A Cdo–Bnip-2–Cdc42 signaling pathway regulates p38α/β MAPK activity and myogenic differentiation

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The p38α/β mitogen-activated protein kinase (MAPK) pathway promotes skeletal myogenesis, but the mechanisms by which it is activated during this process are unclear. During myoblast differentiation, the promyogenic cell surface receptor Cdo binds to the p38α/β pathway scaffold protein JLP and, via JLP, p38α/β itself. We report that Cdo also interacts with Bnip-2, a protein that binds the small guanosine triphosphatase (GTPase) Cdc42 and a negative regulator of Cdc42, Cdc42 GTPase-activating protein (GAP). Moreover, Bnip-2 and JLP are brought together through mutual interaction with Cdo. Gain- and loss-of-function experiments with myoblasts indicate that the Cdo–Bnip-2 interaction stimulates Cdc42 activity, which in turn promotes p38α/β activity and cell differentiation. These results reveal a previously unknown linkage between a cell surface receptor and downstream modulation of Cdc42 activity. Furthermore, interaction with multiple scaffold-type proteins is a distinctive mode of cell surface receptor signaling and provides one mechanism for specificity of p38α/β activation during cell differentiation.

Introduction

Differentiation of vertebrate skeletal myoblasts into multinucleated myofibers is a multistage process that involves the coordinated activation of a cell type–specific transcriptional program and morphological changes that include elongation, alignment, and cell–cell fusion (Pownall et al., 2002; Tapscott, 2005). Members of the myogenic bHLH family (MyoD, Myf5, myogenin, and MRF4) are lineage-specific transcription factors that direct the differentiation process in conjunction with nonskeletal muscle–specific transcription factors such as Mef2 (Pownall et al., 2002; Tapscott, 2005). The activity of such factors is under tight posttranslational control by signal transduction pathways, including the p38α/β MAPK pathway (LLuis et al., 2006).

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Abbreviations used in this paper: β-gal, β-galactosidase; DM, differentiation medium; GAP, GTPase-activating protein; GM, growth medium; MHC, myosin heavy chain.
The online version of this paper contains supplemental material.

p38α/β is activated during myogenic differentiation in vitro, and differentiation is blocked by chemical inhibitors of p38α/β (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000). p38α-null myoblasts are deficient in cell cycle arrest, expression of muscle-specific proteins, and myotube formation, and mice lacking p38α display delayed myofiber growth and maturation (Perdiguero et al., 2007). p38α/β directly phosphorylates several proteins that regulate myogenesis, including Met2 isoforms, the myogenic bHLH heterodimeric partner E47, the SWI–SNF chromatin remodeling complex subunit BAF60, and the RNA decay-promoting factor KSRP (Wu et al., 2000; Simone et al., 2004; Briata et al., 2005; Lluis et al., 2005).

RD rhabdomyosarcoma cells (cancer cells of the muscle lineage that have low differentiation capability) are deficient in p38α/β activity in response to differentiation-inducing culture conditions, and enforced p38α/β activation in these cells by expression of activated MKK6 (an immediate upstream activating
Cdc42 were expressed in different myoblast cell systems have produced contradictory results but, in general, both mutants interfered with myogenesis (Takano et al., 1998; Gallo et al., 1999; Meriane et al., 2000), suggesting that major perturbations in Cdc42 activity are not tolerated.

Like other small GTPases, Cdc42 cycles between an inactive GDP-bound state and an active GTP-bound state. The activity cycle is directly regulated by its interaction with stimulatory guanine nucleotide exchange factors and inhibitory GTPase-activating proteins (GAPs) and by interaction with additional proteins (Van Aelst and D’Souza-Schorey, 1997; Jaffe and Hall, 2005). A candidate regulator of Cdc42 is Bnip-2 (Low et al., 1999, 2000a,b). Bnip-2 is a 314-aa protein that harbors a single recognizable motif, a BCH domain that spans its C-terminal half. Cdc42GAP (also known as p50RhoGAP/ARHGAP1) contains a BCH domain in its noncatalytic N-terminal region, and Bnip-2 and Cdc42GAP interact via their respective BCH domains (Low et al., 1999, 2000b). The Bnip-2 BCH domain also binds Cdc42 itself (Low et al., 2000a). Transient expression of Bnip-2 in several cell types induces cellular elongation and membrane protrusions in a manner dependent on its ability to bind Cdc42 and on cellular Cdc42 activity (Zhou et al., 2005). That Bnip-2 binds Cdc42GAP, a negative regulator of Cdc42, yet has the ability to induce morphological alterations that require Cdc42 binding and Cdc42 activity, suggests that Bnip-2 might function as a scaffold for dynamic regulation of Cdc42 signaling. However, modulation of cellular Cdc42 activity by Bnip-2 has not been demonstrated.

Bnip-2 is a cell surface receptor of the Ig superfamily that promotes myogenesis in vivo and in vitro (Kang et al., 1998, 2003; Cole et al., 2004). Primary myoblasts from Cdo−/− mice and C2C12 myoblasts that express Cdo siRNA differentiate defectively in culture, producing reduced levels of muscle-specific kinesins and various types of cellular stress are activators of the p38a/b and other pathways (Zarubin and Han, 2005); however, treatment of RD cells with TNFα, sorbitol or UV light failed to rescue differentiation despite activation of p38a/b, revealing that differentiation- and stress-induced programs are distinct (Puri et al., 2000). Furthermore, the p38a/b pathway functions as a switch in muscle satellite cells, required initially to phosphorylate unidentified substrates that activate cell cycle entry and subsequently targeting substrates named in the previous paragraph to promote cell differentiation (Jones et al., 2005). Presumably, these distinct roles of p38a/b signaling require appropriate concentrations of p38a/b to become activated at specific times and subcellular locations. However, the spatiotemporal regulatory mechanisms by which reiteratively used signaling pathways (like p38a/b) achieve such specificity are, in most cases, unclear.

The Rho family of small GTPases regulates many biological processes, including cytoskeletal dynamics, cell polarity, signal transduction, and transcription (Van Aelst and D’Souza-Schorey, 1997; Jaffe and Hall, 2005). They are therefore well positioned to coordinate the changes in both gene expression and cell morphology that characterize cell differentiation. The role in vertebrate myogenesis of one such GTPase, Cdc42, is controversial. The concentration of active GTP-bound Cdc42 is relatively low in proliferating myoblasts and increases severalfold in differentiating cells (Travaglione et al., 2005). Studies in which constitutively active and dominant-negative mutants of Cdc42 interact with Bnip-2. (A) Yeast transformed with the indicated vectors for Gal4 DNA binding domain (BD) fused to the transmembrane (TM) plus intracellular regions (ICR) of Cdo or Necl-2 and Gal4 activation domain (AD) fused to Bnip-2 or Pal2 were plated on two-hybrid interaction-dependent selective medium. (B) Lysates of 293T cells transiently transfected with flag-tagged Bnip-2, Cdo, or control (−) expression vectors as indicated were immunoprecipitated (IP) with flag antibodies and then Western blotted with flag or Cdo antibodies. (C) Lysates of C2C12 cells cultured in GM to ~80% confluence and then transferred to DM for the indicated times were subjected to Western blot analysis with the indicated antibodies. (D) Lysates of C2C12 cells that were proliferating in GM (G) or transferred to DM for the indicated times were immunoprecipitated with Bnip-2 antibodies and then Western blotted with Cdo or Bnip-2 antibodies.
encoding Bnip-2 (Fig. 1 A). The intracellular region of another Ig protein, Necl-2, did not interact as efficiently with Bnip-2, nor did Cdo interact as efficiently with a Necl-2 binding protein, Pals-2 (Shingai et al., 2003; Takaesu et al., 2006). Consistent with their interaction in yeast, Cdo and flag epitope–tagged Bnip-2 coimmunoprecipitated when transiently expressed in 293T cells (Fig. 1 A). Like Cdo, Bnip-2 is expressed endogenously in C2C12 myoblasts, and Bnip-2 levels increased during a 4-d time course of differentiation (Fig. 1 C). On SDS-PAGE of C2C12 cell lysates, Bnip-2 migrated as a series of bands (Fig. 1, C and D) that likely represent alternatively spliced variously phosphorylated isoforms (Low et al., 1999; unpublished data).

To assess whether endogenous Cdo and Bnip-2 interact, C2C12 cells were harvested while proliferating in growth medium (GM) or over a 3-d time course after transfer to differentiation medium (DM). Cell lysates were immunoprecipitated with Bnip-2 antibodies and probed with Cdo antibodies (Fig. 1 D). Cdo coimmunoprecipitated with Bnip-2, with maximal interaction occurring over the first 2 d in DM, which is similar to Cdo’s interaction with JLP (Takaesu et al., 2006).

To identify the Cdo binding site in Bnip-2, a series of previously characterized flag-tagged Bnip-2 deletion mutants (Fig. 2 A; Low et al., 2000b; Zhou et al., 2005) were tested for their ability to coimmunoprecipitate Cdo in transiently transfected COS7 cells (Fig. 2 B). A construct comprising only the Bnip-2 BCH domain (aa 167–314) was as effective as full-length Bnip-2 in coimmunoprecipitating Cdo, but a construct lacking this domain failed to do so. Therefore, Bnip-2 binds to Cdo via its BCH domain. Boc is a protein that forms cis complexes with Cdo. Their respective ectodomains have strong sequence similarity but their intracellular regions are unrelated (Kang et al., 2002). Boc also coimmunoprecipitated with Bnip-2, but the interaction appeared less efficient and was only observed with full-length Bnip-2 (Fig. 2 B). A series of Bnip-2 mutants with small deletions in the BCH domain, which disrupt specific protein–protein interactions (Fig. 2 A; Low et al., 2000b; Zhou et al., 2005), was tested in an analogous manner. The deletion constructs Δ261–269, Δ264–284, and Δ285–292 each strongly diminished, but did not fully prevent, Cdo binding (Fig. 2 C). In contrast, Δ217–221, Δ235–239, and Δ251–263 bound Cdo normally, suggesting that a 31-aa region (261–292) near the C terminus of Bnip-2 encompasses the site of Cdo binding.

Bnip-2 positively regulates myogenic differentiation

To explore a role for Bnip-2 in myogenesis, C2C12 cells with enhanced or diminished Bnip-2 levels were constructed by stable expression of a Bnip-2 cDNA or Bnip-2 siRNAs, respectively. Forced expression of a Bnip-2 cDNA generally increased overall immunoreactivity of Bnip-2 bands about fourfold (Fig. 3 A). Overexpression of Bnip-2 did not significantly alter the morphology or proliferation of C2C12 cells cultured in GM (unpublished data). However, when triggered to differentiate, cells that overexpressed Bnip-2 (C2C12/Bnip-2 cells) displayed a greater fraction of cell nuclei in myosin heavy chain–positive (MHC+) myotubes than did vector control cells (Fig. 3 B and C). Furthermore, some of the myotubes formed by C2C12/Bnip-2 cells had much larger numbers of nuclei than were observed with the vector control cells (Fig. 3 C, arrow). When analyzed for DM-induced expression of muscle-specific proteins, C2C12/Bnip-2 cells showed accelerated and enhanced levels of the differentiation markers myogenin, MHC, and troponin T relative to the control cultures (Fig. 3 A).

To assess the effects of diminished Bnip-2 levels on differentiation, three independent Bnip-2 sequences placed into the pSilencer siRNA vector were individually and stably expressed in C2C12 cells. Each sequence substantially reduced Bnip-2 protein levels (Fig. 4 A). Note that the most slowly migrating band was more variably affected than the faster migrating forms in Fig. 4 A and in later figures. This could represent a more
stable species of Bnip-2 that is relatively resistant to RNAi-mediated knockdown or revelation of a protein nonspecifically recognized by the Bnip-2 antibody that comigrates with the top Bnip-2 band. C2C12 cells that expressed each siRNA sequence displayed a smaller percentage of cell nuclei in MHC+ myotubes, many (often most) of the nuclei in the myotube became positive for β-gal activity because the cytoplasmically translated protein diffuses within the myotube (Kang et al., 2004; Takaesu et al., 2006). Expression of wild-type Bnip-2 stimulated MHC expression and production of multinucleated myotubes by β-gal+ transfectants (Fig. 5, A and B). The Δ217–221 mutant functioned similarly to wild-type Bnip-2, indicating that interaction with Cdc42GAP is not required for Bnip-2’s promyogenic activity. In contrast, the Δ264–284 and Δ285–292 mutants lost the ability to promote myogenesis, suggesting that interaction with Cdc42 and/or Cdo are required for this activity (Fig. 5, A and B).

Cdo and Bnip-2 promote Cdc42 activity, and Cdc42 promotes myogenesis

The concentration of GTP-bound (active) Cdc42 rises, and is maintained, when C2C12 cells are transferred into DM (Fig. 6 A; Travaglione et al., 2005). We therefore tested whether modulation of Bnip-2 levels altered the amount of active Cdc42 in these cells. C2C12/Bnip-2 cells displayed elevated levels of GTP-bound Cdc42 relative to vector control cells in both GM and DM (Fig. 6 B), whereas stable expression of Bnip-2 siRNA largely prevented the DM-induced increase in GTP-bound Cdc42 (Fig. 6 C). The concentration of active Cdc42 therefore correlates with Bnip-2 protein levels.

Because Cdo binds Bnip-2 and Bnip-2 binds Cdc42, we asked whether Cdc42 was present in Cdo immunoprecipitates from differentiating C2C12 cell lysates. Indeed, Cdc42 coprecipitated with Cdo with a maximal interaction 1 d after transfer of cultures to DM (Fig. 6 D). This is similar to Cdo’s interaction with Bnip-2 and suggests that formation of a Cdo–Bnip-2–Cdc42 complex promotes Cdc42 activation. To test this possibility further, C2C12 cells that express Cdo siRNA and cultured Cdo−/− myoblasts were analyzed for Cdc42 activity. In C2C12 cells that express Cdo siRNA, the DM-associated elevation in GTP-bound Cdc42 levels seen in control transfectants was almost completely blunted (Fig. 6 E). Primary myoblasts must be cultured in GM plus basic FGF to remain in a proliferative nondifferentiated state, and both Cdo+/+ and Cdo−/− myoblasts had abundant GTP-bound Cdc42 in this medium, likely because of the added growth factor; however, when transferred to DM, the Cdo−/− cells had a lower concentration of active Cdc42 than did the Cdo+/+ cells (Fig. 6 F).

Both Cdo and Bnip-2 positively regulate myogenic differentiation and promote activation of Cdc42, suggesting that Cdc42 is itself promyogenic. However, previous studies in which constitutively active and dominant-negative mutants of Cdc42 were expressed in myoblasts failed to provide evidence for this notion, as both mutants blocked differentiation (Meriane et al., 2000). Expression of siRNA against Cdc42 in C2C12 cells also inhibited differentiation, but these cells acquired a strongly altered morphology (unpublished data). Therefore, to gain information on the role of Cdc42 in myogenesis, we sought to alter Cdc42 activity in a more subtle manner. It was reasoned...
To enhance Cdc42 activity, three independent siRNA sequences against Cdc42GAP were stably expressed in C2C12 cells. Two of these sequences effectively diminished Cdc42GAP protein levels, and this was associated with an increase in the amount of GTP-bound Cdc42 in these cells (Fig. 8, A and B). When cultured in DM, cells with diminished levels of Cdc42GAP produced larger myotubes (Fig. 8 C), had a higher percentage of nuclei present in MHC+ cells (Fig. 8 D), and expressed more MHC protein than did control transfectants (Fig. 8 E). Collectively, the results indicate that Cdc42 activity promotes myogenesis.

Cdo brings JLP and Bnip-2 together to regulate p38α/β activity

Cdc42 signaling activates p38α/β MAPK in several cell systems (Coso et al., 1995; Minden et al., 1995; Molnár et al., 1996). To test the hypothesis that Cdc42 activity promotes myogenesis through JLP/Bnip-2-mediated p38α/β activation, the concentration of GTP-bound Cdc42 and, perhaps more importantly, the amount of time Cdc42 proteins spend in the active state could be altered by modulating the amount of Cdc42GAP protein expressed by C2C12 cells. Two GAP proteins for Cdc42, Cdc42GAP and BPGAP (Shang et al., 2003), were each stably overexpressed in C2C12 cells (Fig. 7 A). In both cases, this resulted in lower steady-state levels of GTP-bound Cdc42 in both GM and DM, relative to control vector transfectants (Fig. 7 B). When cultured in DM, C2C12/Cdc42GAP cells and C2C12/BPGAP cells each displayed a similar phenotype: relative to control cells, a smaller percentage of cell nuclei were found in MHC+ myotubes, and the myotubes that formed were shorter and thinner (Fig. 7 C and D). Furthermore, overexpression of Cdc42GAP or BPGAP resulted in delayed induction of the differentiation markers myogenin and MHC (Fig. 7 E).

Figure 4. RNAi-mediated depletion of Bnip-2 reduces myogenic differentiation. (A) Lysates of C2C12 cells stably transfected with pSilencer containing one of three independent Bnip-2 siRNA sequences (designated 1, 3, and 4) or pSilencer containing an irrelevant sequence (−) were Western blotted with Bnip-2 or, as a control, pan-cadherin antibodies. (B) Photomicrographs of C2C12 cells that express Bnip-2 siRNA sequences or an irrelevant sequence (pSilencer) as indicated, cultured in DM, fixed, and stained with an antibody to MHC. Bar, 0.5 mm. (C) Quantification of myotube formation. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results. Asterisks indicate difference from pSilencer control, *P < 0.01. A level of myotube formation by control cells (~80% nuclei in MHC+ cells) was selected so as to permit visualization of diminished differentiation by Bnip-2 siRNA. (D) Western blot analysis of C2C12 cells that express Bnip-2 siRNA sequences (+) or an irrelevant sequence (−) cultured in GM (G) or in DM for the indicated times.

Figure 5. Bnip-2 deletion mutants defective in binding Cdc42 and Cdo do not promote myogenic differentiation. (A) C2C12 cells were cotransfected with control, Bnip-2 or Bnip-2 deletion mutant expression vectors, and pQ-lacZ (a vector that drives expression of nuclear-localized β-gal to mark transfectants). Differentiated cultures were double stained for β-gal activity (blue) and for MHC expression (brown). Bar, 0.1 mm. (B) Quantification of C2C12 cell differentiation shown in A. Cultures were scored as MHC− or MHC+, with MHC+ cells further scored as having a single (1) nucleus, two to four nuclei, or greater than or equal to five nuclei. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results.
Bnip-2 and Cdo regulate Cdc42 activity. (A) GTP-bound Cdc42 (Cdc42*) in C2C12 cells proliferating in GM (G) or cultured for 48 h in DM (D) was pulled down from cell lysates with GST-PAK-1 PBD beads and Western blotted with Cdc42 antibodies (top). Total levels of Cdc42 were determined by blotting straight lysates with Cdc42 antibodies (bottom). (B) Levels of GTP-bound and total Cdc42 in C2C12/Bnip-2 (+) or vector control (−) cells in GM or DM were as assessed as described in A. (C) Levels of GTP-bound and total Cdc42 in C2C12 cells that express Bnip-2 siRNA (+) or an irrelevant sequence (−) in GM or DM were assessed as described in A. (D) Lysates of C2C12 cells cultured in GM to ~80% confluence and then transferred to DM for the indicated times were immunoprecipitated with Cdo antibodies and then Western blotted with Cdc42 or Cdo antibodies. (E) Levels of GTP-bound and total Cdc42 in myoblasts of the indicated Cdo genotype cultured in GM or DM assessed as described in A.

1997; Bourdoulous et al., 1998). The ability of Cdo to bind Bnip-2 and JLP, which in turn bind Cdc42 and p38α/β, respectively, suggests that Cdo might coordinate Cdc42→p38α/β signaling. The possibility that Bnip-2 and JLP bind the same Cdo complexes was tested initially. COS7 cells were transfected with expression vectors encoding S epitope–tagged JLP (JLP-S), JLP-S and Bnip-2, or JLP-S, Bnip-2, and Cdo. Cell lysates were then precipitated with anti–S agarose and blotted with antibodies to each protein (Fig. 9 A). JLP coprecipitated Bnip-2 in the presence, but not the absence, of coexpressed Cdo. Therefore, JLP and Bnip-2 did not directly interact but were brought together by a mutual interaction with Cdo. To assess whether such complexes form endogenously, lysates of Cdo−/− and Cdo−/− myoblasts were immunoprecipitated with antibodies to Cdo or to Bnip-2 and then Western blotted with antibodies to Cdo, Bnip-2, and JLP. As expected, Bnip-2 and JLP both coprecipitated with Cdo (Fig. 9 B; Takaesu et al., 2006). Furthermore, JLP coprecipitated with Bnip-2 from Cdo−/− cell lysates but not Cdo−/− cell lysates, indicating that Bnip-2 and JLP associate in myoblasts in a Cdo-dependent manner (Fig. 9 C).

C2C12/Bnip-2 cells, which had elevated Cdc42 activity in GM and DM (Fig. 6), also had elevated levels of the dually phosphorylated (active) form of p38α/β (pp38α/β) in GM and DM (Fig. 9 D). Conversely, C2C12 cells that stably express Bnip-2 siRNA, which poorly activated Cdc42 in response to DM (Fig. 6), were also deficient in DM-induced pp38α/β production (Fig. 9 E). Furthermore, expression of Cdc42GAP siRNA increased pp38α/β levels in C2C12 cells in both GM and DM (Fig. 9 F). Collectively, these results are consistent with a model in which Cdo brings Bnip-2 and JLP together to coordinate Cdc42→p38α/β signaling.

To assess whether pp38α/β is functionally downstream of Bnip-2, we asked whether coexpression of the p38α/β activator MKK6(EE) could rescue the differentiation defect associated with expression of Bnip-2 siRNA. The transient C2C12 cell myogenesis assay described earlier (Fig. 5) was used in this case. Approximately ~83% of double control-vector transfectants were MHC+, and these cells were categorized as those that were mononucleolated (~35% of total cell nuclei), those that had between two and five nuclei (~45%), and those that had greater than five nuclei (5%; Fig. 9, G and H). The expression of Bnip-2 siRNA decreased the percentage of MHC+ cells to ~66%, with multinucleated cells representing only ~16%, none of which were in the greater-than-five-nuclei category. Therefore, transient expression of Bnip-2 siRNA reduced myogenesis, which is similar to stable knockdown of Bnip-2. Expression of MKK6(EE) alone enhanced myogenesis relative to control transfectants, resulting in ~94% MHC+ cells, with ~30% of the total having greater than five nuclei. Cells that coexpressed MKK6(EE) and Bnip-2 siRNA underwent robust myogenesis, though not quite to the level observed with expression of MKK6(EE) alone (~95% MHC+ cells, but only ~15% with greater than five nuclei). These results are consistent with the notion that Bnip-2’s promyogenic function is exerted mainly, though perhaps not exclusively, via activation of p38α/β.

We also assessed the effects of MKK6(EE) expression on cells that coexpressed the Bnip-2 deletion mutants A264–284 and A285–292. Similar to the results shown in Fig. 5, expression of either of these two mutants did not dramatically alter myogenesis relative to control transfectants, suggesting a loss of function (Fig. 9, G and H). Coexpression of MKK6(EE) enhanced differentiation of cells expressing these mutants, but the effect was seen more in the production of multinucleated MHC+ cells, with a smaller effect on the percentage of MHC+ versus MHC− cells. The results with the deletion mutants plus or minus MKK6(EE) are somewhat distinct from the effects seen with Bnip-2 siRNA plus or minus MKK6(EE) and suggest that these mutants may have a subtle inhibitory effect made apparent by coexpression of MKK6(EE).

Cdo/Bnip-2 signaling does not account for all p38α/β activity in differentiating myoblasts, and other p38α/β-activating stimuli function in its absence. C2C12 cells that express siRNA against Bnip-2 or Cdo, and Cdo−/− myoblasts, each display a partially defective differentiation program accompanied by lower than normal levels of DM-induced pp38α/β (Takaesu et al., 2006; this study). However,
myoblasts cultured in the presence of the p38α/β inhibitor SB203580 have a more dramatic blockade to differentiation (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000). It seems likely, therefore, that Cdo/Bnip-2–independent pathways also contribute to p38α/β activation during myogenesis. To examine this point more closely, C2C12 cells that stably express Bnip-2 or Cdo siRNA were treated with SB203580 and assessed for myotube formation and expression of MHC (Fig. 10, A–C). The percentages of control cell nuclei that were present in MHC+ cells was selected so as to permit visualization of diminished differentiation by Cdc42GAP proteins. (E) Western blot analysis of muscle-specific proteins by C2C12 cell transfectants cultured in GM or DM for the indicated times.

Discussion

Many ubiquitous signaling pathways perform cell type–specific functions. Furthermore, such pathways can regulate disparate functions in the same cell type. Clearly, this requires that the expression of Cdo/Bnip-2 is not 100%, ~40% of Cdo−/− myoblasts, which express no Cdo protein, are also MHC+. (Takaesu et al., 2006). These results suggest that other pathways that activate p38α/β are functional in Cdo- and Bnip-2–depleted myoblasts.

To assess directly whether additional stimuli can activate p38α/β in cells depleted of Cdo or Bnip-2, Cdo−/− myoblasts and C2C12 cells expressing Cdo or Bnip-2 siRNA were treated with TNFα or with hyperosmotic stress and analyzed for production of pp38α/β. Such cells produced pp38α/β at levels similar to those of control cells in response to these stimuli (Fig. 10, D–F). Collectively, the results suggest that Cdo/Bnip-2 signaling is neither the sole mechanism by which p38α/β becomes activated during myogenesis nor a general requirement for full p38α/β activation but is likely to be specific for differentiation-mediated signaling.
Figure 9. Bnip-2 and JLP bind the same Cdo complexes, and Bnip-2 regulates p38α/β activity to promote myogenesis. (A) Lysates of COS7 cells transiently transfected with JLP-S, Bnip-2, Cdo, or control (−) expression vectors were immunoprecipitated (IP) with anti-S agarose and then Western blotted with JLP, Bnip-2, or Cdo antibodies. (B) Lysates of myoblasts of the indicated Cdo genotype cultured in DM for 48 h were immunoprecipitated with Bnip-2 antibodies and then Western blotted with Cdo, Bnip-2, or JLP antibodies. Total cell lysates were also Western blotted with Bnip-2 or JLP antibodies. (C) Lysates of myoblasts of the indicated Cdo genotype cultured in DM for 48 h were immunoprecipitated with Bnip-2 antibodies and then Western blotted with Cdo, Bnip-2, or JLP antibodies. Total cell lysates were also Western blotted with Cdo or JLP antibodies. The Cdo band below the one indicated by the arrow is a partial degradation product. (D) Lysates of C2C12/Bnip-2 (+) or vector control (−) cells in GM or cultured for 48 h in DM were Western blotted with anti–phospho p38α/β (pp38) or p38α/β (p38) antibodies. (E) Lysates of C2C12 cells stably expressing a Bnip-2 siRNA sequence (+) or an irrelevant sequence (−) in GM or cultured for 48 h in DM were Western blotted with pp38 or p38 antibodies. (F) Lysates of C2C12 cells stably expressing a Cdc42GAP siRNA sequence (+) or an irrelevant sequence (−) in GM or cultured for 48 h in DM were Western blotted with pp38 or p38 antibodies. As the lanes were somewhat unevenly loaded in f, the pp38 and p38 signals were quantified by densitometry, and the pp38/p38 ratio reported under each lane in arbitrary units with the control transfectants in GM set to 1. (G) C2C12 cells were cotransfected with control, Bnip-2 siRNA, or Bnip-2 deletion mutant expression vectors, control or MKK6(EE) expression vectors, and pGlaCZ (a vector that drives expression of nuclear-localized β-gal to mark transfectants). Differentiated cultures were double-stained for β-gal activity (blue) and for MHC expression (brown). Bar, 0.1 mm. (H) Quantification of C2C12 cell differentiation shown in G. Cultures were scored as having a single (1) nucleus, two to five nuclei, or greater than five nuclei. Values represent means of triplicate determinations ± 1 SD. The experiment was repeated three times with similar results.

In this paper, it is demonstrated that Cdo also associates with the Cdc42 binding protein Bnip-2, identifying a novel linkage between a cell surface receptor and downstream modulation of Cdc42 activity. This interaction stimulates Cdc42 and p38α/β activities and regulates myogenic differentiation. Cdo, therefore, appears to promote activation of p38α/β by assembly of multiprotein signaling modules for Cdc42 and p38α/β via direct binding to scaffold proteins for each (i.e., Bnip-2 and JLP, respectively; Fig. 10G). JLP and Bnip-2 associate in a Cdo-dependent manner, implying that Cdc42 bound to Cdo via Bnip-2 activates p38α/β bound to Cdo via JLP and that this represents a pool of p38α/β specifically activated during differentiation. We speculate that binding of Bnip-2–Cdc42 to Cdo allows Cdc42 to interact with a specific guanine nucleotide exchange factor, promoting nucleotide exchange on Cdc42 and subsequent binding to effector proteins that initiate a kinase cascade resulting in activation of p38α/β. Alternatively, Cdc42 may be activated before association with Cdo, and subsequent interaction between Cdo, Bnip-2, and Cdc42 stabilizes Cdc42 in the active state and brings it into proximity with components of the p38α/β pathway.

All the described components of this complex are ubiquitously expressed except Cdo, which, though not muscle specific, is highly enriched in muscle precursor cells and differentiating muscle (Kang et al., 1998; Mulieri et al., 2000), suggesting that Cdo itself may provide some level of cell type specificity to this signaling pathway. Cdo is also expressed at high levels in neuronal precursors, and similar results to those reported here have been obtained in experiments on neuronal differentiation of a muscle cell line.
Active cycling of Cdc42 may be required for efficient myogenesis, as the results presented in this paper demonstrate that Cdc42 activity is important for this process, but expression of constitutively active or dominant-negative mutants of Cdc42 each block differentiation (Meriane et al., 2000). The ability of Cdc42 to trigger p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) activation fits well with the known importance of p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) in myogenic differentiation (Lluis et al., 2006). However, Cdc42 regulates additional processes that are also likely to be relevant to differentiation, such as the formation of filopodia, which may be required for cell–cell interactions in preparation for fusion, and regulation of cell polarity (Jaffe and Hall, 2005). That forced expression of the p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) activator MKK6(EE) has a somewhat greater effect on differentiation of control myoblasts than on myoblasts depleted of Bnip-2 is consistent with the notion that promyogenic pathways other than p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) lie downstream of Cdc42, but additional work will be required to establish that this is the case.

In addition to binding Cdc42, Bnip-2 binds its negative regulator, Cdc42GAP (Low et al., 1999). However, loss- and gain-of-function experiments in myoblasts indicate that Bnip-2 stimulates Cdc42 activity. These data suggest that Bnip-2 may function as a scaffold for dynamic signaling through Cdc42, modulating the balance or kinetics of its activity cycle. This is the first paper to find an endogenous function for Bnip-2, and we are unaware of another protein with this type of activity for Cdc42.

neural precursor line (unpublished observations). It is also clear, however, that not all p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) pathway activity during myoblast differentiation is Cdo- or Bnip-2-dependent, as residual pp38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) is detected in cells depleted for Cdo or Bnip-2, and treatment of such cells with the p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) inhibitor SB203580 further inhibits their differentiation. Potential additional mechanisms for activation of p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) in differentiating myoblasts include low-level autocrine TNF\(^{\text{H9251}}\) signaling and signaling by semaphorin 4C (Ko et al., 2005; Chen et al., 2007; Riuzzi et al., 2007; Wu et al., 2007).

In Figure 10, Other mechanisms of p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) activation occur in the absence Cdo/Bnip-2 signaling. (A) Photomicrographs of C2C12 cells stably transfected with control vector or vectors expressing Cdo or Bnip-2 siRNA that were cultured in DM plus SB203580 (+SB) or DMSO vehicle (−SB), fixed, and stained with an antibody to MHC. Bar, 0.1 mm. (B) Quantification of the percentage of nuclei in cultures shown in A that were in MHC\(^{\text{H9251}}\) or MHC\(^{\text{H9252}}\) cells. The experiment was repeated three times with similar results. Asterisks indicate difference from control vector, P < 0.01. (C) Western blot analysis of cultures shown in A. Extracts were probed with antibodies to MHC or total p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) (p38). (D) Myoblasts of the indicated Cdo genotype were treated with 0.9 M NaCl or 10 ng/ml TNF\(^{\text{H9251}}\) for 15 min and analyzed for pp38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) and p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\). (E) C2C12 cells stably expressing siRNA against Cdo were treated with NaCl or TNF\(^{\text{H9251}}\) and analyzed as in D. (F) C2C12 cells stably expressing siRNA against Bnip-2 were treated with NaCl or TNF\(^{\text{H9251}}\) and analyzed as in D. (G) Model of Cdo-mediated p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) activation during myogenic differentiation. Cdo binds directly to JLP and, via JLP, to p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\). Cdo also binds to Bnip-2 and, via Bnip-2, Cdc42. Formation of a Cdo–Bnip-2–Cdc42 complex promotes or stabilizes activation of Cdc42, which in turn triggers signals culminating in phosphorylation and activation of p38\(^{\text{H9251}}\)/H9252\(^{\text{H9252}}\) bound to Cdo via JLP. Cdo interacts with itself (Kang et al., 2003) and is shown as a dimer. JLP and Bnip-2 are shown as binding to different Cdo proteins of the dimer for convenience and does not preclude the possibility that JLP and Bnip-2 bind the same Cdo protein simultaneously. See text for additional details.
Collectively, these results reveal a distinctive mechanism of signaling: the Cdo intracellular region binds to scaffold proteins that in turn bind multiple components of specific pathways. This is different from other Ig/FNIII repeat receptors related to Cdo, such as the Robo proteins, which bind to the nonreceptor tyrosine kinase, c-Abl, the Rho family GAPs srGAP and Vile, the SH2/SH3 adaptor Nck, and the actin regulator Ena/Vasp (Dickson and Gilestro, 2006). Furthermore, Cdo functions in multiple contexts at the extracellular face of the cell surface. In myoblasts, it forms cis complexes with N-cadherin and neogenin, the latter a receptor for the netrin and RGM families of ligands (Kang et al., 2003, 2004; Cole et al., 2007). Additionally, Cdo binds Sonic hedgehog, possibly as a coreceptor with Patched1 (Tenzen et al., 2006; Yao et al., 2006; Martinelli and Fan, 2007). The mechanism by which binding of JLP and Bnip-2 to Cdo is induced during myogenesis is not clear, but a connection with N-cadherin is likely. Expression in C2C12 cells of a Cdo deletion mutant that is specifically deficient in its ability to bind N-cadherin blocks differentiation (Kang et al., 2003). N-cadherin–based adhesion enhances p38 activity in C2 myoblasts (Lovett et al., 2006), and preliminary results suggest that Cdo, JLP, and Bnip-2 can be recruited to sites of N-cadherin ligation (unpublished data). Cdo may function to link cadherin-based adhesion to the p38α/β pathway, which promotes the muscle-specific transcriptional program. That Cdc42, a known regulator of actin dynamics, is directly involved in this process provides a mechanism by which such changes in gene expression can be coordinated with the alterations in cell morphology that are also required for cell differentiation.

Materials and methods

Yeast two-hybrid screen

Bnip-2 was identified in a previously reported screen in which the trans-membrane plus intracellular region of mouse Cdo was used as bait (Takaesu et al., 2006). Two independent clones were isolated a total of eight times. Both clones started in the 5' UTR, were in frame, and encoded the entire Bnip-2 ORF.

Cell culture

C2C12, 293T, COS7, and myoblasts derived from Cdo−/− and Cdo+/− mice were cultured as previously described (Kang et al., 1998, 2004; Cole et al., 2004). To induce differentiation of C2C12 cells, cultures were transferred from DME containing 15% FBS (GM) to DME containing 2% horse serum (DM). Myotube formation in stable and transient assays was performed and quantified as previously described (Kang et al., 2004). Stable overexpression of Cdo in myoblasts, pX40 vectors encoding flag-tagged forms of Bnip-2, Cdc42GAP, or BPGAP (Low et al., 1999; 2000a,b) were cotransfected with pBabePuro (Morgenstern and Land, 1990) into C2C12 cells with FuGene6 (Roche), and cultures were selected in puromycin-containing medium. Drug-resistant cells were pooled and analyzed. Multiple such pools were studied in each case.

Where indicated, Cdo−/− and Cdo+/− myoblasts and C2C12 cell derivatives were treated with 10 ng/ml TNFα [Sigma-Aldrich] or 0.9 M NaCl for 15 min. C2C12 cell derivatives were treated with 2.5 μM SB203580 (EMD) in DM, replenished every 12 h, and harvested after 48 h for analysis.

For Cdo-Bnip-2 interaction studies, expression vectors encoding Cdo, myc-tagged Boc, Bnip-2, and Bnip-2 deletion mutants (Low et al., 2000b; Kang et al., 2002; Zhou et al., 2005) were transiently transfected into COS7 or 293T cells with FuGene6 or calcium phosphate, respectively. 48 h later, cells were harvested for Western blot and immunoprecipitation analyses as described in the next section.

Western blot, immunoprecipitation, and Cdc42 activity analyses

Western blot analyses were performed as previously described (Kang et al., 2004). For immunoprecipitations, cells were lysed in extraction buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 10 mM NaF, 2 mM DTT, 1 mM Na3VO4, and 0.5% Triton X-100 supplemented with one tablet/40 ml of Complete protease inhibitor cocktail [Roche]). 1 mg of whole cell extract from each sample was precleared with protein G-Sepharose (GE Healthcare) conjugated with 1 μg of normal rabbit IgG (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C, followed by immunoprecipitation with 1 μg of Cdo, flag epitope, or Bnip-2 antibodies for 2 h at 4°C. Immunoprecipitates were washed three times with, and suspended in, extraction buffer, and samples were analyzed by Western blotting. For S-agarose pulldown experiments, whole cell extracts were incubated with 20 μl of 50% slurry S-protein agarose beads (EMD) for 3.5 h at 4°C. Beads were washed three times with, and suspended in, extraction buffer, and samples were analyzed by Western blotting. Levels of GTP-bound Cdc42 were analyzed with the Cdc42 Activation Assay kit with PAK-1 B-DB-agarose (Millipore), according to the manufacturer’s instructions.

Antibodies used were the following: anti-Cdo (Invitrogen), anti-p38α/β (Sigma-Aldrich), anti-p38α/β (Cell Signaling Technology), anti-S-probe (Santa Cruz Biotechnology, Inc.), anti-MHC (MF-20; Developmental Studies Hybridoma Bank), anti-TnT1 (Sigma-Aldrich), antimyogenin (Santa Cruz Biotechnology, Inc.), anti-Cdc42 (Millipore), anti-Cdc42GAP (Abnova), anti-Bnip-2 (Low et al., 1999), anti-flag epitope (Sigma-Aldrich), anti-pan-cadherin (Sigma-Aldrich), anti-Boc, and anti-myc (9E10; Mount Sinai Hybriddoma Core Facility).

Microscopy

 Cultures were fixed and processed for MHC expression and/or β-gal activity as described in the previous sections and examined on a phase contrast microscope (Eclipse TS100; Nikon) with Plan Fluor 10x/0.3 NA and 20x/0.45 NA objectives [Nikon] at room temperature. Images were captured with a camera [Spot RT Color model 2.2.1; Diagnostic Instruments, Inc.] using Spot software (version 3.5.9; Diagnostic Instruments, Inc.) and Photoshop 7.0 [Adobe].

We thank Rishma Taneja, Jeanne Hirsch, and members of the Krauss laboratory for critical reading of the manuscript and David Glass for helpful discussions. This work was supported by grants from the National Institutes of Health (AR46207) and the T.J. Martell Foundation to R.S. Krauss, the Samsung Bio-medical Research Institute (B-A7-002) to J.S. Kang, and the Biomedical Research Council (Singapore R154000271305) to B.C. Low.

Submitted: 18 January 2008
Accepted: 7 July 2008

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