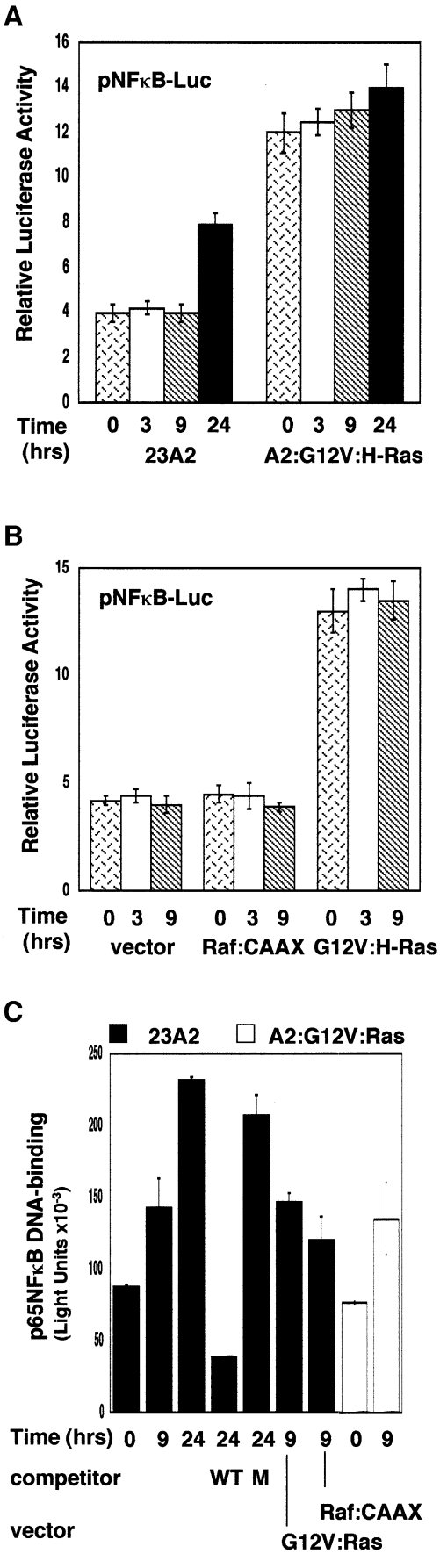

Fig. 1. Elevated NFκB activity in differentiating 23A2 myoblasts and in myoblasts expressing constitutively active G12V:H-Ras but not Raf:CAAX. A: Equal cell numbers of 23A2 myoblasts or A2:G12V:H-Ras myoblasts were plated and the next day transfected as described in Section 2, with 1 μg of the reporter construct pNFκB-Luc (5× κB sites) from Stratagene and 2 ng of the plasmid encoding *R. reniformus* luciferase. B: Equal cell numbers of 23A2 myoblasts were plated and the next day transfected as described in Section 2, with 1 μg of pNFκB-Luc, 2 ng of the plasmid encoding *R. reniformus* luciferase, and 0.5 μg of the indicated test plasmids. The next day, myoblasts were switched from GM to DM for the indicated times prior to the detection of luciferase activity as described in Section 2. Shown in each are the average of duplicates from a single experiment, which are representative of three independent experiments. C: Equal cell numbers of 23A2 myoblasts or A2:G12V:H-Ras myoblasts were plated. The next day, cultures of 23A2 myoblasts were transfected as indicated with 0.5 μg of either plasmid encoding G12V:H-Ras or Raf:CAAX. The next day, cultures were switched to DM for the indicated times prior to the preparation of nuclear extracts and the detection of p65-containing NFκB DNA-binding activity as described in Section 2. Shown in each are the average of duplicates.

dase was transfected with or without pS177E, S181E:IKK-2. Once again, however, an increase in NFκB activity in 23A2 myoblasts to the level observed in G12V:H-Ras myoblasts (Fig. 2A), was not sufficient to decrease their apoptosis during differentiation (Fig. 2D). A lesser increase in NFκB activity is sufficient to inhibit differentiation [37]. It is also worthwhile to note that this increase in NFκB activity did not enhance apoptosis, as has been reported in neurons and epithelial cells [38,39]. We were unable to determine if elevating NFκB activity to levels greater than that observed in the A2:G12V:H-Ras myoblasts affected the survival of 23A2 myoblasts, since transient transfection of more than 50 ng of pS177E, S181E:IKK-2 did not result in higher levels of NFκB activity (Fig. 2A).

3.3. *Elevated NFκB transcriptional activity is not required for the survival of differentiating myoblasts or for the abrogated apoptotic phenotype of myoblasts expressing constitutively active G12V:H-Ras*

The finding that signaling through NFκB is not sufficient to

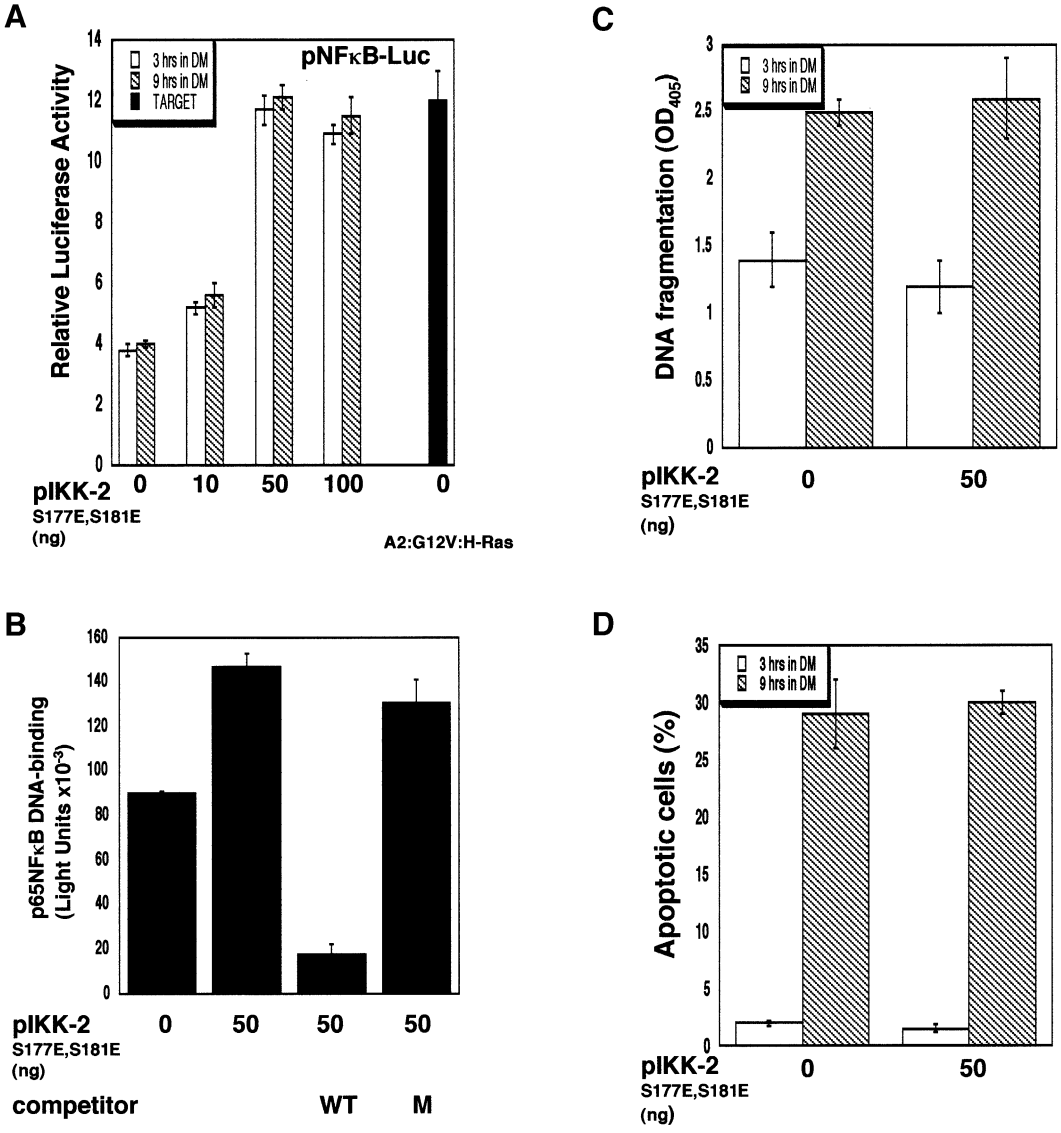

Fig. 2. Constitutive signaling through NFκB is not sufficient to alleviate the apoptosis associated with 23A2 myoblast differentiation. A: Equal cell numbers were plated and, the next day, transfected with 1μg of the reporter construct pNFκB-Luc, 2 ng of the plasmid encoding *R. reniformus* luciferase and the indicated amount of the plasmid encoding constitutively active IKK-2 (pS177E,S181E:IKK-2, a gift from Frank Mercurio). The next day, myoblasts were switched from GM to DM for 3 or 9 h prior to the detection of luciferase activity. Shown in each are the average of duplicates from a single experiment, which are representative of three independent experiments. B: Equal cell numbers were plated and, the next day, transfected with the indicated amount of pIKK-2. Nuclear extracts were prepared the next day and p65-containing NFκB DNA-binding activity was determined. Shown in each are the average of duplicates. C: Equal cell numbers were plated and the next day transfected with the indicated amount of pIKK-2. On the third day, myoblasts were switched to DM. After either 3 or 9 h, cytosolic nucleosomes were detected using Cell Death Detection ELISA Plus Kit (Roche Diagnostics) per manufacturer's instructions as described in Section 2. Shown in each are the average of duplicates from a single experiment, which are representative of three independent experiments. D: Eual cell numbers were plated and the next day transfected with the indicated amount of pIKK-2 and 1 μg of the plasmid encoding β-galactosidase. On the third day, after culture in DM for either 3 or 9 h, cells were stained for β-galactosidase as described in Section 2. Apoptotic cells were scored and the percentage of apoptotic cells was calculated as described in Section 2. Shown are the averages from duplicate plates for each condition.

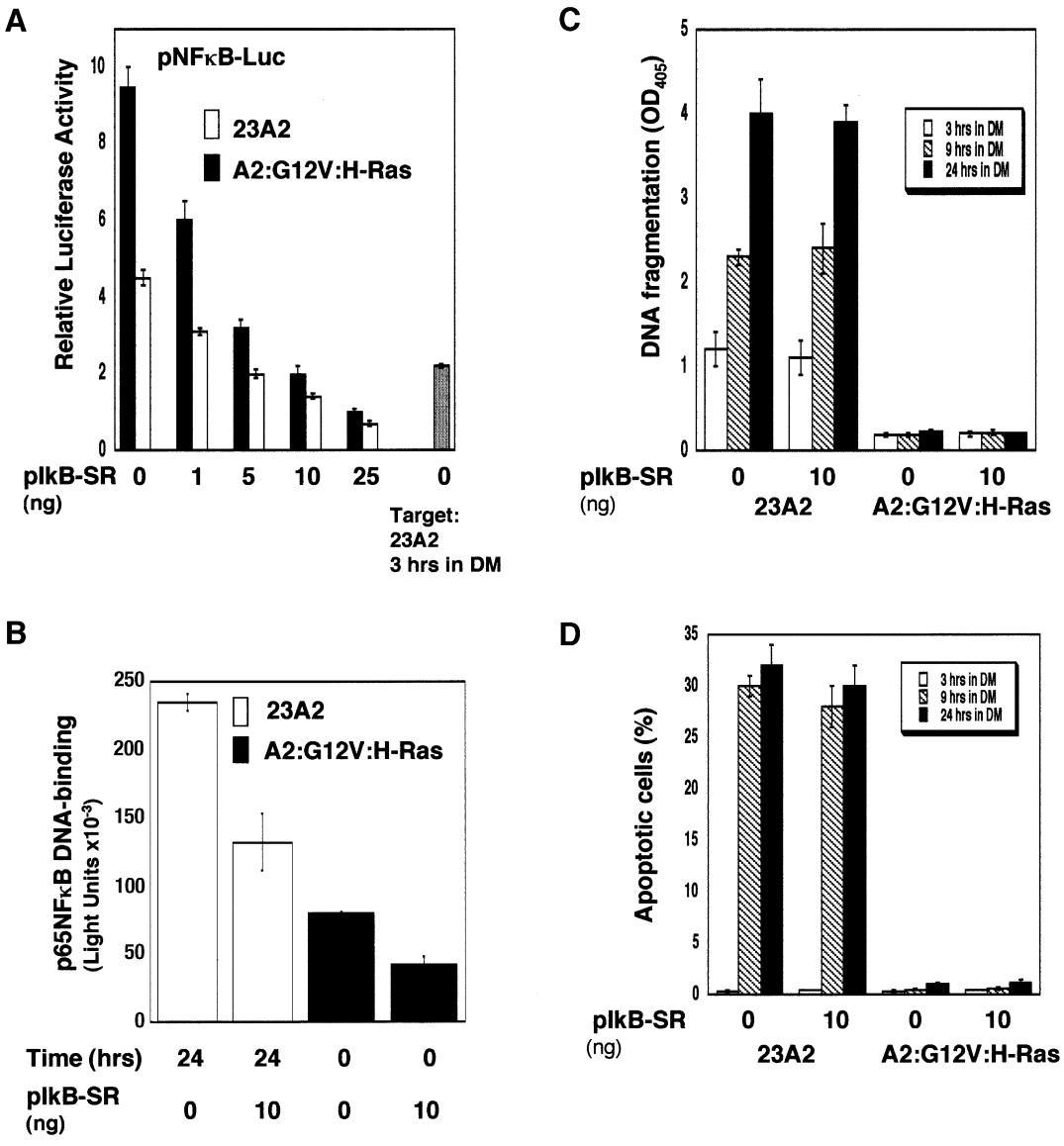

Fig. 3. Inhibition of NFκB activity does not alter the survival of 23A2 myoblasts or A2:G21V:H-Ras myoblasts. A: Equal cell numbers were plated and the next day transfected with 1 μg of the reporter construct NFκB-Luc, 2 ng of the plasmid encoding *R. reniformus* luciferase and the indicated amount of the plasmid encoding IκB-SR (pIκB-SR, a gift from Dr. K. Brand, Munich, Germany). The next day, myoblasts were switched from GM to DM for 24 h, or, as indicated, prior to the detection of luciferase activity. Shown in each is the average of duplicates from a single experiment, which are representative of three independent experiments. B: Equal cell numbers were plated and, the next day, transfected with the indicated amount of pIkB-SR. The next day, myoblasts were switched to DM for the indicated times prior to the preparation of nuclear extracts and the determination of p65-containing NFκB DNA-binding activity. Shown in each is the average of duplicates. C: Equal cell numbers were plated and, the next day, transfected with the indicated amount of pIkB-SR. The next day, myoblasts were switched to DM for the indicated times prior to the detection of cytosolic nucleosomes using Cell Death Detection ELISA Plus Kit. Shown in each is the average of duplicates from a single experiment, which are representative of three independent experiments. D: Equal cell numbers were plated and the next day transfected with the indicated amount of pIκB-SR and 1 μg of the plasmid encoding β-galactosidase. On the third day, cultures were switched to DM for the indicated times. Cells were then stained for β-galactosidase. Apoptotic cells were scored and the percentage of apoptotic cells was calculated as described in Section 2. Shown are the averages from duplicate plates for each condition.

increase myoblast survival during the differentiation process does not eliminate the possibility that signaling through NFκB may still be necessary for the survival of the roughly 70% of myoblasts that do complete the process of differentiation. To investigate this possibility, we utilized a dominant negative form of IκB that cannot be phosphorylated (IκB-SR) and thus is not targeted for degradation by IKK-mediated signaling [35,40]. Again, since the luciferase reporter assay allows detection of NFκB activity in only transfected cells, we utilized this assay to determine that the inhibition of NFκB activity by IκB-SR was dose dependent (Fig. 3A). Transfection of 10 ng of the plasmid encoding IκB-SR (pIκB-SR) was sufficient to prevent the increase in NFκB activity normally observed in 23A2 myoblasts that have survived the apoptosis associated with differentiation (Fig. 3A). A decrease in p65-containing NFκB DNA-binding activity is also achieved with transfection of 10 ng of pIκB-SR (Fig. 3B). Preventing the increase in NFκB activity that occurs during

differentiation, however, failed to increase the amount of DNA fragmentation (Fig. 3C) or the apoptosis of these myoblasts (Fig. 3D). As reported previously [4], although the final percentage of apoptotic myoblasts is determined between 8 and 12 h of culture in DM, the DNA fragmentation per apoptotic cell continues to increase with time in DM (Fig. 3C,D).

Since IκB can relocate NFκB from the nucleus into the cytosol [41], and has been previously used to decrease NFκB activity to determine the significance of NFκB signaling in Ras-mediated phenotypes in fibroblasts [20,42], we employed this same strategy to decrease NFκB activity and assess the contribution of NFκB signaling to the abrogated apoptosis observed in A2:G12V:H-Ras myoblasts. Using the luciferase reporter assay to assess NFκB activity in only transfected cells, a reduction of the constitutively elevated NFκB activity in A2:G12V:H-Ras myoblasts, to a level slightly less than that found in parental 23A2 myoblasts still in the process of undergoing the apoptosis associated with differentiation, was also achieved with 10 ng of pIκB-SR (Fig. 3A). Furthermore, a decrease in the basal p65-containing NFκB DNA-binding activity detected in nuclear extracts of A2:G12V:H-Ras myoblasts could be observed with 10 ng of pIκB-SR (Fig. 3B). Once again, however, this reduction in NFκB activity did not affect the abrogated apoptosis observed in A2:G12V:H-Ras myoblasts (Fig. 3C,D).

3.4. Conclusions

Although NFκB transcriptional activity is elevated in myoblasts that have survived and are in the process of expressing muscle-specific genes, abrogation of this activity does not affect survival. Furthermore, although apoptosis is induced following abrogation of elevated NFκB transcriptional activity in fibroblasts expressing a constitutively active Ras [42], elevated NFκB transcriptional activity is not necessary to maintain the survival of 23A2 myoblasts expressing constitutively active Ras. Finally, constitutive signaling through NFκB is not sufficient to protect myoblasts from the apoptosis associated with differentiation.

Since both differentiation and the associated apoptosis are induced by the same culture conditions, and both can be abrogated by culture in fresh serum or by expression of constitutive active Ras [4] or Raf [17] signaling, the question arises as to whether these processes can be separated. NFκB signaling now represents a pathway capable of inhibiting differentiation without inhibiting the associated apoptosis. Despite advances which minimize immune responses [43,44], myoblast therapy as an approach to the treatment of inherited muscular dystrophies is still severely impaired by the poor survival of transplanted myoblasts [45,46]. Identification of a signaling pathway(s) that does not affect differentiation but that does inhibit the apoptosis associated with differentiation should prove useful. Demonstrating that abrogated differentiation and abrogated apoptosis are separable phenotypes is an important step toward this goal. The search for such candidate pathways is ongoing.

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