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Merosin (Laminin-2/4)-Driven Survival Signaling: Complex Modulations of Bcl-2 Homologs

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Abstract We have shown previously that the promotion of myofiber survival by the basement membrane component merosin (laminin-2 [α2β1γ1]/laminin-4 [α2β2γ1]) is dependent on the activity of the tyrosine kinase Fyn, whereas myofiber anoikis induced by merosin deficiency is dependent on the stress-activated protein kinase p38α. To further understand such merosin-driven survival signaling, we analyzed the expression of five Bcl-2 homologs (Bcl-2, Bcl-X_L, Bax, Bak, Bad) and one non-homologous associated molecule (Bag-1) in normal and merosin-deficient myotubes, with or without pharmacological inhibitors for Fyn and p38. Herein, we report that (1) merosin deficiency induces anoikis and causes decreased Bcl-2, Bcl-X_L, and Bag-1 levels, increased Bax and Bak levels, and decreased Bad phosphorylation; (2) Bcl-2, Bcl-X_L, Bag-1, and Bad phosphorylation are also decreased in anoikis-dying, Fyn-inhibited myotubes; (3) the inhibition of p38α in Fyn-inhibited and/or merosin-deficient myotubes protects against anoikis and increases Bcl-2 levels above normal, in addition to restoring Bad phosphorylation and Bag-1 levels to normal; (4) the overexpression of merosin in deficient myotubes also rescues from anoikis and increases Bcl-2 levels and Bad phosphorylation above normal, in addition to restoring Bcl-X_L, Bag-1, Bax, and Bak levels to normal; and (5) Bcl-2 overexpression is sufficient to rescue merosin-deficient myotubes from anoikis, even though the expression/phosphorylation levels of the other homologs analyzed are not restored to normal. These results indicate that merosin-driven myofiber survival signaling affects complex, differential modulations of individual Bcl-2 homologs. These further suggest that Bcl-2 can play a major role in suppressing myofiber anoikis. J. Cell. Biochem. 89: 1115–1125, 2003.

Key words: anoikis; apoptosis; Fyn; laminin; myofiber; p38; programmed cell death; signal transduction

Programmed cell death (or apoptosis) is an intricaly regulated process, which plays a central role in tissue homeostasis [Jacobson et al., 1997]. The Bcl-2 family of proteins constitutes a critical decisional checkpoint in cell survival and death [Reed et al., 1996a,b; Adams and Cory, 1998; Reed, 1998; Cory and Adams, 2002]. At least 20 family members have been identified so far in mammalian cells, functioning either as anti-apoptotic (e.g., Bcl-2, Bcl-X_L) or pro-apoptotic (e.g., Bax, Bad, Bak) regulators. Bcl-2 homologs are well known to interact among themselves, as well with an expanding repertoire of associated molecules (e.g., Bag-1), allowing for the titration of pro- and anti-apoptotic functions [Gajewski and Thompson, 1996; Reed et al., 1996a,b; Adams and Cory, 1998; Reed, 1998; Cory and Adams, 2002]. It is now well established that the suppression or induction of apoptosis in different cell types does not necessarily depend on the activity of single Bcl-2 homologs, but often on a balance of anti- and pro-apoptotic activities from multiple homologs [Reed et al., 1996a,b; Adams and Cory, 1998; Reed, 1998; Cory and Adams, 2002]. Incoming extracellular signals determine in large part if a cell lives or die, and involve signaling events that ultimately affect

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the expression and/or functions of multiple anti- and pro-apoptotic Bcl-2 homologs: as example, the phosphorylation of Bad results in the neutralization of its pro-apoptotic functions [Ruoslahti and Reed, 1994; Gajewski and Thompson, 1996; Anderson, 1997; Frisch and Ruoslahti, 1997; Reed, 1998; Boudreau and Jones, 1999]. Such survival signals are provided by various stimuli, including growth factors, and cell adhesion [Ruoslahti and Reed, 1994; Anderson, 1997; Frisch and Ruoslahti, 1997; Giancotti, 1997; Meredith and Schwartz, 1997; Boudreau and Jones, 1999; Giancotti and Ruoslahti, 1999; Frisch and Screaton, 2001; Cory and Adams, 2002]. To this effect, the loss of cell extracellular matrix (ECM) interactions induces a form of apoptosis that is termed "anoikis" [Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997; Frisch and Screaton, 2001].

The basement membrane of mammalian skeletal myofibers is predominantly rich in merosin (laminin-2 [$\alpha2\beta1\gamma1$]/laminin-4 [$\alpha2\beta2\gamma1$]). Not surprisingly, merosin acts as a critical promoter of cell survival and suppression of anoikis in myotubes [Vachon et al., 1996, 1997; Kuang et al., 1998a,b; Laprise et al., 2002]. In addition, mutations affecting the merosin $\alpha2$ chain gene have been identified as causes of severe forms of muscular dystrophy [Campbell, 1995; Hebling-Leclerc et al., 1995; Vachon et al., 1996, 1997; Wewer and Engvall, 1996; Kuang et al., 1998a,b; Burkin and Kaufman, 1999]. Such a role for merosin in the suppression of anoikis in skeletal muscle is not shared by other laminins: for example, merosin-deficient myotubes are rescued from anoikis by overexpression of the $\alpha2$ chain of merosin, but not with the $\alpha1$ chain of laminin-1 [Vachon et al., 1996, 1997; Laprise et al., 2002]. It is now well acknowledged that integrins (transmembrane receptors for ECM constituents) that bind merosin mediate in large part merosin's functions in the promotion of myofiber survival [Vachon et al., 1997; Burkin and Kaufman, 1999; Laprise et al., 2002]. In this respect, we have shown recently that the myofiber survival signaling generated by merosin–integrin interactions involve the tyrosine kinase Fyn, whereas a deficiency in merosin and/or the inhibition of integrins induce myofiber anoikis through a p38α stress-activated protein kinase (SAPK)-dependent pathway [Laprise et al., 2002]. To this effect, the inhibition of Fyn by pharmacological compounds (such as herbimycin A (HA) or PP2), or by merosin deficiency, induce myofiber anoikis; conversely, the pharmacological inhibition of p38α (with SB202190 or 203580) protects Fyn-inhibited and/or merosin-deficient myotubes from this form of cell death [Laprise et al., 2002]. Thus, within this context of merosin-dependent survival signaling, the question remains as to how Bcl-2 homologs are affected in merosin-deficient myotubes, as opposed to their merosin-expressing counterparts.

In the present study, we investigated this question by analyzing the expression of five Bcl-2 homologs (Bcl-2, Bcl-X_L, Bax, Bak, Bad), and one non-homologous associated molecule (Bag-1), using the C2C12 cell model of differentiated myotubes and its merosin- or laminin-deficient derivatives that we previously generated and characterized [Vachon et al., 1996, 1997; Laprise et al., 2002]. Cultured myotubes were also exposed to pharmacological inhibitors of Fyn and/or of p38. Herein, we report that merosin deficiency and/or Fyn inhibition result in a balance that is distinctively in favor of pro-apoptotic regulators. In contrast, the inhibition of p38α in merosin-deficient (or Fyn-inhibited) myotubes restores a balance of Bcl-2 homologs in favor of anti-apoptotic regulators. In addition, Bcl-2 overexpression is sufficient to rescue merosin-deficient myotubes from anoikis, even if the expression levels of other homologs are not restored to normal levels. Altogether, these data suggest that merosin-driven myofiber survival signaling affects complex, differential modulations of individual Bcl-2 homologs, and associated non-homologous molecules, generally favoring anti-apoptotic regulators.

MATERIALS AND METHODS

Myogenic Cell Lines and Cell Culture

The C2C12 myogenic cell line was kindly provided by Dr. E. Engvall (The Burham Institute, La Jolla, CA). C2C12 differentiated myotubes express merosin abundantly but little or no other laminins [Schuler and Sorokin, 1995; Vachon et al., 1996]. Myogenic differentiation-defective clonal variants of C2C12 cells were generated and characterized previously [Vachon et al., 1996, 1997]. The C2C12.B4 cells are unable to fuse to form myotubes; these cells express the $\beta1$ and $\gamma1$ chains of laminin but are deficient in the expression of the $\alpha1$ and $\alpha2$

chains [Vachon et al., 1996]. C2C12.B4 cells were stably transfected with the human laminin α1 chain (pLam) or with the human merosin α2 chain cDNA (pMer), in the pCDNA3 expression vector [Vachon et al., 1996, 1997; Laprise et al., 2002]. Laminin/merosin cDNA constructs were a kind gift from Dr. U.M. Wewer (Institute of Molecular Pathology, Copenhagen, Denmark). C2C12.B4/pLam cells are rescued from the phenotype of fusion deficiency, but the myotubes generated undergo anoikis due to merosin deficiency [Vachon et al., 1996, 1997; Laprise et al., 2002]. C2C12.B4/pMer derivatives are rescued from the phenotype of merosin deficiency and thus form viable myotubes [Vachon et al., 1996, 1997; Laprise et al., 2002]. C2C12.B4/pLam cells were further transfected stably with either the puro-pBabe vector (pBabe) containing or not the human Bcl-2 cDNA (pBcl-2) [Vachon et al., 1997]. In any event, cells were grown and induced to differentiate into myotube cultures as already described [Vachon et al., 1996, 1997; Laprise et al., 2002].

In some experiments, differentiated cultures were exposed as previously described [Gauthier et al., 2001a; Laprise et al., 2002] to 1 μM HA (Sigma, Oakville, Ont., Canada), 5 μM PP2 (Calbiochem, San Diego, CA), 20 μM SB202190 (Calbiochem), or 20 μM SB203580 (Calbiochem). HA and PP2 inhibit numerous tyrosine kinases of the Src family [Keely and Parise, 1996; Parsons and Parsons, 1997; Abram and Courtneidge, 2000], including Fyn [Keely and Parise, 1996; Parsons and Parsons, 1997; Abram and Courtneidge, 2000; Laprise et al., 2002] which is the predominant Src family tyrosine kinase in myotubes [Laprise et al., 2002]. The SB compounds inhibit p38α/β SAPK catalytic activity [Boucher et al., 2000; English and Cobb, 2002; Laprise et al., 2002; Vachon et al., 2002]. Note that results were similar, if not identical, whether we used HA or PP2 to inhibit Fyn, and whether we used SB202190 or SB203580 to inhibit p38 (not shown; see Laprise et al., 2002). The determination of the working concentrations for the compounds used herein was performed previously [Boucher et al., 2000; Gauthier et al., 2001a,b; Laprise et al., 2002; Vachon et al., 2002].

DNA Laddering Assays

DNA was isolated according to the method previously described [Vachon et al., 1996; Gauthier et al., 2001a; Laprise et al., 2002]. DNA contents of all samples were estimated by OD_{260}. The visualization of anoikis-associated DNA fragmentation (DNA laddering) was performed on 2% agarose gels (20 μg DNA/lane) as described previously [Vachon et al., 1996, 2002; Gauthier et al., 2001a; Laprise et al., 2002].

Protein Expression Analyses

Differentiated myotube cultures were harvested in sample buffer (2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue, in 125 mM Tris-HCl pH 6.8) as described [Vachon et al., 1996, 1997; Laprise et al., 2002]. Total proteins (25–50 μg/well) were separated by SDS–PAGE, then electrotransferred to nitrocellulose membranes (Amersham Life Science, Piscataway, NJ) for subsequent immunoblotting [Vachon et al., 1996, 1997, 2002; Gauthier et al., 2001a,b; Laprise et al., 2002]. Specific antibodies directed to Bcl-2, Bcl-X_L, Bag-1, Bax, Bak, or Bad are well characterized and were used as described [Krajewska et al., 1996; Wang et al., 1996; Boucher et al., 2000; Vachon et al., 2000, 2001; Gauthier et al., 2001a,b]. An antibody directed to the serine-112 phosphorylated form of Bad (New England Biolabs, Mississauga, Ont., Canada) was also used. Immunoreactive bands were visualized by the enhanced chemiluminescence method (Amersham) according to the manufacturer's instructions. Band intensities were semi-quantified using an Alpha Imager 1200 Documentation and Analysis system (Alpha Innotech Corp., San Leondro, CA). Actin was used as a reference protein to establish the relative expression levels of the molecules analyzed, as previously described [Vachon et al., 1996, 1997, 2002; Gauthier et al., 2001b; Laprise et al., 2002]. Data were in turn compared to those of control cultures (normal C2C12 myotubes), ×100 (expressed as "% of control").

Data Processing

Results, values shown represent mean ± SEM for at least three ($n \geq 3$) separate experiments and/or cultures. Statistically significant differences were determined with the Student's *t*-test. Data were compiled, analyzed, and processed with Excel (Microsoft, Redmond, WA) and Cricket Graph (Computer Associates, Islandia, NY). Except otherwise specified, images from blots, gels, and scans were processed

with Vistascan (Umax Technologies, Fremont, CA), Photoshop (Adobe, San Jose, CA), and PowerPoint (Microsoft).

RESULTS

Loss of Merosin-Driven Survival Signaling Results in a Balance of Expression/Function in Favor of Pro-Apoptotic Regulators

To ascertain whether there is a link between loss of viability in merosin-deficient myotubes and changes in Bcl-2 homologs, we performed comparative analyses of the expression levels of five homologs (anti-apoptotics: Bcl-2, Bcl-X_L; pro-apoptotic: Bax, Bak, Bad), and one non-homologous associated molecule (anti-apoptotic: Bag-1), between control (normal C2C12) myotubes and merosin-deficient (C2C12.B4/pLam) ones. As we reported previously [Vachon et al., 1996, 1997; Laprise et al., 2002], C2C12.B4/pLam myotubes undergo spontaneous anoikis due to merosin deficiency (Fig. 1, lanes 5 and 7), in contrast to normal myotubes (Fig. 1, lane 1). This lack of viability in merosin-deficient myotubes was found to be associated with significant decreases in the steady-state levels of Bcl-2, Bcl-X_L, and Bag-1 (~64, ~61, and ~40% of control, respectively; Fig. 2A, lane 2 vs. 1; Fig. 2B, dark columns). Conversely, the expression levels of Bax and Bak were increased significantly (~160 and ~143% of control, respectively; Fig. 3A, lane 2 vs. 1; Fig. 3B, dark columns). Although the steady-state levels of

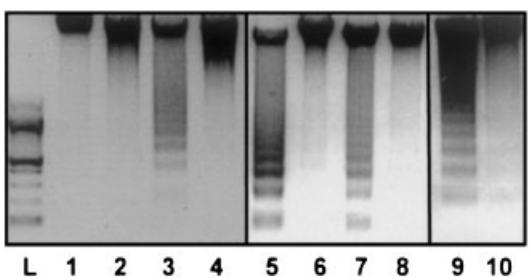

Fig. 1. Merosin-driven signaling in myogenic cell survival. Representative DNA laddering assays from differentiated myotube cultures of C2C12 cells (**lanes 1–4**), merosin-deficient (C2C12.B4/pLam) cells (**lanes 5**, **7**, and **8**), merosin-rescued (C2C12.B4/pMer) cells (**lane 6**), and merosin-deficient (C2C12.B4/pLam) cells stably transfected with the empty pBabe vector (C2C12.B4/pLam/pBabe; **lane 9**) or with pBabe containing the cDNA for Bcl-2 (C2C12.B4/pLam/pBcl-2; **lane 10**). Following differentiation, cultures were maintained 24 h serum-free (lanes 1, 5–7, 9, and 10) or with the addition of 20 μM SB202190 (or 20 μM SB203580; lanes 2 and 8), 1 μM herbimycin A (HA) (or 5 μM PP2; lane 3), or a combination of SB202190 and HA (lane 4), prior to DNA extraction. DNA (20 μg/lane) was isolated from treated cultures, then separated by agarose gel electrophoresis and stained with EtBr for the visualization of apoptosis/anoikis-associated internucleosomal DNA fragmentation (DNA laddering); **lane L**, 100-bp DNA size markers.

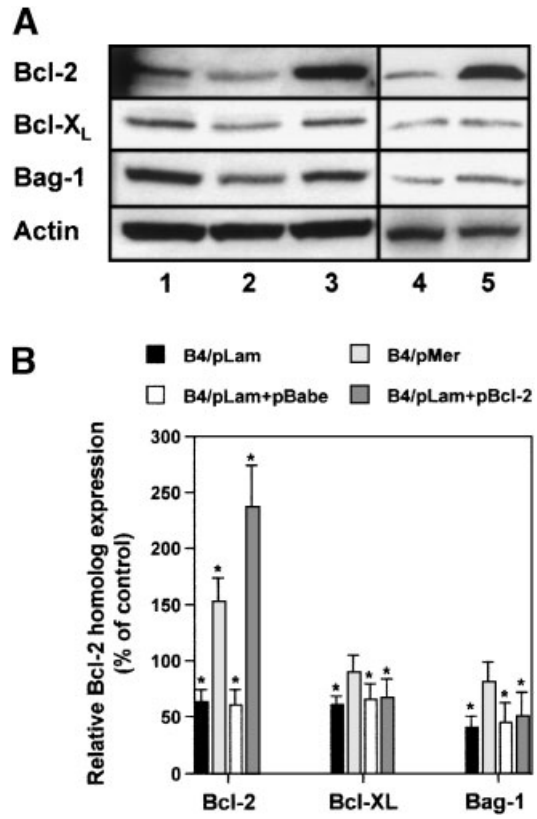

Fig. 2. Merosin deficiency and modulation of anti-apoptotic Bcl-2 homologs. **A**: Representative Western blot analyses of Bcl-2, Bcl-X_L, Bag-1, and actin expression levels from differentiated myotube cultures of C2C12 cells (control; **lane 1**), merosin-deficient (C2C12.B4/pLam) cells (**lane 2**), merosin-rescued (C2C12.B4/pMer) cells (**lane 3**), and merosin-deficient (C2C12.B4/pLam) cells stably transfected with the empty pBabe vector (C2C12.B4/pLam/pBabe; **lane 4**) or with pBabe containing the cDNA for Bcl-2 (C2C12.B4/pLam/pBcl-2; **lane 5**). Following differentiation, cultures were maintained 24 h serum-free prior to harvesting. Total proteins (50 μg/well) were separated by SDS–PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, and then probed with specific antibodies for the detection of Bcl-2 (~26 kDa), Bcl-X_L (~28–30 kDa), or Bag-1 (~32–34 kDa); detection of actin was used as a standard of protein quantities analyzed. **B**: Same as in (A), except that immunoreactive bands for Bcl-2, Bcl-X_L, and Bag-1 detected from merosin-deficient (C2C12.B4/pLam) myotubes (dark columns), merosin-rescued (C2C12.B4/pMer) myotubes (light-grey columns), and merosin-deficient (C2C12.B4/pLam) myotubes stably transfected with the empty pBabe vector (B4/pLam + pBabe; open columns) or with pBabe containing the cDNA for Bcl-2 (B4/pLam + pBcl-2; dark grey columns), were semi-quantified and compared to those of control (C2C12) myotubes, ×100 (expressed as ''% of control''). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control values are indicated by (*).

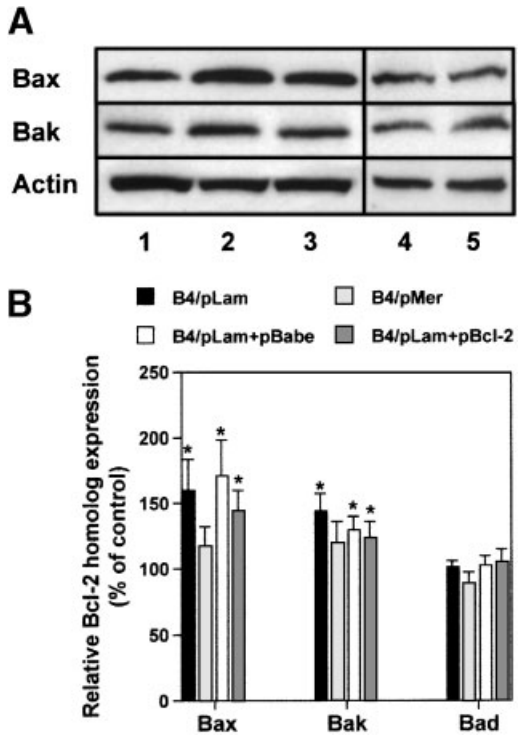

Fig. 3. Merosin deficiency and modulation of pro-apoptotic Bcl-2 homologs. **A**: Representative Western blot analyses of Bax, Bak, and actin expression levels from differentiated myotube cultures of C2C12 cells (control; **lane 1**), merosin-deficient (C2C12.B4/pLam) cells (**lane 2**), merosin-rescued (C2C12.B4/pMer) cells (**lane 3**), and merosin-deficient (C2C12.B4/pLam) cells stably transfected with the empty pBabe vector (C2C12.B4/pLam/pBabe; **lane 4**) or with pBabe containing the cDNA for Bcl-2 (C2C12.B4/pLam/pBcl-2; **lane 5**). Following differentiation, cultures were maintained 24 h serum-free prior to harvesting. Total proteins (50 μg/well) were separated by SDS–PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, and then probed with specific antibodies for the detection of Bax (~21 kDa), Bak (~25–28 kDa), or Bad (~28–32 kDa; see Fig. 4); detection of actin was used as a standard of protein quantities analyzed. **B**: Same as in (A), except that immunoreactive bands for Bax, Bak, and Bad detected from merosin-deficient (C2C12.B4/pLam) myotubes (dark columns), merosin-rescued (C2C12.B4/pMer) myotubes (light-grey columns), and merosin-deficient (C2C12.B4/pLam) myotubes stably transfected with the empty pBabe vector (B4/pLam + pBabe; open columns) or with pBabe containing the cDNA for Bcl-2 (B4/pLam + pBcl-2; dark grey columns), were semi-quantified and compared to those of control (C2C12) myotubes, ×100 (expressed as ''% of control''). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control values are indicated by (*).

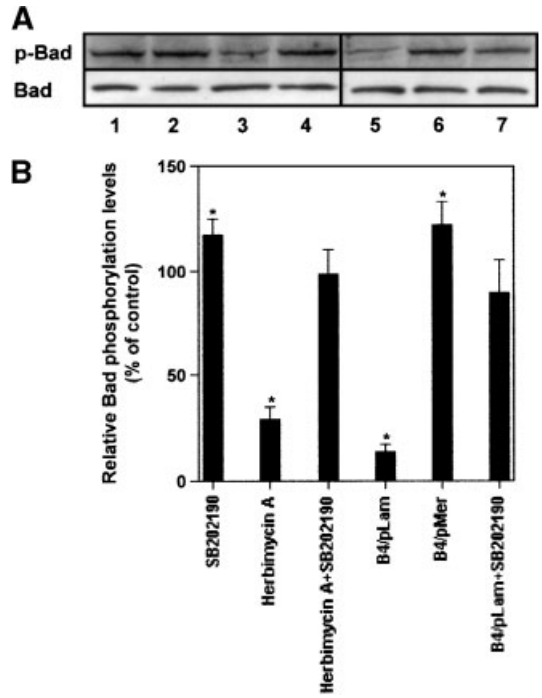

Fig. 4. Loss of merosin-driven survival signaling and modulation of Bad phosphorylation and expression. **A**: Representative Western blot analyses of Bad phosphorylation (p-Bad; **upper panel**) and protein expression (Bad; **lower panel**) levels from differentiated myotube cultures of C2C12 cells (**lanes 1–4**), merosin-deficient (B4/pLam) cells (**lanes 5** and **7**) and merosin-rescued (B4/pMer) cells (**lane 6**). Following differentiation, cultures were maintained 24 h serum-free (lanes 1, 5, and 6) or with the addition of 20 μM SB202190 (or 20 μM SB203580; lanes 2 and 7), 1 μM HA (or 5 μM PP2; lane 3), or a combination of SB202190 and HA (lane 4), prior to harvesting. Total proteins (50 μg/well) were separated by SDS–PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, and then probed with specific antibodies for the detection of Bad (~28–32 kDa) and its serine-112 phosphorylated form. **B**: Same as in (A), except that immunoreactive bands for p-Bad and Bad were semi-quantified in order to establish the relative Bad phosphorylation levels (p-Bad/total Bad), which were in turn compared to those of control/serum-free (C2C12) myotubes, ×100 (expressed as ''% of control''). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control values are indicated by (*).

Bad in merosin-deficient myotubes did not vary significantly from those of normal ones (Fig. 3B, dark column), its phosphorylation levels were drastically reduced (~14% of control; Fig. 4A, lane 5 vs. 1; Fig. 4B, B4/pLam). Thus, these observations indicate that merosin deficiency in myotubes results in a balance of expression and/or function that is clearly in favor of pro-apoptotic regulators (Table I).

The tyrosine kinase Fyn is the predominant Src family member expressed in myotube cultures [Laprise et al., 2002]. Furthermore, merosin deficiency and/or exposure of myotubes to HA (or PP2) results in the inhibition of Fyn activity [Laprise et al., 2002] and, consequently, in myotube anoikis (Fig. 1, lane 3 vs. 1; [Laprise et al., 2002]). Considering this involvement of Fyn in merosin-driven survival signaling, we

TABLE I. Complex Modulations of Bcl-2 Homolog Expression/Function by Merosin-Driven Survival Signaling in Skeletal Muscle Myotubes

		Homolog[c]					
Culture[a]	Anoikis[b]	Bcl-2	Bcl-X_L	Bag-1	Bax	Bak	pBad
Merosin-deficient	+++	↓	↓	↓	↑	↑	↓
Merosin-deficient/p38α-inhibited	− to ±	↑	↓	≈	↑	↑	≈
Merosin rescued	−	↑	≈	≈	≈	≈	↑
Fyn-inhibited	+++	↓	↓	↓	≈	≈	↓
Fyn/p38α-inhibited	− to ±	↑	↓	≈	≈	≈	≈
p38α-inhibited	−	↑	≈	≈	≈	≈	↑
Merosin-deficient/Bcl-2 rescued	− to ±	↑	↓	↓	↑	↑	↓

[a]Differentiated myotube cultures maintained 24 h in serum-free medium before analysis; merosin-deficient, C2C12.B4/pLam myotubes; merosin-deficient/p38α-inhibited, C2C12.B4/pLam myotubes exposed to SB202190 (or 203580); merosin rescued, C2C12.B4/pMer myotubes that overexpress merosin; Fyn-inhibited, normal C2C12 myotubes exposed to herbimycin A (HA) (or PP2); Fyn/p38α-inhibited, normal C2C12 myotubes exposed to a combination of HA (or PP2) and SB202190 (or 203580); p38α-inhibited, normal C2C12 myotubes exposed to SB202190 (or 203580); merosin-deficient/Bcl-2 rescued, C2C12.B4/pLam/pBcl-2 myotubes that overexpress Bcl-2.
[b]Qualitative assessment of anoikis-associated cell death in cultured myotubes analyzed, as observed from DNA laddering assays (see Fig. 1).
[c]Qualitative assessment of modulations in the expression (Bcl-2, Bcl-X_L, Bag-1, Bax, Bak) or phosphorylation levels (pBad) of Bcl-2 homologs (Bcl-2, Bcl-X_L, Bax, Bak, Bad) and associated molecules (Bag-1), in comparison to untreated/control C2C12 myotubes, as observed from Western blot analyses (see Figs. 2–6); ↓, significant down-modulation; ↑, significant up-regulation; ≈, no significant changes as compared to control cultures.

analyzed the expression/function of Bcl-2 homologs in C2C12 myotubes exposed to HA (or PP2) in comparison to untreated (control) myotubes. We found that such pharmacological inhibition of Fyn produced similar effects on Bcl-2 homologs as when Fyn is naturally inhibited within the context of merosin deficiency. Indeed, Fyn-inhibited myotubes exhibited significantly decreased Bcl-2, Bcl-X_L, and Bag-1 levels (~85, ~46, and ~65% of control, respectively; Fig. 5A, lane 2 vs. 1; Fig. 5B, dark columns), as well as decreased Bad phosphorylation (~29% of control; Fig. 4A, lane 3; Fig. 4B, HA). However, the steady-state protein levels of Bax, Bak, and Bad did not change significantly (Fig. 6A, lane 2; Fig. 6B, dark columns), in contrast to merosin-deficient myotubes (Fig. 3A, lane 2 vs. 1; Fig. 3B, dark columns). Nonetheless, the balance of expression/function of Bcl-2 homologs in Fyn-inhibited myotubes is clearly in favor of pro-apoptotic regulators, as in the case of merosin-deficient myotubes (Table I).

Inhibition of p38α Protects Against Anoikis by Restoring a Balance of Expression/Function in Favor of Anti-Apoptotic Regulators

We have shown previously that a deficiency in merosin and/or the inhibition of Fyn by pharmacological compounds induces myofiber anoikis through a transient stimulation of p38α [Laprise et al., 2002]. By contrast, the pharmacological inhibition of p38α (with SB202190 or 203580) protects Fyn-inhibited (Fig. 1, lane 4 vs. 3) and/or merosin-deficient (Fig. 1, lane 8 vs. 5) myotubes from anoikis [Laprise et al., 2002]. Considering this involvement of p38α in anoikis induced by loss of merosin-driven survival signaling, we analyzed the expression/function of Bcl-2 homologs in myotubes exposed to SB202190 (or 203580). Exposure of normal myotubes to either p38 inhibitors did not affect their survival status (Fig. 1, lane 2), as expected [Laprise et al., 2002]. Interestingly, the steady-state levels of Bcl-2 (~132% of control; Fig. 5A, lane 3; Fig. 5B, grey columns) and Bad phosphorylation (~117% of control; Fig. 4A, lane 2; Fig. 4B, SB202190) were increased, whereas the other molecules studied did not change significantly (Figs. 5 and 6). Similarly, treatment of Fyn-inhibited or merosin-deficient myotubes with either SB202190 or SB203580 resulted in an increase of Bcl-2 (Fig. 5A, lane 4; Fig. 5B, white column; Table I), as well as a restoration of Bag-1 (Fig. 5A, lane 4; Fig. 5B, white column; Table I) and Bad phosphorylation (Fig. 4A, lanes 4 and 7; Fig. 4B, HA + SB202190 and B4/pLam + SB202190; Table I) to control levels. However, Bcl-X_L levels were not restored to normal in Fyn-inhibited or merosin-deficient myotubes exposed to p38 inhibitors (Fig. 5A, lane 4; Fig. 5B, white column; Table I); furthermore, the inhibition of p38 failed to restore normal levels of Bax and Bak in merosin-deficient myotubes (not shown; Table I). Nonetheless, these results altogether show that the inhibition of p38α protects myotubes against

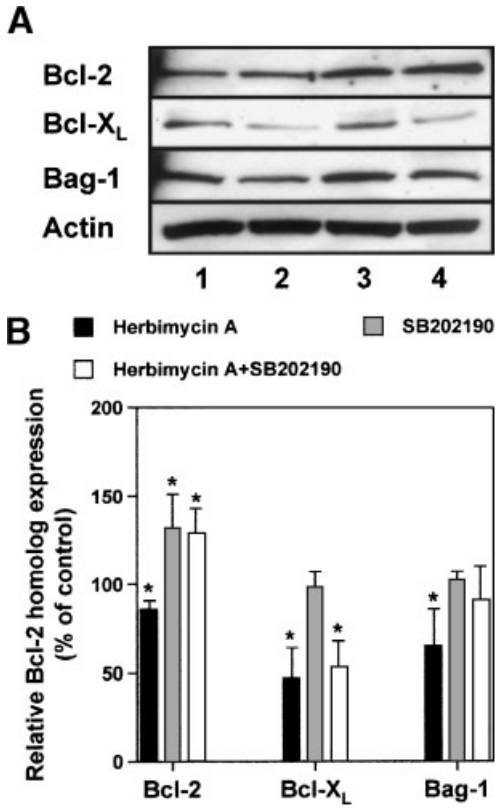

Fig. 5. Inhibition of merosin-driven survival signaling and modulation of anti-apoptotic Bcl-2 homologs. **A**: Representative Western blot analyses of Bcl-2, Bcl-X_L, Bag-1, and actin expression levels from differentiated myotube cultures of C2C12 cells (**lanes 1–4**). Following differentiation, cultures were maintained 24 h serum-free (control; lane 1) or with the addition of 1 μM HA (or 5 μM PP2; lane 2), 20 μM SB202190 (or 20 μM SB203580; lane 3), or a combination of SB202190 and HA (lane 4), prior to harvesting. Total proteins (50 μg/well) were separated by SDS–PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, and then probed with specific antibodies for the detection of Bcl-2 (~26 kDa), Bcl-X_L (~28–30 kDa), or Bag-1 (~32–34 kDa); detection of actin was used as a standard of protein quantities analyzed. **B**: Same as in (A), except that immunoreactive bands for Bcl-2, Bcl-X_L, and Bag-1 detected from myotubes exposed to HA (dark columns), SB202190 (light-grey columns), and to a combination of HA and SB202190 (open columns), were semi-quantified and compared to those of control/serum-free (C2C12) myotubes, ×100 (expressed as ''% of control''). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control values are indicated by (*).

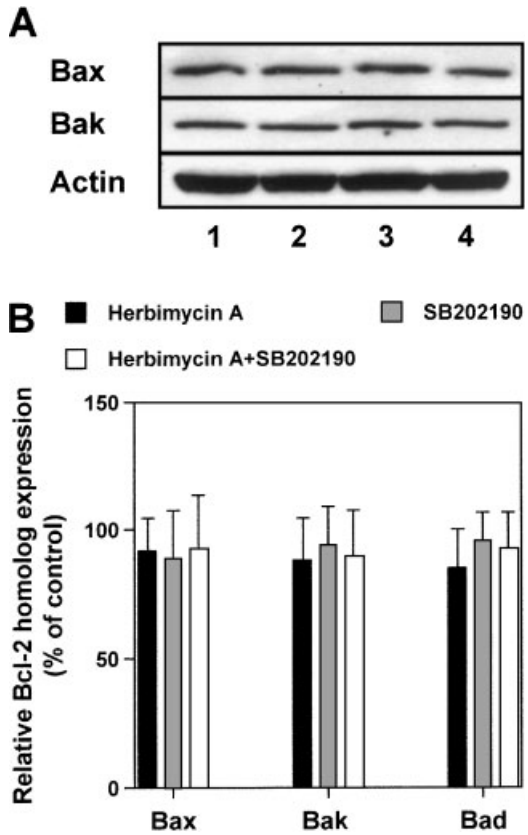

Fig. 6. Inhibition of merosin-driven survival signaling and modulation of pro-apoptotic Bcl-2 homologs. **A**: Representative Western blot analyses of Bax, Bak, and actin expression levels from differentiated myotube cultures of C2C12 cells (**lanes 1–4**). Following differentiation, cultures were maintained 24 h serum-free (control; lane 1) or with the addition of 1 μM HA (or 5 μM PP2; lane 2), 20 μM SB202190 (or 20 μM SB203580; lane 3), or a combination of SB202190 and HA (lane 4), prior to harvesting. Total proteins (50 μg/well) were separated by SDS–PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, and then probed with specific antibodies for the detection of Bax (~21 kDa), Bak (~25–28 kDa), or Bad (~28–32 kDa; see Fig. 4); detection of actin was used as a standard of protein quantities analyzed. **B**: Same as in (A), except that immunoreactive bands for Bax, Bak, and Bad detected from myotubes exposed to HA (dark columns), SB202190 (light-grey columns), and to a combination of HA and SB202190 (open columns), were semi-quantified and compared to those of control/serum-free (C2C12) myotubes, ×100 (expressed as ''% of control''). Statistically significant ($0.001 \leq P \leq 0.01$) differences with control values are indicated by (*).

anoikis induced by loss of merosin-driven signaling by restoring an overall balance of Bcl-2 homologs seemingly in favor of anti-apoptotic regulators (Table I). This in turn indicates that the stimulation of p38α during induction of myotube anoikis contributes to a balance of Bcl-2 homologs that ends up in favor of pro-apoptotic regulators.

Merosin Overexpression Rescues Merosin Deficiency by Restoring a Balance of Expression/Function in Favor of Anti-Apoptotic Regulators

We then performed the same analyses in merosin deficient myotubes that were stably transfected to overexpress merosin. As expected

[Vachon et al., 1996, 1997; Laprise et al., 2002], C2C12.B4/pMer myotubes are rescued from merosin deficiency and thus are fully viable like normal myotubes (Fig. 1, lane 6 vs. 1). We found that such rescue of viability by merosin overexpression resulted in a profile of Bcl-2 homolog expression/function similar to that observed for normal myotubes. Indeed, the levels of Bcl-X_L and Bag-1 were restored to normal (Fig. 2A, lane 3; Fig. 2B, light grey columns), while the levels of Bax and Bak were down-modulated as to not differ significantly from those of normal myotubes (Fig. 3A, lane 3 vs. 1; Fig. 3B, light grey columns). However, the expression of Bcl-2 (~153% of control; Fig. 2A, lane 3 vs. 1; Fig. 2B, light grey column) and the levels of Bad phosphorylation (~122% of control; Fig. 4A, lane 6 vs. 1; Fig. 4B, B4/pMer) were significantly increased above normal. Nonetheless, these observations show that the rescue of merosin deficiency and myotube viability by overexpression of merosin results in a restoration of a balance of expression/function that is in favor of anti-apoptotic regulators (Table I), thus corroborating our results from merosin-deficient and Fyn-inhibited myotubes.

Overexpression of Bcl-2 Is Sufficient to Protect Myotubes Against Anoikis Induced by Merosin Deficiency

We observed that merosin deficiency or the inhibition of Fyn result in a significant drop of Bcl-2 expression levels in myotubes (Table I). In contrast, the rescue of merosin deficiency by overexpression of merosin, as well as the inhibition of p38 in either normal, merosin-deficient, or Fyn-inhibited myotubes, result in increased steady-state levels for Bcl-2 (Table I). Consequently, we further analyzed the role of Bcl-2 as a protector against anoikis caused by loss of merosin-driven survival signaling. As expected [Vachon et al., 1997], C2C12.B4/pLam myotubes transfected stably to overexpress Bcl-2 (C2C12.B4/pLam/pBcl-2) show drastically reduced DNA laddering as opposed to C2C12.B4/pLam myotubes transfected with an empty expression vector (C2C12.B4/pLam/pBabe) (Fig. 1, lane 10 vs. 9). However, and with the obvious exception of Bcl-2 levels (~237% of control; Fig. 2A, lane 5 vs. 1; Fig. 2B, dark grey column), the steady-state levels of all other molecules analyzed (i.e., Bcl-X_L, Bag-1, Bax, Bak, Bad) in C2C12.B4/pLam/pBcl-2 myotubes remained similar to those observed in the merosin-deficient C2C12.B4/pLam/pBabe myotubes (Table I; Fig. 2A, lanes 5 vs. 4; Fig. 2B, dark grey columns vs. open columns; Fig. 3A, lanes 5 vs. 4; Fig. 3B, dark grey columns vs. open columns). Likewise, Bad phosphorylation levels were not restored in C2C12.B4/pLam/pBcl-2 myotubes (not shown; Table I) despite an obvious increase in survivability. Therefore, these results indicate that the overexpression of Bcl-2 in sufficient to protect myotubes against anoikis caused by loss of merosin-driven survival signaling, even without restoring a balance of expression and/or function in favor of anti-apoptotic molecules (Table I).

DISCUSSION

In the present study, we investigated the impact of merosin-driven survival signaling on the expression and/or function of five Bcl-2 homologs (Bcl-2, Bcl-X_L, Bax, Bak, Bad) and one non-homologous associated molecule (Bag-1), using the established C2C12 cell model of differentiated myotubes and its merosin- or laminin-deficient derivatives. Because the promotion of myotube survival by merosin is dependent on the activity of Fyn, whereas myofiber anoikis induced by merosin deficiency is dependent on p38α [Laprise et al., 2002], we also performed analyses in normal and merosin-deficient myotube cultures exposed to pharmacological inhibitors for Fyn and/or p38. Herein, we report that: (1) merosin deficiency induces anoikis and causes decreased Bcl-2, Bcl-X_L, and Bag-1 levels, increased Bax and Bak levels, and decreased Bad phosphorylation; (2) Bcl-2, Bcl-X_L, Bag-1, and Bad phosphorylation are also decreased in anoikis-dying, Fyn-inhibited myotubes; (3) the inhibition of p38α in Fyn-inhibited and/or merosin-deficient myotubes protects against anoikis and increases Bcl-2 levels above normal, in addition to restoring Bad phosphorylation and Bag-1 levels to normal; (4) the overexpression of merosin in deficient myotubes also rescues from anoikis and increases Bcl-2 levels and Bad phosphorylation above normal, in addition to restoring Bcl-X_L, Bag-1, Bax, and Bak levels to normal; and (5) Bcl-2 overexpression is sufficient to protect merosin-deficient myotubes from anoikis.

In recent years, it has become increasingly evident that the regulation of expression/function of individual Bcl-2 homologs can involve

numerous stimuli and pathways, each acting in synergy or independently, thus allowing for a complex and fine-tuned control of cell life and death [Zhang et al., 1995; Reed et al., 1996a,b; Anderson, 1997; Adams and Cory, 1998; Reed, 1998; Townsend et al., 1998; Boudreau and Jones, 1999; Scheid et al., 1999; Boucher et al., 2000; Cross et al., 2000; Danilkovitch et al., 2000; Gauthier et al., 2001a,b; Cory and Adams, 2002]. Numerous lines of evidence from the present study indicate that myofibers appear to be part of this rule as well: (1) the inhibition of Fyn affects all apoptotic regulators analyzed in a similar way as in the case of merosin deficiency, with the exception of Bax and Bak (Table I); (2) the inhibition of p38 in merosin-deficient myotubes fails to restore normal Bcl-X_L, Bax, and Bak levels, in addition to increasing Bcl-2 levels above normal (Table I); (3) the inhibition of p38 in Fyn-inhibited myotubes results in above-normal levels of Bcl-2 and fails to restore Bcl-X_L to normal levels (Table I); (4) the inhibition of basal p38 activity in normal myotubes results in the upregulation of Bcl-2 expression and Bad phosphorylation, without affecting the other apoptotic regulators analyzed (Table I); and (5) the overexpression of merosin in merosin-deficient myotubes restores normal levels of all apoptotic regulators analyzed, with the exception of Bcl-2 expression and Bad phosphorylation levels which become above normal (Table I). Hence, these data illustrate well the inherent complexity that is required in the regulation of the expression/function of Bcl-2 homologs. Furthermore, these also underline the potential implication of other, yet-to-be-identified signaling molecules in merosin-driven myofiber survival, aside from Fyn and p38 [Laprise et al., 2002].

It is noteworthy that the relationship between the balance of anti-apoptotic versus pro-apoptotic regulators and the resulting impact on myofiber survival was not always definite from some of the experiments performed herein. For instance, the inhibition of p38 in merosin-deficient myotubes resulted in an unclear balance of apoptotic regulators, despite its protective effect against anoikis (Table I). Considering that the normal regulation of cell survival implicates not just one homolog but rather complex interactions between numerous Bcl-2 homologs, as well as post-translational modifications/interactions to further modulate their functions [Reed et al., 1996a,b; Adams and Cory, 1998; Reed, 1998; Cory and Adams, 2002], such apparent discrepancies are, therefore, likely due to the additional involvement of homologs other than those analyzed herein (e.g., Bid, Bcl-w, Mcl-1, Bim, etc.) and/or other Bcl-2 homolog post-translational modification events (e.g., Bid phosphorylation/proteolytic activation, Bim sequestration, etc.) not focused upon in the present study. However, this in turn raises the question as to how the overexpression of a single Bcl-2 homolog can be sufficient to impact significantly on cell survival and/or death. Indeed, in addition to previous studies that have identified Bcl-2 as a potential major player in myofiber survival [Behrens et al., 1997; Vachon et al., 1997; Dominov et al., 1998], we have shown herein for the first time that the overexpression of Bcl-2 is enough to protect myotubes from anoikis induced by merosin deficiency, even if the expression/function of other apoptotic regulators were not restored to normal levels (see Table I). It is now becoming well acknowledged that the ability of any given anti- or pro-apoptotic Bcl-2 homolog to singly influence cell survival is largely dependent on the specific functions it performs normally within a cell [Adams and Cory, 1998; Reed, 1998; Cory and Adams, 2002]. Hence, because of Bcl-2's well known role as a 'mitochiondrial guardian' to inhibit Bax/Bak and prevent the activation of the apoptosome (and thus the initiator caspase caspase-9) [Adams and Cory, 1998; Reed, 1998; Cory and Adams, 2002], in addition to its increasingly evident ability to prevent the activation of other initiator caspases (e.g., caspase-2, -12) from the cytosol or the endoplasmic reticulum [Reed, 1998; Cory and Adams, 2002], it therefore follows that its overexpression alone can be sufficient to prevent apoptosis/anoikis in numerous cell types (this study; [Zhang et al., 1995; Reed et al., 1996a,b; Frisch and Ruoslahti, 1997; Vachon et al., 1997; Adams and Cory, 1998; Reed, 1998; Boudreau and Jones, 1999; Frisch and Screaton, 2001; Cory and Adams, 2002]).

In conclusion, the present findings provide further understanding of how merosin-driven signaling promotes myofiber survival. Additional studies will be required to better characterize the expression/function repertoire of Bcl-2 homologs in merosin-deficient, anoikis-dying myotubes, in comparison to normal ones. Likewise, more analyses of the involvement of

signaling pathways, as well as specific functions enacted by Bcl-2 homologs, should provide further insights into the complex regulatory mechanisms that govern myofiber cell survival. Ultimately, these could serve to identify putative pharmacological targets for inhibition or stimulation as potential treatments for degenerative muscular diseases, such as merosin-deficient congenital muscular dystrophy.

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