RIP2, a Checkpoint in Myogenic Differentiation

Barbara Munz,1 Eberhard Hildt,2 Matthew L. Springer,1 and Helen M. Blau1*

Baxter Laboratory for Genetic Pharmacology, Stanford University Medical Center, Stanford, California 94305-5175,1 and Robert Koch Institute, D-13353 Berlin, Germany2

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Using a subtractive cDNA library hybridization approach, we found that receptor interacting protein 2 (RIP2), a tumor necrosis factor receptor 1 (TNFR-1)-associated factor, is a novel early-acting gene that decreases markedly in expression during myogenic differentiation. RIP2 consists of three domains: an aminoterminal kinase domain, an intermediate domain, and a carboxy-terminal caspase activation and recruitment domain (CARD). In some cell types, RIP2 has been shown to be a potent inducer of apoptosis and an activator of NF-κB. To analyze the function of RIP2 during differentiation, we transduced C2C12 myoblasts with retroviral vectors to constitutively produce RIP2 at high levels. When cultured in growth medium, these cells did not show an enhanced rate of proliferation compared to controls. When switched to differentiation medium, however, they continued to proliferate, whereas control cells withdrew from the cell cycle, showed increased expression of differentiation markers such as myogenin, and began to differentiate into multinucleated myotubes. The complete RIP2 protein appeared to be necessary to inhibit myogenic differentiation, since two different deletion mutants lacking either the aminoterminal kinase domain or the carboxy-terminal CARD had no effect. A mutant deficient in kinase activity, however, had effects similar to wild-type RIP2, indicating that phosphorylation was not essential to the function of RIP2. Furthermore, RIP proteins appeared to be important during myogenic differentiation in vivo, as we detected a marked decrease in expression of the RIP2 homolog RIP in several muscle tissues of the dystrophic mdx mouse, a model for continuous muscle degeneration and regeneration. We conclude that RIP proteins can act independently of TNFR-1 stimulation by ligand to modulate downstream signaling pathways, such as activation of NF-κB. These results implicate RIP2 in a previously unrecognized role: a checkpoint for myogenic proliferation and differentiation.

Myogenic differentiation is a complex and highly organized process: during embryonic development, multipotent mesodermal precursor cells become committed to the myogenic lineage under the influence of various extracellular signals. The resulting myoblasts that are committed, but not yet differentiated, are capable of migrating and of differentiating into multinucleated myotubes. Under the influence of a number of intracellular muscle regulatory factors, including myogenin and members of the MEF2 family of myogenic transcription factors, they eventually exit the cell cycle and differentiate to produce mature muscle cells (myocytes), which fuse to form large multinucleated myotubes (reviewed in references 1, 18, 24, and 35).

For many years, it has been known that several myogenic cell lines, as well as primary myoblasts, can also be induced to differentiate in vitro by switching them to low-serum medium. This process closely resembles myogenic differentiation in vivo, in terms of transcription factors and other regulatory proteins involved, and also in terms of the phenotype of the differentiated, multinucleated myotube. Although in the past few decades a large number of proteins have been implicated as crucial modulators of myogenic differentiation, the complexity of this process strongly suggests that more remain to be discovered.

Since most of the known regulators are encoded by genes that have temporally and spatially restricted expression patterns, we sought to find novel genes that are differentially expressed early in myogenesis. To this end, we used a subtractive cDNA library hybridization approach comparing the gene expression pattern in C2C12 myoblasts differentiated in vitro for a short time period with that in C2C12 cells that had not differentiated. We identified a number of genes which increased or decreased in expression after the induction of differentiation. Here, we present evidence that expression of the gene encoding receptor interacting protein 2 (RIP2), an early-acting intracellular protein previously implicated in apoptosis and a component of the tumor necrosis factor 1 (TNFR-1) signaling complex (11, 16, 30), is markedly decreased during myogenic differentiation. Furthermore, sustained RIP2 expression mediated by transduction with constitutive retroviral vectors prevents differentiation from occurring. These data provide evidence that, in myogenesis, RIP2 does not have a role in apoptosis but instead serves a critical function as a checkpoint between proliferation and differentiation.

MATERIALS AND METHODS

Tissue culture. Murine myoblasts of the C2C12 cell line were cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (growth medium). Primary mouse C57 and C3H myoblasts were isolated and cultured as previously described (23). To induce differentiation, cells were grown to 80 to 90% confluency and then switched to differentiation medium (Dulbecco's modified Eagle's medium containing 2% horse serum).

Generation of myoblasts constitutively expressing high levels of RIP2. The human wild-type RIP2 cDNA and the RIP2 mutants K47A, 1-454, and 311-541...
were a generous gift from V. Druit (Genentech, South San Francisco, Calif.). They were cloned into the retroviral vector pBluescript. To transfect the resulting constructs by using Fugene (Roche). C2C12 cells were subsequently infected with the viral supernatants at high efficiency, as previously described for primary myoblasts (27).

Subtractive cDNA library hybridization. Total cellular RNA was extracted with the RNeasy RNA isolation kit (Qiagen), and poly(A) RNA was isolated by using oligo(dT) cellulose spin columns (Clontech) according to the instructions of the manufacturer. For subtractive cDNA hybridization, the PCR Select kit (Clontech) was used.

Northern blot analysis. Eight micrograms of total RNA isolated as described above was separated on a 1% agarose gel containing formaldehyde (2%). Subsequently, RNA was transferred to a nylon membrane. Ten micrograms of total protein was loaded on a sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked in 3% powdered milk in Tris-buffered saline containing 0.5% Tween 20 (TBS-T) for 30 min; incubated with a dilution of the first antibody, a mouse monoclonal anti-myHC antibody (Pharmpingen), a mouse monoclonal anti-myosin heavy chain antibody (anti-MyHC; 4A.1025) (26), or a rabbit polyclonal anti-I\textsubscript{B} antibody (Santa Cruz) in blocking solution; washed three times with TBS-T; incubated for 30 min with a 1:2,500 dilution of the second antibody, a peroxidase-conjugated anti-mouse or anti-rabbit monoclonal antibody (Amersham); and developed with DAB (4-chloro-1-naphthol) detection system (Amersham).

Cell proliferation assay. Cells were seeded in chamber slides and incubated at room temperature for 30 min. Gel electrophoresis was carried out on a nonnaturating, 4% polyacrylamide gel, and after drying, the dry gel was exposed to an autoradiography film.

Cell proliferation assay. Cells were seeded in chamber slides, pulse-labeled with bromodeoxyuridine (BrdU) for 20 min, and stained with the 5\textsuperscript{-}Bromo-2\textsuperscript{-}Deoxy Labeling and Detection Kit II (Boehringer).

Immunoassays. Cells were seeded in chamber slides and induced to differentiate as described above. After 24 to 48 h, the medium was aspirated and cells were washed with phosphate-buffered saline (PBS), fixed with ethanol for 20 min at \(-20^\circ\)C, and air dried. After rehydration in PBS, they were blocked for 30 min at room temperature in blocking buffer (10% bovine serum albumin in PBS, 0.05% Tween 20). Subsequently, they were incubated with the first antibody, a monoclonal anti-myHC antibody (4A.1025) (26), for 60 min at room temperature. After being washed with PBS, they were incubated with an Alexa 488-coupled anti-mouse antibody (Molecular Probes), diluted 1:500 in blocking buffer, for 30 min at room temperature. After washing with PBS and addition of 1 μg of Hoechst 33258/ml to the washing buffer, they were mounted and analyzed with a Zeiss Axiohot fluorescence microscope.

RESULTS

RIP2 expression declines during differentiation of cultured myoblasts. To identify novel genes that are differentially expressed during early myoblast differentiation, cultured C2C12 myoblasts were induced to differentiate with and without prior addition of 1 ng of transforming growth factor β1 (TGF-β1)/ml, a potent inhibitor of the differentiation process. Rather than simple comparison of cells in growth and differentiation medium, this approach was chosen to minimize the detection of nonspecific changes in gene expression due to numerous differences in the composition of growth and differentiation medium. After 16 h, RNA was isolated and assayed for differential gene expression by a subtractive cDNA library hybridization approach. Increased myogenin mRNA levels after 16 to 24 h and increased MyHC protein expression after 48 h confirmed that the cells underwent differentiation. Several genes were identified which were increased or decreased in expression after the induction of differentiation. Differential expression was confirmed by Northern blot analysis. All transcript levels were normalized to mRNA levels of rpl32, a ubiquitous ribosomal protein. One of the genes which exhibited markedly decreased expression within a few hours of induction of differentiation was identified as RIP2, a protein that is involved in TNF-R1 signaling and has been implicated in apoptosis (11, 16, 30). More detailed analysis revealed that RIP2 expression had decreased as early as 9 to 12 h after the induction of differentiation (data not shown). After 24 h, mRNA levels were reduced approximately fivefold (Fig. 1A). In addition, in differentiation medium containing TGF-β, RIP2 expression was also lower than in growth medium, indicating that this growth factor only partially inhibited differentiation. The two bands evident on the Northern blot at approximately 2 and 2.5 kb obtained for RIP2 (and also RIP and RIP3; see below) have been described before and are likely to reflect differential polyadenylation of RIP transcripts (11).

The expression patterns of the other two members of the RIP family, RIP and RIP3, were also analyzed during myoblast differentiation. Both RIP and RIP3 were expressed in proliferating myoblasts. However, whereas RIP2 expression was weak and remained more or less constant throughout the differentiation process (data not shown), RIP, like RIP2, declined in expression, although to a lesser degree than RIP2 (Fig. 1B).

RIP proteins are components of the signaling complex that associates with TNFR-1 and mediates downstream responses. All RIP proteins consist of an amino-terminal serine-threonine kinase domain and a central intermediate domain (Fig. 2A). The carboxy-terminal domains, however, differ. Whereas RIP and RIP2 harbor a death domain and a caspase activation and recruitment domain (CARD), both of which are known mediators of apoptosis, the carboxy-terminal domain of RIP3 does not display any homology to known proteins (29, 34). Nevertheless, all three RIP proteins have been shown previously to induce apoptosis and to modulate activation of NF-κB (11, 14–16, 22, 28–31, 34). Furthermore, RIP2 has recently been shown to act as a Raf1-activated mitogen-activated protein kinase kinase (MAPKK) (20).

Generation of myoblasts transduced to constitutively express high levels of RIP2. To gain insight into the function of RIP2 during the differentiation process, we generated retroviral constructs to constitutively express high levels of the recombinant protein in myoblasts. Furthermore, a series of various RIP2 mutants which had previously been shown to have differential effects on apoptosis and NF-κB activation (16) as well as on the MAPKK activity of RIP2 (20) were expressed (Fig. 2A). Wild-type RIP2 and all mutants were fused with a C-terminal myc-His tag, to allow detection of the recombinant proteins. As shown in Fig. 2B, all constructs were highly expressed at the mRNA level, and expression levels were comparable for all mutants compared to the mRNA loading con-
trol rpl32. Hybridization of a parallel blot with the mouse RIP2 probe confirmed that expression levels of recombinant RIP2 were at least 100-fold higher than those of the endogenous mRNAs (data not shown). At the protein level (Fig. 2C), a weak signal was detected for recombinant wild-type RIP2 and the phosphorylation-deficient mutant K47A, whereas the truncation mutants, especially the 1-454 variant which lacks the CARD, were expressed at higher levels.

Increased expression of wild-type RIP2 maintains myoblast proliferation in differentiation medium. To determine whether RIP2 is a regulator of myoblast proliferation, the cells transduced to express elevated levels of RIP2 were pulse-labeled with BrdU for 20 min and stained with an anti-BrdU antibody. All nuclei were made visible by staining with Hoechst 33258, and the number of BrdU-labeled versus total nuclei was determined in the same fields. A quantitative analysis of these data is shown in Fig. 3B. In growth medium, myoblasts expressing RIP2 at high levels did not exhibit an enhanced proliferation rate compared to wild-type myoblasts. By contrast, C2C12 myoblasts or LacZ-transduced (data not shown) cells in differentiation medium ceased proliferating, withdrew from the cell cycle, and began to fuse to form myotubes, whereas the myoblasts expressing high levels of recombinant RIP2 continued to proliferate at almost the same rate as in growth medium.

Increased RIP2 expression inhibits myogenic differentiation. To study the effects of elevated RIP2 expression on myogenic differentiation, we analyzed myogenin mRNA expression 24 h after the induction of differentiation in myoblasts transduced with retroviral vectors encoding RIP2 compared to control cells. Based on previous studies (Fig. 1 and data not shown), myogenin transcript levels are at their maximum at this time point. Indeed, high levels were found in control myoblasts, whereas increased expression of wild-type RIP2 or the K47A phosphorylation-defective mutant almost completely suppressed myogenin induction (Fig. 4A). By microscopic analysis, no myotubes were visible at 24 or 48 h in myoblasts expressing high levels of RIP2 (Fig. 4B, right panel), whereas in controls, small myotubes could be detected after 24 h, and after 48 h numerous large, MyHC-positive myotubes were evident (Fig. 4B, left panel). These data were corroborated by Western blot analysis; 44 h after the induction of differentiation, control myoblasts or myoblasts expressing LacZ or one of the truncated RIP2 proteins expressed high levels of MyHC, whereas MyHC was barely detectable in cells expressing elevated levels of wild-type RIP2 or the K47A mutant (Fig. 4C). The low levels of myogenin (data not shown) and of MyHC observed in the wild-type or K47A mutant RIP2 could be due to the fact that myoblast populations, rather than clones, were analyzed; thus, some cells may have been included that expressed little or none of the recombinant protein.

NF-κB activity is high in RIP2 myoblasts transduced to express high levels of RIP2. Recent reports have shown that NF-κB activity is inhibited in differentiating myoblasts and that this inhibition is necessary for differentiation to occur (8). We hypothesized that RIP2 could act as an inhibitor of differentiation in normal myoblasts, since RIP2 levels are high in growth medium, leading to NF-κB activation. Accordingly, once cells were switched to differentiation medium, RIP2 expression should decline, leading to reduced NF-κB activation. Alternatively, if high levels of RIP2 were still present, NF-κB activity should not decrease, which would prevent differentiation from occurring.

To test this hypothesis, we measured NF-κB activity under growth conditions and at different time points after the induction of differentiation. For this purpose, an enzyme-linked immunosorbent assay (ELISA) specific for active NF-κB was used. In growth medium, NF-κB activity in cells expressing high levels of wild-type RIP2 or of the K47A mutant was approximately three times as high as in control cells or in cells expressing one of the truncation mutants. After the induction of differentiation, NF-κB activity was reduced in all cells but was still higher in cells expressing high levels of recombinant RIP2. Forty-eight hours after the induction of differentiation, NF-κB activity was significantly reduced in all cell lines (Fig. 5A).

The expression of IκB, an inhibitor of NF-κB activity, was
induction of differentiation (in hours), with 0 h corresponding to cells
numbers at the bottom of each lane indicate time points after the
did not change considerably after 24 h in differentiation medium.
 Transcript levels give the weakest signal with the shortest transcript. Transcript levels
entire RIP2 coding sequence was used, which would be expected to
311-541 is probably due to the fact that a probe directed against the
were expressed at high levels. The slightly weaker signal for mutant
ern blotting, with a probe directed against human RIP2. All transcripts
pressed as fusion proteins with a carboxy-terminal myc-His tag (shown
in grey). (B) Expression of recombinant RIP2 was analyzed by North-
shows the domain structure of the recombinant proteins: the kinase
domain is shown as a hatched pattern, and the phosphorylation site is
indicated. The intermediate domain is shown as a line, and the car-
domain is shown as a hatched pattern, and the phosphorylation site is
studied by Western blot analysis with an antibody to the carboxy-
in growth medium. (C) Expression of recombinant RIP2 was also
in growth medium. (C) Expression of recombinant RIP2 was also
studied by Western blot analysis with an antibody to the carboxy-
terminal myc tag which was included in all constructs. Bands of the
expected molecular weight for each mutant are indicated by arrows;
the additional bands at lower molecular weights are commonly ob-
erved and are probably degradation products. Molecular weights are
shown at left in thousands. As a loading control for total protein, the
same filter was reprobed with an antibody specific for α-tubulin.

FIG. 2. Transduction of myoblasts with wild-type and mutant RIP2.
C2C12 myoblasts were transduced with retroviral vectors to express
LacZ (control), wild-type RIP2 (WT), a phosphorylation-defective
mutant (K47A), a carboxy-terminal deletion mutant (1-454), or an
amino-terminal deletion mutant (311-541). (A) The schematic drawing
shows the domain structure of the recombinant proteins: the kinase
domain is shown as a hatched pattern, and the phosphorylation site is
indicated. The intermediate domain is shown as a line, and the car-
boxy-terminal CARD is labeled as such. All the proteins were ex-
pressed as fusion proteins with a carboxy-terminal myc-His tag (shown
in grey). (B) Expression of recombinant RIP2 was analyzed by North-
ern blotting, with a probe directed against human RIP2. All transcripts
were expressed at high levels. The slightly weaker signal for mutant
311-541 is probably due to the fact that a probe directed against the
entire RIP2 coding sequence was used, which would be expected to
give the weakest signal with the shortest transcript. Transcript levels
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Numbers at the bottom of each lane indicate time points after the
induction of differentiation (in hours), with 0 h corresponding to cells
in growth medium. (C) Expression of recombinant RIP2 was also
studied by Western blot analysis with an antibody to the carboxy-
terminal myc tag which was included in all constructs. Bands of the
expected molecular weight for each mutant are indicated by arrows;
the additional bands at lower molecular weights are commonly ob-
served and are probably degradation products. Molecular weights are
shown at left in thousands. As a loading control for total protein, the
same filter was reprobed with an antibody specific for α-tubulin.

also analyzed. An inverse correlation between IκB levels and
NF-κB activity is typically observed, since IκB is degraded
when NF-κB becomes activated (reviewed in reference 13). In
growth medium, IκB levels in control cells and cells expressing
high levels of RIP2 were similar. However, after 48 h in dif-
ferentiation medium, IκB levels were lower in cells that ex-
pressed high levels of wild-type RIP2 or the K47A mutant,
indicating that NF-κB activity remained elevated in these cells.
By contrast, myoblasts that expressed high levels of the dele-
tion mutants showed IκB levels on a par with control myoblasts
in which RIP2 was not expressed or in which only LacZ was
expressed at high levels (Fig. 5B). Similar results were obtained
when NF-κB nuclear translocation was analyzed as a function
of its binding to its consensus sequence by EMSA (Fig. 5C).
These findings are consistent with our observations that these
cells differentiated in a manner indistinguishable from that of
control cells.

Of note was the finding that a higher level of NF-κB was
detected in myoblasts expressing elevated levels of RIP2 ex-
posed to growth medium, whereas there was no difference in
IκB levels under these conditions. One explanation for this
observation could be that the ELISA used here was specific for
NF-κB p65 and that other NF-κB isoforms that were not assa-
yed could have contributed to the physiological effect on
myogenensis.

RIP2 expression is decreased during primary myoblast dif-
ferentiation. Since C2C12 myoblasts are a transformed cell
line, we analyzed the physiological relevance of our findings by
studying RIP2 expression in primary myoblasts isolated from
two different mouse strains, C57BL/6 and C3H. We found a
strong and early decrease in RIP2 expression in primary myo-
blasts upon induction of differentiation, albeit to different ex-
tents (2- to 10-fold after 22 h), with the magnitude depending
on the mouse strain (Fig. 6). These data indicate that modu-
lation of RIP2 expression during differentiation is a property
not only of an established cell line but also of primary myo-
blasts isolated directly from mouse muscle tissues.

RIP expression is reduced in different skeletal muscle tis-
sues of the mdx mouse. Since several skeletal muscle diseases,
including muscular dystrophy, are characterized by aberrant
expression of myogenic regulatory factors (32), we tested
whether RIP2 expression might also be altered. Skeletal muscle
tissues of various origins (diaphragm, quadriceps, and tib-
ials anterior) were dissected from mdx mice and from sex- and
age-matched control mice between 3 and 14 weeks of age. It is
during that time period that mdx mouse muscles undergo con-
tinuous cycles of muscle degeneration and regeneration (ref-
ence 21 and references therein). RIP2 expression was unde-
tectable in the diverse muscles from mdx or control mice
analyzed by Northern blotting. In contrast, the RIP2 homolog
RIP was readily detected in control animals and markedly
reduced in levels in all tissues from the mdx mice in which
regeneration and differentiation are highly active (Fig. 7).

DISCUSSION

Here, we identify a novel function for the TNFR-1-associated
factor RIP2 as a checkpoint in myogenic differentiation.
Several studies have shown that increased expression of
TNFR-1-associated factors often mimics activation of TNFR-1
physiological conditions has not previously been described. Means of modulating cell growth and differentiation under the expression of TNFR-1-associated factors such as RIP2 as a means of modulating cell growth and differentiation under physiological conditions has not previously been described. To our knowledge, however, regulation of ex-

The RIP family of TNFR-1-associated factors consists of three proteins, RIP, RIP2 (also named RICK or CARDIAK), and RIP3. They share a highly homologous amino-terminal serine-threonine kinase domain, but their carboxy termini differ. Whereas RIP contains a death domain (28) and RIP2 harbors a CARD, both of which can induce apoptosis (11, 16, 30), the carboxy-terminal domain of RIP3 has no homology to known proteins (29, 34). The amino- and carboxy-terminal domains are linked via an intermediate domain, which is unique for each of the RIP proteins.

Here we show first that expression of RIP2 decreases profoundly early during myogenic differentiation. Expression of RIP also declines, although not as strongly, whereas RIP3 is only weakly expressed and not regulated during the differentiation process. These findings suggest that both RIP and RIP2 may be involved in the regulation of the differentiation process.

To test this hypothesis, we delivered wild-type RIP2, a kinase-deficient mutant, and two different RIP2 deletion mutants into myoblasts via retroviral transduction in order to express high constitutive levels. At the mRNA level, all constructs were expressed at similarly high levels, whereas at the protein level, the deletion mutants, especially the carboxy-terminally truncated variant which is lacking the CARD, were detected at much higher levels than wild-type RIP2 or the K47A kinase deletion mutant. These results indicate either that the latter are less stable or that their biological activities render cells less tolerant to elevated levels of these proteins.

In growth medium with a high serum concentration, myo-

blasts expressing high levels of RIP2 were phenotypically indistinguishable from control cells. Once switched to differentiation medium with low serum content, however, cells expressing high levels of wild-type RIP2 or the K47A mutant proliferated at essentially the same rate as in growth medium, whereas control myoblasts or cells expressing the truncated RIP2 proteins withdrew from the cell cycle and ceased to proliferate.

Myoblasts expressing high levels of wild-type RIP2 or the K47A mutant expressed only very low levels of various differentiation markers and never formed myocytes or myotubes. By contrast, control cells increased their expression of various differentiation markers, produced elongated myocytes, and eventually fused with each other to form multinucleated myotubes. The fact that the K47A mutant had effects similar to wild-type RIP2, whereas the deletion mutants had no effect, indicates that all three domains of the RIP2 protein function to enhance proliferation and inhibit differentiation. Kinase activity, however, does not appear to be necessary.

RIP2 has been shown elsewhere to activate the transcription factor NF-κB (11, 16, 30). Indeed, a recent report shows that a decrease in NF-κB activity is essential for myogenic differen-
tiation to occur (8). Furthermore, it has long been known that stimulation of myoblasts with TNF-α inhibits differentiation even under low-serum conditions (17), an effect which might also be mediated by NF-κB. Forced expression of RIP2 could lead to prolonged activation of NF-κB, thereby inhibiting differentiation even under low-serum conditions. Indeed, in cells expressing elevated levels of recombinant wild-type RIP2 or of the K47A mutant, we detected a high degree of NF-κB activity. Furthermore, IκBα, an NF-κB inhibitor protein that binds inactive NF-κB and becomes phosphorylated and eventually

by ligand, in that it can lead to induction of apoptosis and activation of NF-κB (2, 5, 10). One recent study demonstrates that TNFR-associated factor 1 (TRAF1), a protein that asso-

uates with TNFR-2 and various other members of the TNFR family of receptors, is expressed at high levels in transformed, but not in normal, lymphocytes and may inhibit apoptosis in these cells (6). To our knowledge, however, regulation of expression of TNF-1-associated factors such as RIP2 as a means of modulating cell growth and differentiation under physiological conditions has not previously been described.

The RIP family of TNF-1-associated factors consists of
degraded once NF-κB is activated, was expressed at low levels in these cells.

Surprisingly, although differences in NF-κB expression could be observed only in differentiating cells, NF-κB activity was already altered under growth conditions, although differences with respect to cell proliferation compared to controls could be observed only in differentiating cells. It is possible that, in growth medium in which numerous growth factors and cytokines are present, a variety of other growth-promoting signal-

ing cascades, such as the MAPK pathways, might already be highly activated. As a result, in growth medium, activation of NF-κB by RIP2 would have little effect on cell proliferation. In differentiation medium, however, NF-κB is inhibited in normal myoblasts, whereas its activity remains elevated in myoblasts expressing elevated levels of RIP2.

RIP2 was recently shown to act as a Raf1-activated MAPKK, eventually activating Erk2 (20). Activation of Raf-1 also inhibits myogenesis (4, 25, 33), and inactivation of the Raf-1-MAPKK1/2-ERK1/2 pathway prevents myoblast proliferation (12). Thus, action via this pathway could constitute an additional mechanism by which increased levels of RIP2 inhibit differentiation.

Surprisingly, as assessed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling assay, expression of high levels of RIP2 did not induce apoptosis in our studies of myogenesis (B. Munz and H. M. Blau, unpublished observations). This could be due to cell-type- or species-specific differences in signaling. Previous studies (11, 16, 30) in which apoptosis in response to RIP2 was observed were carried
out in human cell lines that were not derived from a myogenic lineage.

Finally, we investigated whether RIP proteins may also play a role during myogenesis in vivo. As in the C2C12 myogenic cell line, RIP2 expression declines markedly upon differentiation of primary myoblasts isolated from two different mouse strains, albeit to different extents. In addition, whereas RIP2 was not expressed at a detectable level in wild-type as well as mdx mouse muscle, its homolog RIP was expressed in diverse muscle tissues isolated from wild-type mice. Moreover, expression was decreased strongly in mdx mice. These animals undergo continuous cycles of muscle degeneration and regeneration, especially between 3 and 14 weeks of age (reference 21 and references therein). Although the complex pattern of gene expression during muscle regeneration in mdx mice is not fully understood, previous studies have mostly shown higher expression of genes that are normally induced during muscle differentiation, such as troponin I and insulin-like growth factor 2 (32) or the cell cycle inhibitor protein p21 (7). One explanation for this might be that, in mdx mouse muscle, due to continuous degeneration and regeneration, the pool of undifferentiated myogenic stem cells or satellite cells gets exhausted, thus enriching for more differentiated myoblasts which might express higher levels of differentiation-associated proteins (7). Accordingly, a gene which is normally reduced in expression during differentiation, such as RIP, would be expected to be found at lower levels in mdx mouse muscle.

Indeed, differential expression of RIP proteins in degenerating and regenerating skeletal muscle is especially interesting in the context of a recent publication (9) showing that increased activation of NF-κB leads to the muscle decay associated with cachexia, characteristic of many cancer patients. In contrast to the mdx mouse model, a very low rate of muscle regeneration is observed in these patients (reference 9 and references therein). Therefore, it would be interesting to study expression levels of RIP proteins in cachetic muscle. Thus, further analysis of mechanisms for altering the complex expression patterns of RIP proteins and other regulators of NF-κB in normal and diseased skeletal muscle may have important therapeutic implications.

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