The GEF Bcr activates RhoA/MAL signaling to promote keratinocyte differentiation via desmoglein-1

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The stratification and differentiation of keratinocytes is a crucial process required for the maintenance and regeneration of healthy skin (Delva et al., 2009; Simpson et al., 2011). In keratinocytes destined for terminal differentiation, structural and signaling cues instruct cells to halt proliferation, transit into the superficial layers, and undergo transcriptional reprogramming to produce the structural and chemical products required for creating the epidermal barrier. Many of these cues arise from proteins involved in maintenance of cell–cell junctions. Whereas adherens junction proteins have been identified as key players in epidermal growth, polarity, and barrier formation (Tunggal et al., 2005; Müller et al., 2008; McCaffrey and Macara, 2011), desmosomal proteins have emerged as critical modulators of signaling pathways involved in differentiation (Green and Simpson, 2007; Thomason et al., 2010).

The desmosome is composed of the transmembrane cadherins (desmogleins, desmocollins), armadillo proteins (plakophilins and plakoglobin), and the cytoskeletal linker protein desmoplakin (DP). Desmosomal cadherins physically link cells together in the extracellular space (Dusek et al., 2007). Armadillo proteins serve as the bridge linking the cadherins to DP, but they also have several nonjunctional roles (Hatzfeld, 2007; Bass-Zubek et al., 2009; Wolf and Hatzfeld, 2010). In turn, DP is responsible for tethering the junctional complex to intermediate filaments (Hatsell and Cowin, 2001). Multiple desmosomal proteins such as desmoglein-1 (Dsg1), plakophilin-1 (PKP1), and DP are required for epidermal integrity, as evidenced by the cutaneous diseases caused by mutations in these proteins (McGrath et al., 1997; McGrath, 2005; Kottke et al., 2006; Amagai and Stanley, 2012). We have recently shown that Dsg1 is a crucial mediator of differentiation, as its loss reduces expression of multiple differentiation markers (Getsios et al., 2009).

Other studies have pointed to the importance of Rho GTPases in epidermal differentiation. Rho GTPases (RhoA, Rac1, and Cdc42) are multifunctional proteins that regulate many biological processes such as cell migration and morphogenesis (Etienne-Manneville and Hall, 2002; Burridge and Wennerberg, 2004; Jaffe and Hall, 2005). These proteins cycle between an active GTP-bound form and an inactive GDP-bound form.
Guanylate nucleotide exchange factors (GEFs) activate GTPases by catalyzing the exchange of GDP for GTP, whereas GTPase activating proteins (GAPs) inactivate GTPases by stimulating the intrinsic ability of GTPases to hydrolyze GTP (Moon and Zheng, 2003; Rossman et al., 2005). The increased focus on studying GEFs and GAPs in recent years has served to considerably increase our understanding on how Rho GTPase signaling is propagated during different biological processes (Rossman et al., 2005).

RhoA activates effector proteins such as Rho kinase (ROCK) and Dia, which promote actomyosin contractility and F-actin polymerization, respectively (Bishop and Hall, 2000). In addition, the transcription factor SRF (serum response factor) is activated by Rho GTPases and is responsible for expression of multiple different proteins (Posern and Treisman, 2006; Busche et al., 2008; Olson and Nordheim, 2010). The ability of Rho GTPases to regulate SRF-mediated transcription depends on a family of myocardin-related transcription factors (MRTFs), which include Myocardin, MAL/ MRTF-A, and MRTF-B. Rho-mediated F-actin polymerization drives MAL nuclear localization and consequent SRF-dependent transcription (Posern and Treisman, 2006; Olson and Nordheim, 2010). Rho GTPases and SRF have emerged as key signaling players in the process of epidermal differentiation. Blocking the Rho–ROCK pathway inhibits the differentiation of keratinocytes, and expression of active ROCK-II promotes differentiation (Sugai et al., 1992; McMullan et al., 2003).

SRF and MAL have also been shown to positively regulate epidermal differentiation in both animal and in vitro models (Koegele et al., 2009; Connelly et al., 2010; Verdoni et al., 2010; Luxenburg et al., 2011). In contrast to RhoA, Rac1 is required for maintenance of basal proliferating stem cell populations, and loss of Rac1 was shown to promote terminal differentiation (Benitah et al., 2005; Nikolova et al., 2008). Together these data suggest that activities of RhoA and Rac1 need to be dually coordinated for proper differentiation to occur. Unique among the GEF/GAP families are the proteins Bcr and Abr because they contain DH-PH domains capable of activating RhoA, as well as a RhoGAP domain, which selectively inactivates Rac/Cdc42 (Chuang et al., 1995; Vaughan et al., 2011). Although previous work on Bcr suggests that it predominantly functions as a Rac GAP in certain cell types (Cho et al., 2007; Oh et al., 2010), the ability of Bcr to regulate RhoA or Rac1 in keratinocytes has not been investigated.

In this study we demonstrate that Bcr silencing significantly reduces RhoA activity (and stress fibers/local adhesions) in keratinocytes, with relatively minor effects on global Rac1 or Cdc42 activity. Loss of Bcr decreased the ability of keratinocytes to differentiate, and also decreased MAL nuclear localization and SRF activity. We demonstrate that Dsg1 mRNA levels are reduced upon Bcr and MAL KD, and restoring Dsg1 expression rescues Bcr-induced differentiation defects. These data therefore highlight the importance of Dsg1 in coordinating the process of epidermal differentiation downstream of Bcr-induced RhoA/MAL signaling.

Results

**Bcr is required for maintaining RhoA activity in epidermal keratinocytes**

Previous work has suggested that Bcr functions mainly as a Rac GAP in certain cell types, but the net effect of Bcr on RhoA and Rac1 activity has not been determined in keratinocytes. We therefore used three different siRNA oligonucleotides specific for Bcr to knockdown (KD) the protein, each resulting in >80% reduction in protein levels (Fig. 1A). Bcr KD resulted in a significant decrease in RhoA activity in SCC9 cells (as tested by G-LISA assays), indicating that the DH domain of Bcr has functional exchange activity on RhoA in these cells (Fig. 1A). All three siRNAs demonstrated a reduction in RhoA activity, indicating that this effect is not due to nonspecific effects of a single siRNA. In contrast to RhoA, there was no significant change in global Rac1 activity upon KD of Bcr (Fig. 1B). The sensitivity of the G-LISA assay to detect changes in Rac1 activity in SCC9s was tested using EGF, which produced an ~1.8-fold increase in Rac activity (Fig. 1B).

To further examine a role for Bcr in regulating RhoA activity, we performed a calcium switch assay (Godsel et al., 2010). Control and Bcr KD SCC9s were placed in low calcium media for 12–16 h, followed by reintroduction of normal calcium media to trigger reformation of junctional complexes (Fig. 1C). RhoA activity was assayed using traditional GST-tagged Rhotekin Rho-binding domain (GST-RBD) pull-downs. As we have previously shown (Godsel et al., 2010), RhoA activity follows a bi-phasic pattern in control SCC9s after a calcium switch, where a transient activation at 5 min is followed by a decrease in activity by 15–30 min. In contrast to control cells, we observed a defect in the ability of Bcr KD cells to activate RhoA in response to calcium switch (Fig. 1C).

We next addressed whether Bcr functions similarly in normal human epidermal keratinocytes (NHEKs). Successful (>80%) KD of Bcr in NHEKs was obtained by a pool of oligonucleotides as described in Materials and methods. KD of Bcr significantly decreased RhoA activity in these cells in low calcium media, and at 1, 2, or 24 h after addition of high calcium media (Fig. 1D). Further, overexpression of wild-type (WT) Bcr increased RhoA activity in keratinocytes (Fig. 1E). Importantly, a previously described GEF-dead mutant of Bcr (NE/AA; Cho et al., 2007) was not able to activate RhoA (Fig. 1E). RhoB expression was not detected in NHEKs, and activity of RhoC was minimally affected upon KD of Bcr (Fig. 1F). Although a small but significant increase in Rac1 activity was observed, we did not detect any changes in Cdc42 activity in NHEKs upon loss of Bcr (Fig. 1, G and H). Collectively, these results suggest Bcr plays a major role as a RhoA GEF in human keratinocytes.

**Bcr regulates stress fibers and focal adhesion formation in epidermal keratinocytes**

Considering the effect of Bcr on RhoA activity, we next investigated whether Bcr can regulate the actin cytoskeleton in keratinocytes. In control NHEKs, calcium-induced junction formation...
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stimulated the formation of stress fibers, an effect that was markedly reduced in Bcr KD NHEKs (Fig. 2, A and B). Focal adhesion formation was also impaired in Bcr KD NHEKs, which demonstrated smaller and fewer focal adhesions compared with control NHEKs (Fig. 2 C). Actin cytoskeletal organization and focal adhesion formation was also visualized in SCC9 cells (Fig. 2, D and E). Compared with control cells, Bcr KD SCC9s demonstrated larger membrane protrusions (Fig. 2 D), and a significant decrease in the size and number of peripheral focal adhesions (Fig. 2 E, inset). The increase in membrane protrusion upon loss of Bcr may indicate localized changes in Rac1 activity, which was not detected in the G-LISA for global Rac1 activity. Loss of stress fibers is a common phenotype observed upon loss of RhoA activity (Chrzanoska-Wodnicka and Burridge, 1996). We confirmed that RhoA KD reduces stress fiber formation in human keratinocytes (Fig. S1 A). These data demonstrating a reduction in stress fiber and focal adhesion formation upon Bcr KD is therefore consistent with the effect of Bcr on RhoA activity.

Loss of Bcr impairs differentiation in submerged cultures of epidermal keratinocytes

Previous work has demonstrated that inhibition of all Rho isoforms with C3 causes a decrease in differentiation (McMullan et al., 2003). To extend this analysis, we specifically knocked down RhoA in NHEKs and placed the cells in high calcium-containing media (1.2 mM CaCl2) to induce differentiation (Hennings et al., 1980). An ~50% knockdown of RhoA resulted in reduced expression of multiple differentiation markers, such as the desmosomal cadherins Dsg1 and desmocollin-1 (Dsc1), and the cornified envelope protein loricrin (Fig. S1 C). Importantly, 50% knockdown of...
grown to confluence, placed in high calcium media, and samples were analyzed for differentiation markers at different days (day 0 indicates low calcium). Expression of Dsg1, Dsc1, and PKP1 was induced in control cells within 1–2 d of calcium induction, along with the suprabasal keratins 1 and 10. In contrast, Bcr KD cells exhibited reduced expression of all these differentiation markers (Fig. 3A). By day 4, expression of Dsg1 and Dsc1 had partially recovered, but expression of the cornified envelope protein loricrin was dramatically reduced in Bcr KD cells. These data therefore demonstrate a defect in the ability of NHEKs to differentiate upon Bcr KD (Fig. 3B).

RhoA did not perturb endogenous levels of other GTPases, as has been shown to occur in other cell types in response to competition for RhoGDI1 binding (Boulter et al., 2010). Nevertheless, when greater than 90% knockdown of RhoA was achieved, expression of both RhoC and Rac1 were indeed increased, as previous evidence would predict (Fig. S1D). Under these conditions, the specific effect of RhoA on differentiation is partially lost, likely a result of compensatory changes in expression of these other GTPases.

Considering the effect of Bcr on RhoA activity, we wanted to determine if loss of Bcr would also affect the ability of keratinocytes to differentiate. Control or Bcr KD NHEKs were grown to confluence, placed in high calcium media, and samples were analyzed for differentiation markers at different days (day 0 indicates low calcium). Expression of Dsg1, Dsc1, and PKP1 was induced in control cells within 1–2 d of calcium induction, along with the suprabasal keratins 1 and 10. In contrast, Bcr KD cells exhibited reduced expression of all these differentiation markers (Fig. 3A). By day 4, expression of Dsg1 and Dsc1 had partially recovered, but expression of the cornified envelope protein loricrin was dramatically reduced in Bcr KD cells. These data therefore demonstrate a defect in the ability of NHEKs to differentiate upon Bcr KD (Fig. 3B).
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Control and Bcr KD NHEKs were grown to confluence and switched to high calcium media for 1–4 d to induce differentiation. (A) Control and Bcr KD samples were blotted for Bcr, GAPDH, and a panel of differentiation markers including Dsg1, Dsc1, PKP1, keratin 10, and keratin 1. (B) Samples from lysates taken at 4 d after induction of differentiation were also lysed for Dsg1, Dsc1, loricrin, Bcr, E-cad, and GAPDH. Fold-change values over control quantified by densitometry are noted below blots. These data indicate that Bcr KD causes a decrease in expression of all differentiation markers tested. (C) Samples were also blotted for a range of proteins in the desmosome and adherens junction families, such as PKP2, DP, PG, E-cad, and β-catenin, indicating that expression of cell–cell adhesion proteins is not compromised upon loss of Bcr. All Western blots shown are representative of three independent experiments. (D) Control or Bcr KD NHEKs expressing WT CFP-tagged Bcr or a GEF-dead Bcr mutant (NE/AA) were grown to confluence, induced to differentiate, and samples blotted with Bcr, GFP, Dsc1, loricrin, and GAPDH antibodies [note: anti-Bcr [N-20] antibody does not recognize exogenous expression of CFP-Bcr likely due to interference of the N-terminal CFP tag with the antibody epitope which is at the N terminus of Bcr]. (E) Similar samples using GFP-tagged Bcr constructs were induced to differentiate on coverslips and stained for Dsc1. Re-expression of WT Bcr (but not the GEF-dead mutant) can rescue the loss of loricrin and Dsc1 expression seen upon Bcr KD.

Figure 3. Loss of Bcr causes a delay in the onset of differentiation markers in submerged cultures of epidermal keratinocytes. (A–C) Control and Bcr KD NHEKs were grown to confluence and switched to high calcium media for 1–4 d to induce differentiation. (A) Control and Bcr KD samples were blotted for Bcr, GAPDH, and a panel of differentiation markers including Dsg1, Dsc1, PKP1, keratin 10, and keratin 1. (B) Samples from lysates taken at 4 d after induction of differentiation were also lysed for Dsg1, Dsc1, loricrin, Bcr, E-cad, and GAPDH. Fold-change values over control quantified by densitometry are noted below blots. These data indicate that Bcr KD causes a decrease in expression of all differentiation markers tested. (C) Samples were also blotted for a range of proteins in the desmosome and adherens junction families, such as PKP2, DP, PG, E-cad, and β-catenin, indicating that expression of cell–cell adhesion proteins is not compromised upon loss of Bcr. All Western blots shown are representative of three independent experiments. (D) Control or Bcr KD NHEKs expressing WT CFP-tagged Bcr or a GEF-dead Bcr mutant (NE/AA) were grown to confluence, induced to differentiate, and samples blotted with Bcr, GFP, Dsc1, loricrin, and GAPDH antibodies [note: anti-Bcr [N-20] antibody does not recognize exogenous expression of CFP-Bcr likely due to interference of the N-terminal CFP tag with the antibody epitope which is at the N terminus of Bcr]. (E) Similar samples using GFP-tagged Bcr constructs were induced to differentiate on coverslips and stained for Dsc1. Re-expression of WT Bcr (but not the GEF-dead mutant) can rescue the loss of loricrin and Dsc1 expression seen upon Bcr KD.

Considering the effect of Bcr on expression of Dsg1 and Dsc1, we analyzed whether expression of other junctional proteins are affected by Bcr KD. Compared with control, KD of Bcr did not alter the expression patterns of PKP2, DP, PG, E-cad, or β-catenin (Fig. 3 C), and we have observed no change in the junctional localization of these proteins upon Bcr KD (Fig. S2). In particular, assembly of E-cad at cell–cell borders is not altered in Bcr KD cells (Fig. S2).

Interestingly, knockdown of the closely related protein Abr did not cause any changes in the expression of differentiation markers (Fig. S3). To confirm that the effects of Bcr KD are specific, we rescued Bcr expression using constructs containing siRNA-refractory silent mutations. Re-expression of WT Bcr was able to restore the loss of Dsc1 and loricrin expression seen upon Bcr KD, but the GEF-dead mutant of Bcr could not (Fig. 3, D and E). These data indicate that the GEF activity of Bcr is crucial for its effect on differentiation.

**Bcr deficiency impairs cellular organization and differentiation in an organotypic raft model**

To study differentiation in a more physiologically relevant setting, an organotypic model of human epidermis was used. Control and Bcr KD NHEKs were induced to undergo stratification in 3D by exposing them to an air–medium interface (Asselineau and Prunieras, 1984; Meyers and Laimins, 1994; Getsios et al., 2009). Consistent with data obtained from 2D cultures, loss of Bcr in rafts resulted in a decrease in expression of differentiation-specific proteins (Dsg1, Dsc1, keratin 10, loricrin, and involucrin), without any effect on other cell–cell adhesion proteins (E-cad and DP; Fig. 4 A). Analysis of the morphology of rafts by hematoxylin and eosin staining indicated that Bcr KD resulted in reduced granulation in the superficial layers and impaired stratum corneum development (Fig. 4 B). In addition, although localization of Dsg1 to cell–cell borders was slightly affected, border localization of Dsc1 was dramatically reduced (Fig. 4 C). Junctional localization of PG was marginally perturbed in the suprabasal layers, likely an effect of decreased Dsg1, PG’s primary binding partner in this region of the epidermis. The staining intensity of keratin 1 and loricrin is also reduced upon loss of Bcr (Fig. 4 C). The effects of Bcr on keratinocyte differentiation are not related to alterations in cell proliferation, as we did not observe significant changes in the number of Ki67-positive cells upon loss of Bcr (Fig. S4). Taken together, these data demonstrate that Bcr promotes the differentiation of keratinocytes in organotypic raft cultures.
expression of Dsg1, Dsc1, and keratin 1 without affecting levels of Bcr (Fig. 5A). As expected, we also observed a similar effect on differentiation upon KD of SRF (Fig. S5A). Analysis of MAL/SRF function was also performed using the drug CCG-1423, which is an inhibitor of MAL/SRF-dependent transcriptional signaling (Evelyn et al., 2007). A dose–response analysis revealed that higher doses of CCG-1423 demonstrated a selective inhibition of differentiation-specific proteins Dsg1, Dsc1, and keratin 1 without affecting levels of Bcr (Fig. 5A). As expected, we also observed a similar effect on differentiation upon KD of SRF (Fig. S5A). Analysis of MAL/SRF function was also performed using the drug CCG-1423, which is an inhibitor of MAL/SRF-dependent transcriptional signaling (Evelyn et al., 2007). A dose–response analysis revealed that higher doses of CCG-1423 demonstrated a selective inhibition of differentiation-specific proteins Dsg1, Dsc1,
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keratin 1, keratin 10, and loricrin, but not other adhesion molecules such as E-cad, DP, or PG (Fig. 5 B).

We next investigated whether loss of Bcr alters MAL localization in keratinocytes, as nuclear translocation of MAL occurs in response to Rho-mediated F-actin polymerization (Posern and Treisman, 2006; Olson and Nordheim, 2010). Compared with control, loss of Bcr caused a major defect in the ability of MAL to translocate to the nucleus upon a calcium switch (Fig. 5 C). To determine whether Bcr affects SRF activity in keratinocytes, we performed an SRE-luciferase assay, which measures the transcriptional ability of SRF to drive expression of the luciferase gene under control of a serum response element (SRE). KD of Bcr caused a significant reduction in SRF activity in differentiating keratinocytes at day 1 and 2 (Fig. 5 D). Like Bcr, RhoA KD also caused a decrease in SRF activity in keratinocytes (Fig. S1 B). Previous studies have established that loss of SRF in mouse epidermis results in decreased levels of junctional actin (Koegel et al., 2009; Luxenburg et al., 2011). Analysis of junctional actin staining in rafts indicated that Bcr KD also causes a significant decrease in the amount of junctional actin in suprabasal cells (Fig. 5 E). Taken together, these data demonstrate that loss of Bcr abrogates normal MAL/SRF signaling in keratinocytes, a process crucial for epidermal differentiation.

Loss of Bcr or MAL signaling decreases mRNA transcript levels of the differentiation modulator Dsg1

As shown above, loss of Bcr or MAL results in a reduction in protein levels of Dsg1, which has been shown to be required for proper
also resulted in decreased mRNA levels of all differentiation markers (Dsg1, Dsc1, keratin 1, keratin 10, involucrin, loricrin, filaggrin, and suprabasin), as well as other known SRF gene targets such as ActB, ActG1, and JunB (Fig. 6 D). Expression of the differentiation marker suprabasin was previously shown to be regulated by SRF (Park et al., 2002; Luxenburg et al., 2011). In addition, expression of JunB through MAL/SRF activity has been shown to be required for keratinocyte differentiation (Connelly et al., 2010).

Restoring Dsg1 expression is sufficient to rescue the differentiation defects seen upon loss of Bcr or MAL signaling

We have demonstrated that Bcr is required for differentiation and that Bcr and MAL can regulate mRNA levels of Dsg1. Considering the previously demonstrated importance of Dsg1 differentiation (Getsios et al., 2009), DSG1 was among 72 mRNAs identified as altered in an array analysis of SRF-null mouse keratinocytes, indicating that this gene is under regulation of SRF (Luxenburg et al., 2011). We therefore analyzed whether KD of Bcr or MAL regulates DSG1 mRNA levels. Control or Bcr KD NHEKs were induced to differentiate for 2 d, after which total RNA was collected and mRNA levels analyzed by quantitative real-time PCR (qPCR). Bcr KD caused a significant reduction in the levels of DSG1 mRNA, but not of plakoglobin (JUP; Fig. 6 A). In addition, either KD of MAL or treatment with the MAL transcriptional inhibitor CCG-1423 caused a significant decrease in DSG1 mRNA (but not JUP; Fig. 6, B and C). Loss of SRF also recapitulated these data (Fig. S5 B). These data demonstrate for the first time that both Bcr and MAL are required for DSG1 mRNA expression during the process of differentiation. Bcr KD in rafts
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Discussion

Rho GTPases have been established as important mediators of differentiation in keratinocytes, but little is known about specific Rho GEFs/GAPs that are responsible for coordinating Rho GTPase signaling during differentiation. We have identified the protein Bcr as a major regulator of RhoA activity, cytoskeletal organization, and differentiation in keratinocytes. We demonstrate further that loss of Bcr abrogates signaling through the MAL/SRF transcriptional nexus. KD of either Bcr or MAL reduces mRNA levels of the desmosomal cadherin Dsg1, restoration of which rescues the defects in differentiation seen upon loss of Bcr or MAL signaling. These data reveal the importance of Bcr in epidermal differentiation and establish the functional importance of a RhoA–SRF–Dsg1 pathway in this process.

Previous work has shown that RhoA activity increases in differentiating keratinocytes and is required along with its downstream effector PRK2 for maintenance of cell–cell adhesion...
RhoA (Zheng et al., 2006). In contrast, other studies in macrodemonstrating a dual ability to inactivate Rac1 and activate multiple GTPases, as evidenced from studies in fibroblasts (Chuang et al., 1995). Bcr therefore has the ability to regulate and Cdc42, but not RhoA (Heisterkamp et al., 1985, 1993; and Cdc42, and a GAP domain with intrinsic activity for Rac and its functional activity is regulated by changes in expression (Chardin, 2006). Rac1 expression was shown to increase upon differentiation, and loss of Rac1 expression in keratinocytes results in hyperproliferation and an increase in integrin-mediated adhesion, both of which result in decreased differentiation (Liebg et al., 2009). Other studies have shown that although ROCK II promotes terminal differentiation in HaCaT cells, ROCK I has anti-differentiation effects (Lock and Hotchin, 2009). Taken together, these studies indicate that Rho-mediated signaling specificity in differentiation is tightly regulated and might even play divergent roles at different points during the process of epidermal morphogenesis. How different GEFs or GAPs might control Rho signaling specificity during the process of differentiation is unknown.

Here we show that Bcr regulates the activity of RhoA in epidermal keratinocytes (Fig. 1). The protein Bcr was originally identified in a group of leukemia patients who carried the Philadelphia translocation, where the resulting Bcr–Abl fusion protein is an oncogene characterized as being responsible for different types of leukemia, especially chronic myelogenous leukemia (Daley et al., 1990). Previous work has demonstrated that Bcr and Abl also regulate inflammatory responses and vestibular morphogenesis in mice (Kaartinen et al., 2002; Cunnick et al., 2009).

Among the ~70 GEFs and 80 GAPs that have been identified for Rho GTPases, Bcr and Abl are unusual in having a GEF domain that can catalyze GTP exchange on RhoA, Rac, and Cdc42, and a GAP domain with intrinsic activity for Rac and Cdc42, but not RhoA (Heisterkamp et al., 1985, 1993; Chuang et al., 1995). Bcr therefore has the ability to regulate multiple GTPases, as evidenced from studies in fibroblasts demonstrating a dual ability to inactivate Rac1 and activate RhoA (Zheng et al., 2006). In contrast, other studies in macrophages and neurons have shown that Bcr seems to predominantly regulate Rac activity (Cho et al., 2007; Oh et al., 2010). To our knowledge, the functional activity of Bcr on different GTPases has not been studied in keratinocytes. Our analysis shows that whereas loss of Bcr dramatically reduces RhoA activity, it has a minimal effect on global Rac1 activity in keratinocytes, although local changes in Rac1 cannot be ruled out. These data raise the possibility that Rac1 activity in keratinocytes may be regulated by other GEFs or GAPs, potentially Abr. It is also possible that Bcr activity may be targeted specifically to RhoA via a yet undiscovered scaffold, as has been demonstrated in other cell systems (Jaffe et al., 2005; García-Mata and Burridge, 2007; García-Mata et al., 2007).

We show that loss of Bcr reduces expression of desmosomal cadherins (Dsg1 and Dsc1), suprabasal keratins (keratin 1 and keratin 10), and cornified envelope proteins (loricrin), indicating the importance of this protein for the process of differentiation (Figs. 3 and 4). Loss of Abr does not affect differentiation, suggesting that Abr is not involved in this process (Fig. S1). The ability of Bcr to regulate differentiation depends on its GEF activity, as a GEF-dead mutant of Bcr was unable to rescue the differentiation defect seen upon Bcr KD (Fig. 3). To determine the mechanism via which Bcr regulates differentiation, we investigated signaling pathways downstream of Rho known to be important for differentiation. Specifically, SRF and its cofactor MAL have been shown to be required for shape-induced differentiation of keratinocytes on micropatterned collagen islands (Connelly et al., 2010), and a major role for SRF has been established in development of mouse epidermis (Koegel et al., 2009; Verdoni et al., 2010; Luxenburg et al., 2011). We demonstrate that loss of Bcr abrogates the nuclear translocation of MAL, a process that is required for activation of SRF via Rho-mediated cytoskeletal rearrangements (Fig. 5). We further show that loss of Bcr reduces SRF activity in differentiating keratinocytes, as well as the amount of junctional actin, a commonly studied target of SRF-mediated transcriptional activity (Koegel et al., 2009; Luxenburg et al., 2011). We also confirmed that like Bcr, RhoA KD reduces both stress fibers and SRF activity in keratinocytes (Fig. S1). Taken together, these data indicate that loss of Bcr causes a decrease in MAL/SRF-mediated signaling.

A recent study revealed the desmosomal cadherin Dsg1 as being among the mRNA transcripts that differ between control and SRF-null mouse keratinocytes (Luxenburg et al., 2011). We previously demonstrated that in addition to being critical for intercellular adhesion in the superficial layers of the epidermis, Dsg1 also promotes differentiation, including expression of Dsc1, suprabasal keratins, and loricrin (Getsios et al., 2009). Here we show that KD of Bcr, MAL, or inhibition of MAL signaling using the drug CCG-1423 causes a reduction in mRNA levels of Dsg1, indicating that its expression is regulated upstream by Bcr and MAL (Fig. 6). Transcriptional control of Dsg1 has previously been shown to be regulated by two different transcription factors, p63 and grainyhead-like 1 (Wilanowski et al., 2008; Ferone et al., 2013). Whether MAL and/or SRF regulate Dsg1 transcription directly or in conjunction with these other transcription factors is an area that awaits further investigation.
Considering the importance of Dsg1 for the proper differentiation of keratinocytes, we hypothesized that Dsg1 may be a key intermediary in the regulation of differentiation by a Bcr/MAL signaling pathway. Indeed, restoration of Dsg1 expression was sufficient to rescue the differentiation defect seen upon KD of Bcr or treatment with CCG-1423 (Fig. 7). These data suggest that Bcr regulates differentiation partially through expression of Dsg1. The desmosomal cadherins are targets of auto-antibodies in pemphigus vulgaris and pemphigus foliaceus, which cause severe blistering of the skin in humans (Green and Simpson, 2007). Treatment of keratinocytes with pemphigus antibodies was shown to decrease RhoA activity, and reactivation of RhoA in pemphigus antibody–treated skin was shown to significantly rescue the blistering phenotype (Waschke et al., 2006). These studies demonstrate that RhoA activity can also be modulated by targeting desmogleins, suggesting the possibility of a feedback loop.

In summary, our study has implicated Bcr in the regulation of RhoA activity, MAL/SRF signaling, and epidermal differentiation in keratinocytes. These data highlight a novel pathway linking Bcr and MAL to Dsg1 expression during the process of differentiation. Taken together, these studies further our understanding of how Rho GTPase–mediated signaling pathways contribute to the process of epidermal differentiation.

Materials and methods

Growth and maintenance of cells
The SCC9 cell line (gift of L. Hudson, University of New Mexico, Albuquerque, NM) was maintained in DMEM/F12 medium (Corning) supplemented with 10% FBS (Atlanta Biologicals) and penicillin/streptomycin solution (Sigma-Aldrich). NHEKs were regularly obtained from the Northwestern University Skin Disease Research Center, where they are isolated from neonatal human foreskin as described in Halbert et al. (1992). In brief, foreskins (n = 3) were incubated overnight in 2.4 U/ml dispase (Roche) at 4°C to separate the epidermis from the dermis. Epidermal sheets are trypsinized, and keratinocytes released from the tissue by mechanical dispersion and passage through a 40-µm nylon sieve (BD). The cells were grown in medium 154 containing human keratinocyte growth supplement, gentamycin, and 0.07 mM CaCl2.

Calcium switch and induction of differentiation in submerged and organotypic raft cultures
Calcium switch experiments in SCC9s were performed by switching the cells to low calcium DMEM (0.05 mM CaCl2) for 4–16 h, followed by reintroduction of SCC9 growth media to induce formation of cell–cell junctions (Godosl et al., 2010). For NHEKs, analysis of cell-cell contact–induced cytoskeletal changes was performed by switching cells to high Ca2+ medium. Control for calcium switch experiments was sufficient to induce differentiation of submerged cultures. NHEKs were generated with collagen I (BD) and DMEM, and allowed to polymerize at 37°C. NHEKs are seeded on top of the collagen plugs, and 48 h later, lifted to an air–medium interface and grown for a period of 3–6 d.

DNA constructs, siRNA, and chemical reagents
The retroviral expression constructs LZRS-GFP and C-terminally tagged LZRS-Dsg1-Flag have been described in Getson et al. (2009). N-terminally tagged pECFP-Bcr (WT) and pECFP-Bcr (N689A/E690A) expression constructs were a gift of N. Heisterkamp (University of Southern California, Los Angeles, CA). siRNA refractory silent mutations were introduced into these constructs using the QuikChange mutagenesis kit (Agilent Technologies). The pLPCX retroviral construct for expression of Cterminally tagged HA-MAL was a gift from G. Posern (Max Planck Institute of Biochemistry, Martinsried, Germany). The adenoviral pAd-CMV-shRhoA knockdown construct (Aghajanian et al., 2009) was a gift from K. Burridge (University of North Carolina at Chapel Hill, Chapel Hill, NC). Control siRNA oligonucleotides and those specific for KD of Bcr were purchased from Invitrogen. A pool of three siRNA oligonucleotides were used for >80% KD of Bcr in NHEKs [target sequences: siBcr#1, 5′-CCCTCGAGGGTGGATCTTTGGGTAT-3′; siBcr#2, 5′-CTTGGCATACACCTGCAAAGTC-3′; siBcr#3, 5′-CTTACAGAAGTGTTTGAGAAGTCT-3′], whereas individual oligonucleotides were sufficient for KD of Bcr in SCC9 cells. KD of Abl in NHEKs was also performed using a pool of three siRNA oligonucleotides (target sequences: 5′-CAGAGGAGTGGCGATCTACAGGATA-3′; 5′-GAAGAGTGAAGTCTGCAACCCATGAC-3′; 5′-AACAACGTGTTGCAAACTACTCAT-3′). KD of SRF was performed with two different siRNA oligonucleotides (target sequences: siSRF#1, 5′-CCTGGCAGACCTGTCGATGCTG-3′; and siSRF#2, 5′-AAGGAGTGGCGATCTACAGGATA-3′), and the same was achieved for MAL (target sequences: siMAL#1, 5′-GGAACAGAATGTCTACAAAACGGAAAA-3′; and siMAL#2, 5′-CTAGATTGCATAGTGGCACTGCA-3′). siRNA delivery in SCC9s was achieved by transfection with DharmaFECT (Thermo Fisher Scientific) and by AMAXA nucleoporation (Lonza) in NHEKs, both according to the manufacturers’ instructions. EGFR (CN20) was purchased from Cytoskeleton, Inc. The MAL/SRF transcriptional inhibitor CCG-1423 was purchased from Cayman Chemical and used at the concentrations described during induction of differentiation in NHEKs with 1.2 mM Ca2+ medium 154.

Virus production and infection of keratinocytes
The phoenix packaging cell line was grown in DMEM supplemented with 10% FBS and penicillin/streptomycin solution. For production of GFF, Dsg1, and MAL HA retrovirus, phoenix cells were transfected with the appropriate DNA constructs and placed in 1 µg/ml puromycin selection media 48 h after transfection. After drug selection, phoenix cells were placed at 32°C for 16–24 h for collection of retroviral supernatant. Fresh supernatant with 4 µg/ml polybrene was then used to infect cells for 90 min at 32°C. After retroviral infection, cells were washed twice with 1 x PBS and returned to 37°C with fresh growth media. SRE-luciferase lentivirus was produced by the Northwestern University Skin Disease Research Center using a construct obtained from L. Shea (Northwestern University, Chicago, IL). Luciferase activity was measured using a standard luciferase reporter assay (Promega).

Quantitative real-time PCR
For measurement of mRNA transcript levels using quantitative real-time PCR, RNA was isolated using the RNeasy Mini kit (Qiagen), according to the manufacturer’s instructions. Total RNA concentrations were equalized between samples, and cDNA prepared using the Superscript III First Strand kit (Invitrogen). Quantitative PCR was performed using SYBR Green PCR master mix (Applied Biosystems) and gene-specific primers in a StepOnePlus instrument (Applied Biosystems). Calculations for relative mRNA levels were performed using the ΔΔCt method, normalized to GAPDH and represented as fold-change values compared with control siRNA samples. Statistical analysis was performed using a student two-tailed t test.

Western blotting
To analyze protein expression levels, cells from submerged or raft cultures were washed briefly in phosphate-buffered saline (PBS) and lysed in urea sample buffer (8 M deionized urea, 1% SDS, 10% glycerol, 60 mM Tris, pH 6.8, and 5% β-mercaptoethanol) and equalized for total protein concentration. Samples were subjected to SDS-PAGE on 7.5 or 15% polyacrylamide gels, followed by transfer to polyvinylidene fluoride (PVDF) or nitrocellulose membranes (EMD Millipore). Membranes were probed with specific primary and secondary antibodies (as described below) and visualized using enhanced chemiluminescence and x-ray film (Thermo Fisher Scientific). Western blots were quantified by standard densitometry analysis using ImageJ software (National Institutes of Health, Bethesda, MD). All Western blots shown are representative data obtained from three independent experiments.

Immunofluorescence and immunohistochemical analysis
For immunofluorescence, cells grown on coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Primary and secondary antibody incubations were performed at room temperature for 1 h, interspaced by multiple washes in PBS, and followed by mounting coverslips in polyvinyl alcohol (Sigma-Aldrich). Fixed cells were visualized with a microscope (model DM2; Leica) fitted with a 40x objective (PL APO, NA 1.32). Images were captured with an ORCA-100 CCD camera (model C4742-95; Hamamatsu Photonics) and MetaMorph 6.1 imaging software (Molecular Devices). Confocal imaging was performed at the Northwestern Center for Advanced Light Microscopy.
University Cell Imaging Facility. For quantification of stress fibers, cells were counted based on having either prominent or reduced/no stress fibers. More than 500 cells were counted from randomly imaged fields for each cell condition, and the data represented as percentage of the total cells counted on a bar graph. For quantification of MAL localization, cells from randomly imaged fields were scored for nuclear staining of ectopically expressed HA-tagged MAL, and the data represented as percentage of calculated.

Orogenotypic raft cultures were fixed in 10% neutral-buffered formalin and embedded in paraffin. Antigen retrieval for paraffin-embedded sections was performed by heating samples to 95°C in 0.01 M citrate buffer. Sections were blocked in 10% normal goat serum, and incubated with primary and secondary antibodies for 1 h at 37°C. After mounting in polyvinyl alcohol, sections were visualized as described above. Hematoxylin and eosin staining from paraffin-embedded sections was performed according to established protocols. Quantification of cortical actin staining and E-cadherin was performed by measuring fluorescence intensity per area in regions of interest formed by tracing cell–cell borders in randomly imaged fields. Statistical analysis was performed using a student t-test.

Antibodies
The following primary antibodies were used: 26C4/anti-RhoA, anti-RhoC, anti-Cdc42, anti-Bcr (N-20), anti-SRF, anti-MAL, PY99/anti-phosphotyrosine (Santa Cruz Biotechnology, Inc.), anti-Rac1, anti–β-catenin (BD), 278Z/anti-Dsg1, anti-GFP (Invitrogen), U100/anti-Des1 (RDI), 1407/anti-FG (Aves Laboratories), anti-α-tubulin, anti-Ki67 (EMD Millipore), anti–GAPDH, anti–HA (Sigma-Aldrich), anti-HC (AlkPhos, Roche) was incubated with glutathione Sepharose 4B beads (GE Healthcare) and absorbance measurements for GTPase activity were performed according to the manufacturer’s instructions (Cytoskeleton, Inc.), and absorbance measurements for GTPase activity were performed according to the manufacturer’s instructions (Cytoskeleton, Inc.), and absorbance measurements for GTPase activity were performed according to the manufacturer’s instructions (Cytoskeleton, Inc.).

Rho, Rac, and Cdc42 activity assays
Construction of the pGEX4T-1 prokaryotic expression construct containing Rhoetkin Rho-binding domain (GST-RBD) has been described previously (Liu and Burridge, 2000). Purification of GST-RBD and pull-down assays for active Rho GTPases were performed as described previously (Noren et al., 2000; Dubash et al., 2007). In brief, expression of the fusion proteins in E. coli cells was induced with 100 µM IPTG for 12–16 h at room temperature. Bacterial lysate collected in 50 mM Tris, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 1 mM DTT, and protease inhibitors (Complete; Roche) was incubated with glutathione Sepharose 4B beads (GE Healthcare) for 1 h at 4°C, followed by washes in lysis buffer. For GTPase pull-downs, cells were lysed in 50 mM Tris pH 7.6, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 mM MgCl₂, and protease inhibitors. lysates were clarified by centrifugation, equilized for total protein concentration, and incubated with 30–60 µg of GST-RBD for 30 min at 4°C. Bead pellets were washed in 50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl₂, and protease inhibitors, and further processed for SDS-PAGE and Western blotting. GLUSA assays for RhoA, Rac1, and Cdc42 activity were performed according to the manufacturer’s instructions (Cytoskeleton, Inc.), and absorbance measurements for GTPase activity was obtained using a Synergy 2 plate reader (Biotek Instruments, Inc.).

Online supplemental material
Fig. S1 shows defects in stress fiber formation, SRF activity, and keratinocyte differentiation upon RhoA KD. Fig. S2 highlights localization of Raf effectors upon RhoA KD. Fig. S3 demonstrates no change in differentiation upon Abr KD. Fig. S4 shows Ki67 staining upon Bcr KD. Fig. S5 indicates differentiation defects and loss of Dsg1 mRNA upon KD of Rho. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201304.133.DC1.

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