α6β4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-κB-dependent resistance to apoptosis in 3D mammary acini

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Summary
Malignant transformation and multidrug resistance are linked to resistance to apoptosis, yet the molecular mechanisms that mediate tumor survival remain poorly understood. Because the stroma can influence tumor behavior by regulating the tissue phenotype, we explored the role of extracellular matrix signaling and tissue organization in epithelial survival. We report that elevated (α6)β4 integrin-dependent Rac-Pak1 signaling supports resistance to apoptosis in mammary acini by permitting stress-dependent activation of the p65 subunit of NF-κB through Pak1. We found that inhibiting Pak1 through expression of N17Rac or PID compromises NF-κB activation and renders mammary acini sensitive to death, but that resistance to apoptosis could be restored to these structures by overexpressing wild-type NF-κB p65. We also observed that acini expressing elevated levels of Pak1 can activate p65 and survive death treatments, even in the absence of activated Rac, yet will die if activation of NF-κB is simultaneously inhibited through expression of IκBαM. Thus, mammary tissues can resist apoptotic stimuli by activating NF-κB through α6β4 integrin-dependent Rac-Pak1 signaling. Our data emphasize the importance of the extracellular matrix stroma in tissue survival and suggest that α6β4 integrin-dependent Rac stimulation of Pak1 could be an important mechanism mediating apoptosis-resistance in some breast tumors.

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Key words: α6β4 integrin, Rac, Pak1, NF-κB, Apoptosis resistance, Mammary epithelial cells

Introduction
Apoptosis is essential for tissue development and homeostasis and is required for efficient tumor therapy (Fernandez et al., 2002; Igney and Krammer, 2002; Zakeri and Lockshin, 2002). Aberrant regulation of cell death contributes to the pathogenesis of various diseases in adult tissues, including cancer and neurodegeneration. Moreover, defects in the regulation of apoptosis and the execution of cell death have been implicated in the pathogenesis of treatment-resistant tumors (Fulda and Debatin, 2004; Sethi et al., 1999; Zahir and Weaver, 2004). While we know much about the molecular machinery and events driving the execution of apoptosis at the cellular level, by comparison we know little about how cell-death decisions are controlled in three-dimensional (3D) tissues.

In vivo, epithelial cells are incorporated into 3D tissues that are typically quite resistant to pro-apoptotic treatments such gamma irradiation and chemotherapy (Barros et al., 1995; Bissell and Radisky, 2001; Unger and Weaver, 2003). Similar to tissues in vivo, mammary epithelial cells (MECs) embedded within a reconstituted basement membrane (rBM) assemble 3D tissue-like structures (acini) that are highly resistant to diverse death stimuli. In contrast to tissues in vivo and differentiated 3D mammary acini, non-differentiated MECs grown as two dimensional (2D) monolayers are comparatively sensitive to induction of apoptosis (Weaver et al., 2002). The increased death resistance displayed by 3D multicellular structures in vivo and in culture (acini and spheroids) has been linked to the enhanced cell-cell associations and altered cell-ECM interactions found in these structures (Bates et al., 1994; Hermiston and Gordon, 1995; Kirshner et al., 2003; Santini et al., 2000; Zahir and Weaver, 2004). Cell adhesion can promote the viability of cells grown as non-differentiated monolayers by changing the activity or expression of Bcl2 family-member dimers, or by increasing Erk, phosphoinositide 3-kinase (PI 3-kinase) and Akt signaling to inhibit mitochondria-dependent death, modify cellular metabolism and sustain cell proliferation (Matter and Ruoslahti, 2001; Plas and Thompson, 2002; Vander Heiden et al., 2001). Cell-ECM interactions and integrin engagement can also protect cells grown as
MECs resist death through β4 integrin, Rac and NF-κB

Monolayers from death stimuli by tempering death receptor signaling and altering cell-cycle dynamics (Dalton, 2003; Sethi et al., 1999). Interestingly, ECM-ligated epithelial cells incorporated into 3D tissue-like structures or spheroids are more resistant to death cues in comparison with epithelial cells interacting with the same ECM ligand in 2D where they grow as cellular monolayers (i.e. plated on top of the same ECM ligand) (Igney and Krammer, 2002). It is not clear whether the resistance phenotype of 3D spheroids is linked to previously characterized adhesion-dependent survival signaling or whether novel adhesion-regulated, death-resistance mechanisms become engaged in 3D spheroids.

Cells grown as 3D spheroids often exhibit reduced growth, altered cell-cycle regulation, hypoxia, compromised drug accessibility, increased matrix deposition and modified cell adhesion, and any one of these characteristics could account for the death-resistance phenotype of these structures (Kuh et al., 1999; Sminia et al., 2003; Tagliabue et al., 1998; Tannock et al., 2002). We found that α6β4 integrin and tissue polarity mediate resistance to apoptosis in 3D mammary acini in the absence of hypoxia, and irrespective of the growth status of the cells, by enhancing stress-dependent NF-κB activation (Weaver et al., 2002). The importance of α6β4 integrin-NF-κB signaling in resistance to apoptosis is underscored by the finding that aggressive and treatment-resistant solid tumors frequently show constitutive activation of NF-κB (Baldwin, 2001; Sovak et al., 1997), and NF-κB can protect epithelial cells in vivo from death induced through radiation treatment, stimulation of immune receptors and exposure to chemotherapy (Baeuerle and Baltimore, 1996; Baldwin, 2001). In addition, high-grade tumors with a poor clinical prognosis often express increased levels of α6β4 integrin and secrete abundant quantities of laminin-332 (Davis et al., 2001; Jones et al., 1997; Taylor-Papadimitriou et al., 1993), and elevated α6β4 integrin has been linked to tumor metastasis, which is a process that requires resistance to apoptosis (Lipscomb et al., 2005; Mercurio and Rabinovitz, 2001; Gupta and Massague, 2006). We showed that laminin-ligated α6β4 integrin can enhance anchorage-independent survival in 3D mammary spheroids by facilitating epidermal growth factor (EGF)-dependent activation of Rac and NF-κB (Zahir et al., 2003). Consistently, Rac levels and activity are often elevated in aggressive human epithelial tumors in vivo (Fritz et al., 1999), and Rac has been strongly implicated in tissue morphogenesis and polarity (O’Brien et al., 2001; Akhtar and Streuli, 2006). These findings implicate but do not prove that α6β4 integrin-Rac-NF-κB signaling mediates resistance to apoptosis in tumors. To explore this possibility, we asked whether α6β4 integrin-polarized 3D mammary acini resist diverse exogenous apoptosis stimuli through Rac-dependent NF-κB activation, and if so, how?

Using two non-malignant human MEC models and a reconstituted, laminin-rich rBM (Debnath et al., 2003; Johnson et al., 2007; Weaver et al., 1997), we grew MECs on top of rBM (2D) and compared their death-resistance behavior with that of mammary acini embedded within rBM (3D). We show that laminin-ligated α6β4 integrin confers resistance to apoptosis to 3D mammary acini by increasing the activity of Rac and p21-activated kinase 1 (Pak1 signaling) to enhance stress-induced NF-κB activation. Our findings emphasize the importance of matrix context in the regulation of cell death and implicate α6β4 integrin-dependent Rac-Pak1-NF-κB signaling in the pathogenesis of treatment-resistant tumors.

Results

α6β4 integrin regulates resistance to apoptosis and Rac activity

When compared with cells grown as 2D monolayers, epithelial cells assembled into 3D spheroids are more resistant to apoptosis to a degree that is similar to that exhibited by multidrug-, immune- and radiation-resistant tumors in vivo (Desozie and Jardillier, 2000). 3D spheroids are typically multilayered with a hypoxic core, and cells incorporated into these structures display growth patterns that appear to depend upon their location within the spheroid (reviewed in Zahir and Weaver, 2004; Santini et al., 2000). Accordingly, the increased survival behavior of 3D spheroids has been largely attributed to compromised drug penetration, altered cell-cycle dynamics or hypoxia (Kuh et al., 1999; Sminia et al., 2003; Tannock et al., 2002). By contrast, we found that rBM-generated 3D acini of S1 HMT-3522 and MCF10A non-malignant MECs, consisting of a single layer of non-hypoxic cells interacting directly with the rBM (Weaver et al., 2002) and proliferating at a rate similar to that of MECs ligated with rBM in 2D (monolayer; data not shown), are consistently more resistant to immune receptor stimuli such as Trail (Fig. 1A; top left and right), chemotherapy treatments including taxol (Fig. 1A; bottom left and right) and exposure to gamma irradiation (not shown).

We previously reported that the apoptosis-resistant phenotype of 3D mammary acini depends upon laminin-ligation of α6β4 integrin and NF-κB activation (data not shown) (Weaver et al., 2002). Laminin-ligated α6β4 integrin regulates Rac (Russell et al., 2003; Zahir et al., 2003), and Rac can promote anchorage-independent cell survival (Zahir et al., 2003; Jacquier et al., 2006). We found that the level of GTP-Rac is significantly elevated in BM-ligated 3D MEC acini in comparison with BM-ligated MECs grown as 2D monolayers, consistent with the idea that MECs incorporated into a 3D spheroid acquire death resistance through elevated adhesion-dependent survival signaling (Fig. 1B; quantified in 1C). We additionally observed that ectopic expression of a tail-less EGFP-tagged β4 integrin that competes with the endogenous wild-type β4 integrin to heterodimerize with α6 integrin at the membrane and that mediates laminin-dependent adhesion, but does not permit hemidesmosome formation nor α6β4 integrin-dependent signaling (Zahir et al., 2003; Spinardi et al., 1995), significantly reduces Rac activity in rBM-ligated 2D (data not shown) (Zahir et al., 2003) and 3D cultures of MECs (Fig. 1D; quantified in 1E). Because we demonstrated that expression of the tail-less β4 integrin compromises tissue polarity and renders 3D MEC acini sensitive to exogenous death stimuli (immune receptor and chemotherapy), and Rac can regulate tissue polarity (Akhtar and Streuli, 2006; O’Brien et al., 2001) and cell survival (Zahir et al., 2003), these observations indicate that the elevated laminin-α6β4-integrin-dependent Rac activity displayed by 3D mammary acini could contribute to their apoptosis-resistance phenotype.

Rac activity is necessary for apoptosis resistance

To examine the functional link between resistance to apoptosis and Rac in 3D mammary acini, we reduced GTP-Rac levels through stable retroviral expression of a dominant-negative
EGFP-tagged N17Rac (Fig. 2A). Consistent with the idea that elevated Rac activity contributes to apoptosis resistance, expression of N17Rac substantially reduced GTP-Rac levels (Fig. 2B; quantified in C) in MECs grown in monolayers and the activity of one of its effectors, Pak1, in 3D mammary acini (Fig. 2D; quantified in 2E). Reducing Rac and Pak activity also significantly sensitized the 3D mammary acini to apoptosis induced in response to immune-receptor ligation and chemo-reagent treatment (Trail and taxol-induced apoptosis; Fig. 2F).

Interestingly, although decreasing Rac activity sensitized the 3D mammary acini to death induction, N17 Rac expression did not appear to overly compromise tissue integrity (Fig. 3A; compare top row left phase-contrast image with middle image). N17Rac expression also did not appear to compromise tissue polarity (Fig. 3A; compare first column of confocal images with second column), as illustrated by basally localized β4 integrin, basal deposition of laminin-332 and collagen IV, and maintenance of cellular adhesions, as indicated by cell-cell-localized scribble (Fig. 3A; left) and colocalized E cadherin.

Fig. 1. Tissue differentiation is associated with an increase in (α6)β4 integrin-dependent Rac activity and enhanced resistance to apoptosis. (A) Dose-response curves showing that nonmalignant HMT-3522 S1 and MCF10A MECs acquire resistance to apoptosis induced by chemotherapeutics including taxol and immune receptor activators such as Trail following their rBM-induced differentiation into 3D polarized acini. The percentage apoptosis was calculated by scoring the number of activated caspase-3-positive cells 48 hours after treatment divided by the total cell number. MECs were either plated on top of a 1:100 diluted rBM for 48-96 hours (2D) or differentiated by embedment within rBM for 10-12 days (3D) followed by exposure to increasing doses of apoptotic stimuli.

(B) Representative immunoblot of immunoprecipitated Pak-associated Rac (GTP-Rac), total Rac (Rac) and E-cadherin in MECs plated either on top (2D) as monolayers or within (3D) rBM to assemble acini. The data indicate that total Rac decreases noticeably following rBM-induced differentiation, whereas GTP-loaded Rac increases dramatically. (C) Average relative specific activity of Rac in 3D mammary acini calculated by densitometric analysis of immunoblots of GTP-Rac divided by total cellular Rac following E-cadherin normalization, as shown in B.

(D) Representative immunoblot of GTP-Rac, total Rac (Rac) and E-cadherin in vector control and tail-less β4 integrin (β4Δcyto) 3D mammary acini grown within rBM (10-14 days). The data illustrate that Rac activity diminishes significantly in mammary acini that express the signaling-defective tail-less β4 integrin. (E) Average relative specific activity of Rac in control mammary acini versus mammary tissues expressing the tail-less β4 integrin calculated as above in C and shown in D. Results are the mean±s.e.m. of three to five separate experiments. *P<0.05; **P<0.01; ***P<0.001.
and β-catenin (data not shown). Because tissue polarity has been linked to resistance to apoptosis in mammary tissues (Weaver et al., 2002), and Rac has been strongly implicated in mammary morphogenesis (Akhtar and Streuli, 2006) and polarity (O’Brien et al., 2001), we further explored the effect of Rac on tissue polarity. First, we assessed the efficiency of exogenously expressed N17Rac in reducing GTP-Rac levels in MECs grown as 3D acini in comparison with MECs grown as 2D monolayers. N17Rac effectively reduced GTP-Rac by greater than 80% in MECs grown as 2D rBM-ligated monolayers (Fig. 2B; quantified in 2C), whereas, somewhat surprisingly, despite similar expression of the transgene, N17Rac only reduced Rac activity in 3D MEC acini by 40% (Fig. 3B; quantified in 3C). Nevertheless, a 40% reduction in GTP-Rac substantially reduced Pak1 activity and was sufficient to sensitize 3D MEC acini to death stimuli including Trail and taxol (Fig. 2F), even in the absence of an effect on tissue polarity (Fig. 3A). These data imply that, while Rac

![Fig. 2. Rac activity is necessary for resistance to apoptosis in 3D mammary acini. (A) FACS analysis showing increased EGFP expression in MECs expressing the EGFP-tagged N17 Rac (P4) in comparison with vector control MECs (P3). (B) Representative immunoblot of GTP-Rac, Rac and E-cadherin in vector control MECs grown as 2D monolayers in comparison with MECs expressing EGFP-tagged N17 Rac. The data illustrate that N17Rac significantly reduces GTP-Rac levels in MECs. (C) Average relative specific activity of Rac in MECs calculated by densitometric analysis of immunoblots of GTP-Rac divided by total cellular Rac following E-cadherin normalization of data illustrated in B. (D) Representative immunoblot of phospho-Pak1 and total Pak1 in 2D monolayer cultures of control MECs and MECs expressing EGFP-tagged N17Rac demonstrating how loss of Rac activity also reduces Pak1 activity. (E) Bar graph depicting the average degree of reduction of Pak1 activity in MECs expressing EGFP-N17Rac in comparison with control MECs. (F) Dose-response curves of the percentage apoptosis, as determined by calculating the number of activated caspase-3-positive cells divided by the total cell number, showing how 3D rBM polarized mammary acini with reduced Rac activity are now more sensitive to both chemotherapeutic (taxol) and receptor-mediated (Trail) apoptotic stimuli. Results are the means±s.e.m. of three to five separate experiments. *P<0.05 (C,E,F); **P<0.01 (F).]
mediates resistance to apoptosis of 3D spheroids, it apparently does so through a mechanism independent from that controlling tissue polarity.

Rac elicits its cellular actions by activating a plethora of downstream effectors, each with different activation and turnover dynamics. Accordingly, we reasoned that the Rac-effector pathways driving survival might be distinct from those that mediate tissue polarity. To test this possibility, we treated pre-formed death-resistant acini with pharmacologic inhibitors of RhoGTPase, including the general inhibitor toxin A and the

Fig. 3. Rac-mediates resistance to apoptosis and changes in tissue polarity through distinct mechanisms. (A) (top) Phase-contrast and confocal immunofluorescence images of colonies of control mammary acini (left panel; control), acini expressing EGFP-tagged N17Rac (middle panel; N17Rac) and acini treated with the Rac1 inhibitor NSC23766 (right panel; NSC23766) stained for β4 integrin, laminin-332, collagen IV and scribble (red). The images show that expression of N17Rac affects neither tissue integrity (compare regions highlighted by arrows in control phase-contrast images of acini with regions highlighted by arrows in images of N17Rac-expressing acini; top) nor tissue polarity (lower images; evidenced by similar basally localized β4 integrin; deposition of laminin-332 and collagen IV and intact cell-cell-localized scribble; compare images of control acini with images of acini expressing N17Rac). However, treatment of acini with the Rac inhibitor NSC23766 disrupted the integrity of acini (compare regions of image highlighted by arrows in phase-contrast images of control with images of NSC23766-treated acini) and severely perturbed tissue polarity, as evidenced by disturbed localization of β4 integrin, laminin-332, collagen IV and scribble (compare images of control with images of NSC23766-treated acini). Bar, 50 μm. (B) Representative immunoblot of GTP-Rac, Rac and E-cadherin in vector control MECs grown as 3D acini in comparison to MECs expressing EGFP-tagged N17 Rac and control acini treated with the specific Rac inhibitor NSC23766. The data illustrate that while N17Rac partially reduces GTP-Rac levels in 3D mammary acini, treatment with the Rac inhibitor decreases Rac activity to barely detectable levels. (C) Average relative specific activity of Rac in MECs calculated by densitometric analysis of immunoblots of GTP-Rac divided by total cellular Rac following E-cadherin normalization of data illustrated in B. (D) Bar graphs illustrating increased percentage of apoptotic cells induced by treatment with Trail (1 μg/ml) (left) or Taxol (40 μM) right in 3D rBM differentiated MECs with significantly reduced Rac activity mediated by treatment with the specific Rac inhibitor NSC23766. The percentage apoptosis was calculated by scoring the number of activated caspase-3-positive MECs divided by the total number of MECs 24 hours following treatment with Trail or Taxol. Results are the mean ± s.e.m. of three to five separate experiments. *P<0.05; **P<0.01.
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specific Rac inhibitor NSC23766. We found that directly inhibiting Rac activity with toxin A (see supplementary material Fig. S1) or with NSC23766 (Fig. 3B; quantified in 3C) substantially sensitized 3D mammary acini to induction of death induced through either immune receptor stimuli or chemotherapy treatment (Fig. 3D). More importantly, and consistent with previous data implicating Rac in polarity, reducing Rac activity to less than 10% was sufficient to disrupt mammary tissue integrity (Fig. 3A; compare phase-contrast images top left to far right showing control acini and NSC23766-treated structures) and to compromise tissue polarity, as illustrated by loss of basal β4 integrin localization, perturbed deposition of laminin-332 and collagen IV and aberrant distribution of scribble (Fig. 3A; compare confocal images in the first column with the images in the last column). While these results suggest that Rac does regulate tissue polarity, they also imply that the molecular mechanism(s) whereby Rac mediates resistance to apoptosis and polarity in 3D tissues is most likely mediated through distinct effector pathways.

To further explore the functional link between Rac, tissue polarity and resistance to apoptosis in 3D mammary acini, we ectopically expressed the Rac-specific GAP β2-chimerin, which accelerates the hydrolysis of GTP from Rac, without influencing Cdc42 or RhoA GTP activity (Caloca et al., 2003). Monolayers of non-differentiated MECs and rBM-differentiated 3D mammary acini infected with adenoviral β2-chimerin showed uniform and sustained (8 days) expression of a hemagglutinin (HA)-tagged β2-chimerin transgene (Fig. 4A,E middle images), which was associated with a significant reduction in Rac activity (Fig. 4B; quantified in 4C) and increased death sensitivity in the 3D mammary acini (Fig. 4D). Yet again, however, while β2-chimerin expression substantially reduced Rac activity and reduced death resistance, acini integrity (Fig. 4E; compare phase-contrast images top left to far right). Results are the mean ± s.e.m. of three separate experiments. *P<0.05; **P<0.01.

![Fig. 4. GAP-dependent Rac inactivation sensitizes mammary acini to induction of death.](image-url)
images of control with β2-chimerin-expressing acini; top row) and polarity (demonstrated by the maintenance of basally localized β4 integrin; Fig. 4E; compare confocal images of control with β2-chimerin-expressing acini) remained essentially intact. Collectively, these data indicate that the elevated GTP-Rac observed in 3D mammary acini promotes resistance to apoptosis through a pathway that is independent from that directing polarity.

p21-activated kinase 1 activity is necessary and sufficient for Rac-mediated resistance to apoptosis in 3D mammary acini

The Rho GTPase Rac regulates cell behavior by stimulating the activity of multiple molecular effectors, including members of the Pak family (Boettner and Van Aelst, 1999; Schmitz et al., 2000; Van Aelst and D'Souza-Schorey, 1997). Pak proteins are important modulators of tissue development and homeostasis, they can repress anoikis (Menard et al., 2005) and they have been implicated in malignant transformation (Qu et al., 2001; Wang et al., 2006). Consistent with an important role for Pak proteins in mediating resistance to apoptosis in MEC acini, we found that total levels of Pak1 and activated Pak1 rise significantly following rBM-induced tissue differentiation (acini formation) in both MCF10A (Fig. 5A; quantified in B) and HMT-3522 S1 (data not shown) nonmalignant MECs. By contrast, we noted that the specific activity of Pak4 decreased (Fig. 5A) and Pak3 protein was nondetectable (Fig. 5A). Pak1 activity was also significantly reduced in 3D mammary acini expressing the tail-less β4 integrin (Fig. 5C; quantified in D), which we showed also abrogates Rac activity (Fig. 1D; quantified in E). Inhibiting GTP-Rac through ectopic expression of N17Rac, which sensitizes 3D mammary acini to apoptotic stimuli (Fig. 3D), concomitantly decreased Pak1 activity (data not shown). Consistently, a constitutively active adenoviral V12Rac could restore Pak activity and resistance to apoptosis to increasingly cytotoxic doses of Trail and taxol in N17Rac death-sensitized mammary acini (Fig. 5E). By contrast, infection with V12RacH40, which harbors a mutation that prevents the activated Rac from interacting with and stimulating Pak, could not (Fig. 5F). Indeed, direct inhibition of Pak activity through ectopic expression of the Pak inhibitor domain (PID) rendered 3D mammary acini sensitive to death stimulation by Trail (Fig. 5G) and taxol (data not shown). Finally, infection with a wild-type adenoviral Pak1 (Pak1 WT) restored resistance to apoptosis to the death-sensitive N17Rac-expressing acini, analogous to the resistance achieved through expression of the constitutively active V12Rac (Fig. 5H). These findings strongly implicate Rac-dependent Pak1 activity as being necessary and sufficient for mediating α6β4 integrin-dependent resistance to apoptosis in 3D mammary acini.

Rac activates Pak1 and mediates resistance to apoptosis in 3D mammary acini by stimulating NF-κB

Having established a functional link between Rac, Pak and the increased survival phenotype of 3D mammary acini, we sought to delineate the molecular mechanism(s) whereby Rac activated Pak1 could promote resistance to apoptosis. Pak can regulate cell survival by inhibiting the activity of the pro-apoptotic Bcl2 family member Bad (Cotteret et al., 2003). However, whereas GTP-Rac and Pak activity were consistently and significantly elevated in the death-resistant 3D mammary acini, Bad levels and activity remained unaltered in cultures of rBM-ligated MECs grown in 2D and 3D (data not shown). We therefore reasoned that Pak1 must be driving death resistance through an alternative mechanism. Pak proteins can activate NF-κB to mediate cell survival (Frost et al., 2000), and we showed that α6β4 integrin-polarized 3D mammary acini resist apoptosis by activating NF-κB in response to an exogenous death-promoting stress (Weaver et al., 2002). Consistently, we could show that incubating 3D mammary acini with the membrane-soluble peptide SN50, which inhibits activation of NF-κB, but not with the nonfunctional peptide SN50M, prevented nuclear translocation of the p65 subunit of NF-κB (data not shown) (Zahir et al., 2003) and increased the sensitivity of the structures to death stimuli such as Trail (Fig. 6A). In addition, inhibiting nuclear translocation of p65 (and the subsequent activation of NF-κB), through treatment of the acini with SN50, also prevented exogenously expressed Pak1 from conferring resistance to apoptosis to N17Rac-expressing, death-sensitized mammary acini (Fig. 6B). Indeed, while control mammary acini survived and exhibited high levels of nuclear p65 following incubation with Trail, mammary structures with PID-reduced Pak activity had low to non-detectable nuclear p65 when exposed to Trail (Fig. 6C; quantified in 6D) and died 24 hours following treatment (Fig. 6E). Finally, we found that 3D mammary acini sensitized to apoptosis that constitutively expressed N17Rac showed lower levels of nuclear p65 following Trail exposure, but that nuclear p65 levels in these cells were restored following ectopic expression of a wild-type Pak1 adenoviral construct (Fig. 6C; quantified in 6D).

To implicate more directly Pak activation of NF-κB in the apoptosis-resistance phenotype of 3D mammary acini, we prepared populations of MECs stably expressing a retroviral wild-type p65, which promotes constitutive activation of NF-κB. MEC acini ectopically expressing p65 showed constitutive expression of nuclear p65 (supplementary material Fig. S2) that was not diminished by either reducing cellular GTP-Rac or decreasing Pak activity through coexpression of N17Rac or PID. More importantly, constitutive activation of NF-κB prevented the induction of apoptosis in 3D mammmary acini treated with Trail even when they had reduced Rac or Pak activity (Fig. 6E). Furthermore, coexpression of the NF-κB signaling pathway super-repressor IκBeM, which prevents NF-κB activation by inhibiting nuclear translocation of p65, permitted death induction in 3D polarized mammary acini, even when these MECs expressed elevated levels of exogenous Pak1 (Fig. 6F). These data indicate that Rac-mediated activation of Pak1 supports resistance to apoptosis in 3D mammary acini by regulating NF-κB activation. However, because we determined that inhibiting Rac and Pak1 through expression of N17Rac reduced the levels of phospho-Bad, whereas increasing Pak levels increased the phosphorylation of Bad (see supplementary material), we cannot rule out the possibility that Rac-Pak-dependent modulation of Bad activity also increases the survival behavior of 3D mammary acini (Fig. 7). Nevertheless, we suggest that mammary acini primarily resist apoptotic stimuli through α6β4 integrin-dependent Rac-Pak1-NF-κB signaling (Fig. 7).

Discussion

Using a rBM assay and two non-malignant, immortalized MEC lines, HMT-3522 S-1 and MCF10A, we demonstrated that a
MECs resist death through β4 integrin, Rac, Pak and NF-κB

Fig. 5. Rac-dependent Pak1 activity is necessary and sufficient for the resistance to apoptosis of mammary acini. (A) Representative immunoblots of phospho-Pak1, total Pak1, Pak-3, phospho-Pak4, total Pak4 and E-cadherin in rBM-ligated MECs plated as 2D monolayers or 3D mammary acini. The data indicate that Pak1 activity is significantly higher, although the abundance of Pak4 is higher in 3D the specific activity of Pak4 is substantially lower and Pak3 is non-detectable in 3D mammary acini in comparison with 2D monolayers of MECs. (B) Quantification of averaged experimental data shown in A of Pak1 specific activity, calculated by densitometric analysis of immunoblots of phospho-Pak1 divided by total Pak1 after normalization to E-cadherin. Similar results were obtained for HMT-3522 S1 and MCF10A nonmalignant MECs. (C) Representative immunoblots of phospho-Pak1 and total Pak1 in 3D mammary acini expressing the tailless β4 integrin (β4cyto) in comparison with control acini. The data demonstrate that Pak1 activity, but not expression, is regulated by (o6)β4 integrin signaling. (D) Quantification of averaged experimental data shown in C of Pak1 specific activity calculated as described above in B. (E) Dose-response curves illustrating the percentage apoptosis induced in 3D mammary acini following 24 hours of treatment with increasing concentrations of Trail (left) and taxol (right), calculated by scoring the number of caspase-3-positive cells divided by the total number of cells. Mammary acini with reduced Rac activity were sensitized to Trail and taxol-induced apoptosis (N17Rac vector) but their death-resistance phenotype was restored following ectopic expression of V12Rac. (F) Bar graphs illustrating how expression of V12Rac restores resistance to apoptosis induced by Trail treatment in 3D mammary acini expressing N17Rac, whereas expression of V12Rac H40, which cannot activate Pak, does not restore resistance. The percentage apoptosis was calculated by scoring the number of activated caspase-3-positive cells divided by the total number of cells. (G) Bar graph demonstrating that inhibiting Pak activity, by expressing PID significantly sensitizes mammary acini to Trail-induced death, analogous to that mediated by N17Rac. (H) Bar graph showing how expression of wild-type Pak1 can restore resistance to Trail treatment to 3D mammary acini expressing N17Rac. Results are the mean ± s.e.m. of three to five separate experiments. *P<0.05; **P<0.01.
Fig. 6. Pak permits activation of NF-κB to mediate resistance to apoptosis in mammary acini. (A) Bar graph indicating how inhibiting activation of NF-κB by treating mammary acini with SN50 peptide permits Trail-induced apoptosis. MECs were grown in rBM for ten days and treated with SN50 or inactive, scrambled SN50M peptide. Polarized acini were treated with Trail (1 μg/ml) and after 24 hours the acini were stained and quantified for activated caspase 3. (B) Bar graph illustrating how ectopically expressed wild-type Pak1 can restore resistance to apoptosis to N17Rac-expressing mammary acini treated with Trail (N17Rac), but the acini remain death sensitive if NF-κB activation is prevented by pre-incubation with SN50. 3D mammary acini were infected with adenovirus, pre-incubated for 24 hours with either SN50 to inhibit NF-κB activation or its non-active analogue SN50M, and treated with Trail (1 μg/ml) for 24 hours. The percentage apoptosis for (A) and (B) was calculated by scoring the number of activated caspase-3-positive cells divided by the total cell number. (C) Confocal immunofluorescence microscopy images showing NF-κB p65 nuclear translocation in response to treatment with Trail (90 min) in 3D mammary acini expressing either vector (control), the Pak activity inhibitor (PID), N17Rac (N17Rac) or N17Rac and wild-type Pak1 (N17Rac/Pak1 WT). Note the presence of high nuclear levels of p65, as indicated by “n” and identified by arrow, in response to Trail stimulation in control and N17Rac/Pak1-WT-expressing mammary tissues and decreased levels in acini with reduced Rac or Pak activity. Bar, 10 μm. n, nucleus. (D) Quantification of nuclear p65 in 50-100 representative images as shown in C. (E) Bar graph showing how expression of a wild-type p65 transgene restores Trail-induced death-resistance to apoptosis-sensitized N17Rac- and PID-expressing mammary acini. (F) Bar graph showing how inhibiting activation of NF-κB through expression of the IκBαM super-repressor permits Trail-dependent induction of death in 3D mammary acini despite elevated levels of Pak1. Results are the mean ± s.e.m. of three separate experiments. *P<0.05; **P<0.01; ***P<0.001.
polarized 3D mammary acinus resists diverse exogenous apoptotic stimuli including immune-receptor stimuli and chemotherapeutic agents by promoting Pak1-mediated activation of NF-κB. We determined that α6β4 integrin signaling in 3D mammary acini is necessary to elevate the activity of Rac and Pak1 and that Pak1 enhances activation of NF-κB in response to an exogenous stress, leading to enhanced MEC survival. Although α6β4 integrin can support epithelial survival through ERK and PI 3-kinase (Bachelder et al., 1999), and PI 3-kinase can activate Rac (Shaw et al., 1997), we found that the activity of ERK and PI 3-kinase was significantly diminished in 3D mammary acini, and that inhibiting the activity of these kinases failed to sensitize mammary acini to apoptotic stimuli (data not shown) (Zahir et al., 2003). Although we have no explanation for this finding, transgenic and 3D organoid experiments suggest that Akt might be more crucial for regulating cellular metabolism and tumor invasion in 3D tissues in vivo (Boxer et al., 2006; Hutchinson et al., 2004; Irie et al., 2005; Ju et al., 2007). Furthermore, studies that have linked α6β4 integrin-dependent survival to ERK and PI 3-kinase signaling used transformed breast cells grown as 2D monolayers, whereas we used non-transformed mammary acini grown within a compliant 3D rBM to demonstrate that α6β4 integrin-dependent survival depends upon elevated Rac and Pak1 and NF-κB. The compliance of hydrogels such as the rBM we used for our studies are similar in physical consistency to the extracellular matrix stroma found in tissues in vivo, and both of these microenvironments are considerably softer than the rigid tissue-culture plastic or borosilicate glass surfaces used for traditional signal-transduction experimentation. Indeed, other investigators and ourselves have shown that matrix stiffness profoundly alters ERK and RhoGTPase activity and signaling (Engler et al., 2006; McBeath et al., 2004; Paszek et al., 2005). Whether matrix compliance could modify the molecular mechanisms whereby matrix adhesion regulates cell survival is being investigated.

Similar to metastatic tumors in vivo, tumor cells grown as 3D spheroids can rapidly acquire multidrug resistance in response to acute drug treatment (Durand and Olive, 2001; Kerbel, 1994; Sutherland and Durand, 1972). The treatment resistance behavior of 3D spheroids has been attributed to reduced drug penetration (Jain, 1987; Tong et al., 2004), hypoxia (Sminia et al., 2003) and altered growth or cell-cycle regulation (St Croix et al., 1996). However, we found that the viability of the drug-treated 3D polarized, apoptosis-resistant mammary acini was not modified by proliferation or cell-cycle status, that the structures were not hypoxic, and that the acini could resist even high doses of gamma irradiation, where drug penetration is not an issue (shown by increased resistance to apoptosis and high clonogenic survival; unpublished observations). Instead, our data indicate that the apoptosis-resistance phenotype of 3D acini is due to increased (integrin-dependent) adhesion signaling, similar to the enhanced-survival phenotype of myeloma, cervical, lung and lymphoid tumor cells exposed to pro-death stimuli (Dalton, 2003; Damiano et al., 1999; Sethi et al., 1999; Whitacre and Berger, 1997) and the higher drug-resistance of integrin-ligated monolayers of tumors and 3D spheroids (Damiano et al., 1999; Kerbel et al., 1996; Narita et al., 1998; Santini et al., 2000). To explain this adhesion-mediated resistance, we showed that laminin-mediated ligation of α6β4 integrin promotes immune-receptor and multidrug resistance in mammary acini by permitting stress-induced activation of NF-κB (Weaver et al., 2002). We also reported that α6β4 integrin permits epidermal growth factor-dependent activation of Rac that stimulates NF-κB and mediates anchorage-independent survival of MECs (Zahir et al., 2003). Our current findings extend our earlier observations by demonstrating that laminin-ligated α6β4 integrin enhances Rac and Pak activity in 3D mammary acini resulting in stress-dependent NF-κB activation and increased survival through a mechanism that appears to be independent of tissue polarity. Although we do not know how α6β4 integrin modulates tissue polarity, we did find that α6β4 integrin regulates GTP-Rac and, in accordance with previous work, that Rac regulates tissue morphology and polarity (Akhtar and Streuli, 2006; O’Brien et al., 2001) (Fig. 3A). However, given that we could distinguish between Rac-dependent survival through Pak and Rac-mediated tissue polarity, we suggest that the Rac effectors directing tissue polarity differ from those that promote cell survival. Consistently, we determined that Rac regulates Pak activity to support resistance to apoptosis in mammary acini, but that modulating Pak had no detectable morphological effect on tissue organization or integrity. Clearly, additional experiments will be required to identify the Rac effectors directing tissue polarity and to determine whether these pathways are regulated by α6β4 integrin.
It is not clear why αβ4 integrin activation of Rac and Pak is dramatically enhanced in MECs grown as 3D rBM acini in comparison with 2D rBM monolayers. Plausible explanations for this observation include differences in matrix compliance, integrin adhesions and receptor dynamics or qualitative changes in RhoGTPase signaling linked to tissue organization. For example, other investigators and ourselves have reported that matrix stiffness increases the activity of Rho to promote assembly of focal adhesions (Bershadsky et al., 2006; Paszek et al., 2005) and that Rac activity is inhibited by matrix stiffness and increases significantly in MECs interacting with a compliant substrate (our unpublished observations). The targeting and functioning of RhoGTPase are also modified by tissue morphology such that the localization of activated Rac is restricted to tight junctions following epithelial polarization (Chen and Macara, 2005), and PTEN localizes activated cdc42 to stimulate αPKC-dependent lumen formation in polarized kidney epithelial cysts (Martin-Belmonte et al., 2007).

Rac is often overexpressed in tumors of the breast (Fritz et al., 1999), it can support anchorage-independent growth of MECs (Bouzahzah et al., 2001; Zahir et al., 2003) and protects MDCK cells from anoikis (Coniglia et al., 2001). Depleting Rac in glioblastoma cells and breast cancer cells also strongly inhibits invasion (Chan et al., 2005). Rac elicits its pleiotropic effects on cell function by activating a plethora of cellular targets including the Pak family of signaling molecules. Pak proteins facilitate growth factor signaling and interact with multiple downstream effectors to alter cell growth, survival, migration and differentiation (Bokoch, 2003; Puto et al., 2003; Vadlamudi and Kumar, 2003; Wang et al., 2002), and Pak proteins can promote the tumorigenic behavior of cells (Menard et al., 2005; Schurrmann et al., 2000; Tang et al., 1997). For example, Rac-dependent Pak activation drives cell migration and tumor invasion (Alahari, 2003; Alahari et al., 2004; Brown et al., 2005), and Pak proteins can enhance cell survival (Gnesutta et al., 2001; Johnson and D’Mello, 2005; Qu et al., 2001). Here, we show that the specific activity of Pak1 increases dramatically and significantly in apoptosis-resistant 3D mammary acini, and that Pak1 is essential for immune-receptor and drug-induced death because it permits stress-dependent NF-κB activation (Fig. 6C,D). Our results are consistent with experiments showing how Pak4 sustains anchorage-independent cell survival by stimulating NF-κB (Cammarano and Minden, 2001) and extend these observations to demonstrate that Pak1 can render cells resistant to diverse exogenous apoptotic stimuli by regulating NF-κB activity. Activated Pak can also prevent apoptosis by inducing phosphorylation of Bad on Ser112 to prevent mitochondria-mediated activation of executioner caspases (Cotteret et al., 2003; Deacon et al., 2003; Gnesutta et al., 2001; Schurrmann et al., 2000), and we were able to establish a correlation between Bad phosphorylation, resistance to apoptosis and Pak activity (see supplementary material Fig. S3). However, we found that neither expression of Bad nor its phosphorylation increased in association with death resistance in 3D MEC acini, despite finding that Rac and Pak activity were significantly higher in these same structures. Thus, although we cannot rule out the possibility that decreased phosphorylation of Bad contributes to Rac-Pak-dependent resistance to apoptosis, we suggest that Pak-induced NF-κB activation is more likely the predominant anti-apoptotic mechanism operating in 3D mammary acini.

We established a functional link between laminin-mediated ligation of αβ4 integrin and Rac-dependent activation of Pak1, and β4 integrin and laminins are frequently elevated in tumors (Davis et al., 2001), they cooperate to drive invasion and metastasis (Jones et al., 1997; Lipscomb and Mercurio, 2005; Lipscomb et al., 2005) and their coexpression is associated with a poor prognosis in breast cancer patients (Tagliaabue et al., 1998). Our data offer an explanation for these findings and additionally explain why Pak proteins are often overexpressed in tumors (Vadlamudi and Kumar, 2003) and why hyper-activation of Pak1 efficiently drives mammary gland tumor formation (Wang et al., 2006). Indeed, expression of NF-κB increases during malignant transformation of the breast (Kim et al., 2000), and NF-κB is implicated in malignant tumor pathogenesis (Sovak et al., 1997), breast tumor metastasis (Nakshatri et al., 1997) and resistance to chemotherapy, immune and radiation therapy (Baeuerle and Baltimore, 1996; Baldwin, 2001; Weaver et al., 2002). Moreover, Rac-Pak-dependent activation of NF-κB can drive Kaposi-sarcoma-associated malignant transformation (Dadke et al., 2003). Significantly, by linking matrix-dependent Rac-Pak activation to NF-κB-dependent resistance to apoptosis, our findings underscore the importance of identifying pharmacological Pak inhibitors that could be used as tractable anti-tumor therapies.

Materials and Methods

Antibodies and reagents

We used commercial EHS matrix (MatrigelTM; Collaborative Research) for the reconstituted basement membrane (rBM) assays. The sources of the primary antibodies used in these studies were as follows: β4 integrin, clone 3E1 (Chemicon); phospho-Bad (Ser136), Bad, cleaved caspase 3, phospho-Pak1 (Thr423)/Pak2 (Thr402), Pak1, Pak 3, Pak 4, phospho-Pak4 (Ser474)/Pak5 (Ser602)/Pak6 (Ser560), rabbit sera (all from Cell Signaling); E-cadherin, clone 36, and Rac1, clone 102 (BD Biosciences); HA.11, clone 3E1 (Chemicon); and NF-κB p65, rabbit sera (Santa Cruz Biotechnology). The secondary antibodies used were as follows: HRP-conjugated anti-rabbit and mouse (Amersham Laboratories); and Alexa-Fluor-488 and Alexa-Fluor-555-conjugated anti-mouse and rabbit IgGs (Molecular Probes). Reagents used in the studies were as follows: Rho GTPase inhibitor toxin A Clostridium difficile (10 mM in DMSO; Calbiochem); and Rac1 inhibitor NSC23766 (50 mM in H2O; Calbiochem); NF-κB SN50, active cell-permeable inhibitory peptide (50 μM in H2O), NF-κB SN50M, inactive cell-permeable control peptide (50 μM in H2O), Tra1 (approximately 0.5 μg/ml), and taxol (20 mM in DMSO; Biomol); etoposide (10–100 μM in DMSO; TopoGen); live/dead viability cytotoxicity kit (Molecular Probes); and the in situ cell death detection kit (TUNEL; Roche).

Cell culture

The HMT-3522 and MCF10A mammary epithelial cells (MECs) were grown and maintained exactly as previously described (Paszek et al., 2005).

Flow cytometry

Live cells were isolated, re-suspended in Dulbecco’s PBS with 0.5% Fetal Bovine Serum and immediately sorted for high EGFP expression on a FACScan (Becton Dickinson). All manipulations were conducted at 4°C.

Immunofluorescence analysis

Cells were directly fixed using 100% methanol, and samples were incubated with primary antibody followed by either Alexa-Fluor-488 or Alexa-Fluor-555-conjugated secondary antibody. Nuclei were counterstained with diamidino yellow (DAPI, Sigma-Aldrich). Cells were visualized using a Bio-Rad MRC 1024 laser-scanning confocal microscope attached to a Nikon Diaphot 200 microscope. Images were recorded at 60× magnification.

Apoptosis assay

Apoptosis was assayed by immuno-detection of activated caspase 3. The percentage apoptosis was quantified as the number of cells positive for activated caspase 3 divided by the total number of cells. The minimum number of cells scored was 200-300 per experimental condition. Results were confirmed using live/dead and TUNEL assays. Cells were visualized using a fluorescence microscope (Olympus model U-LH100GHPA0).
Adenoviral and retroviral constructs and studies

EGFP tagged Rab11 was obtained from Dr. J. Rust (1997). Stable MEC populations expressing EGFP-tagged Rab11 were selected and cultured as described previously (1999). In brief, MEC cultures were transduced with a retroviral vector expressing the EGFP-Rab11 fusion protein. Cultures were selected with puromycin and then maintained without selection to ensure that stable integrated transgenes were maintained. These EGFP-Rab11 expressing MECs were then used in the studies described in this report.

Inactivation of Rab11

Rab11 was inactivated by treatment of MEC cultures with 0.1 μM C3 transferase, a specific inhibitor of Rab11. The effect of C3 transferase on Rab11 activity was determined by examining the localization of Rab11 to membrane fractions. Following C3 transferase treatment, MECs were washed twice with PBS and then lysed with a solution containing 1% NP-40, 1% sodium deoxycholate, 0.5% SDS, 1 mM EDTA, and a protease/phosphatase inhibitor cocktail. The lysates were centrifuged at 14,000 x g for 30 min. The supernatants were then immunoblotted with anti-Rab11 antibodies to assess the expression of Rab11.

Role of Rab11 in MEC Function

The role of Rab11 in MEC function was assessed by examining the effects of Rab11 inactivation on MEC migration and invasion. MECs were grown on collagen-coated substrates and then treated with C3 transferase. The migration and invasion of MECs was assessed using Boyden chamber assays. The effect of Rab11 inactivation on MEC migration and invasion was assessed by quantifying the number of MECs that migrated or invaded through the membrane of the Boyden chamber.

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