

Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1

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Tumor cells undergo epithelial-to-mesenchymal transition (EMT) to convert from a benign to a malignant phenotype. Our recent focus has been signaling pathways that promote EMT in response to collagen. We have shown that human pancreatic cancer cells respond to collagen by up-regulating N-cadherin, which promotes tumor growth, invasion, and metastasis. Initial characterization showed that knocking down c-Jun NH₂-terminal kinase prevented N-cadherin up-regulation and limited tumor growth and invasion in a mouse model for pancreatic cancer. The current study was designed to understand

the pathway from collagen to N-cadherin up-regulation. Initiation of the signal requires two collagen receptors, $\alpha 2\beta 1$ integrin and discoidin domain receptor (DDR) 1. Each receptor propagates signals through separate pathways that converge to up-regulate N-cadherin. Focal adhesion kinase (FAK)-related protein tyrosine kinase (Pyk2) is downstream of DDR1, whereas FAK is downstream of $\alpha 2\beta 1$ integrin. Both receptor complexes rely on the p130 Crk-associated substrate scaffold. Interestingly, Rap1, but not Rho family guanosine triphosphatases, is required for the response to collagen I.

Introduction

Epithelial cells transition from an epithelial to a mesenchymal phenotype during normal development (Thiery, 2002; Lee et al., 2006). This phenomenon is referred to as an epithelial-to-mesenchymal transition (EMT) and is tightly regulated by activators and repressors (Affolter et al., 2003). EMTs that drive development are critical for events such as gastrulation, neural crest cell migration, and wound healing (Savagner, 2001; Grunert et al., 2003; Thiery and Sleeman, 2006). During EMT, cells often decrease expression of E-cadherin and increase expression of N-cadherin (Thiery, 2003; Lee et al., 2006). In addition, they lose epithelial polarity, gain the expression of mesenchymal markers, and become highly motile (Thiery, 2003; Lee et al., 2006). We have shown that cadherin switching is necessary for the increased cell motility that accompanies EMT (Maeda et al., 2005)

and that inhibiting N-cadherin up-regulation prevents tubulogenesis (Shintani et al., 2006b).

EMT-like transitions also occur in tumor cells when they change from a benign to an aggressive phenotype, although they often do not fully change to mesenchymal cells (Grunert et al., 2003). The extent of EMT varies and is often transient, occurring at the invasion front of metastatic tumors (Grunert et al., 2003). Although they may not express all the genes that typify full-blown EMT, many tumor cells respond to inducers of EMT by changing their shape, displaying a scattered phenotype, becoming highly motile, and undergoing a cadherin switch (Islam et al., 1996; Pishvaian et al., 1999; Tomita et al., 2000; Feltes et al., 2002; Grunert et al., 2003). A switch to N-cadherin expression by tumor cells promotes motility, invasion, and metastasis (Nieman et al., 1999; Hazan et al., 2000; Hult et al., 2007).

EMT is initiated by signals originating from outside the cell, including growth factors and ECM molecules (Savagner, 2001; Lee et al., 2006). Triggers for normal EMT vary tremendously depending on the tissue and context (Thiery and Sleeman, 2006). For example, hepatocyte growth factor induces EMT in early development and during cardiac cushion formation but

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Abbreviations used in this paper: CAS, Crk-associated substrate; CRIB, Cdc42-Rac interactive binding; DDR, discoidin domain receptor; EMT, epithelial-to-mesenchymal transition; MKK, MAPK kinase; MLK, mixed lineage kinase; pAb, polyclonal antibody; PAK, p21-activated kinase; PI3K, phosphatidylinositol 3-kinase; RIPA, radioimmunoprecipitation assay; SD, substrate domain; shRNA, short hairpin RNA; TNE, Tris/NP40/EDTA.

The online version of this paper contains supplemental material.

inhibits EMT and, thus, prevents fibrosis during repair of adult kidney injury (Zavadil and Bottinger, 2005). The matrix metalloproteinase-28 induces EMT by proteolytically releasing E-cadherin from the cell surface and activating latent TGF β (Zavadil and Bottinger, 2005). Other proteases can induce EMT by activating distinct downstream signals (Radisky et al., 2005; Zhang et al., 2007). Growth factors like FGF and hepatocyte growth factor act through phosphatidylinositol 3 kinase (PI3K) to activate Rac and Cdc42 and inactivate Rho, which results in reorganization of the actin cytoskeleton, leading to EMT-like events (Lee and Kay, 2006; Lee et al., 2006).

Perhaps the best-studied inducer of EMT is TGF β , which binds to serine/threonine receptor kinases that signal through Smads to regulate EMT-specific genes (Shi and Massague, 2003; ten Dijke and Hill, 2004; Zavadil and Bottinger, 2005). TGF β can also participate in Smad-independent pathways that involve Par6- and Smurf1-mediated degradation of RhoA, leading to dissolution of tight junctions and actin reorganization, which contribute to EMT (Barrios-Rodiles et al., 2005; Moustakas and Heldin, 2005; Ozdamar et al., 2005). The signals that initiate EMT-like changes in cells and the downstream pathways are diverse. Because these pathways play a significant role in the behavior of tumor cells, it is critical that we have a complete understanding of the signals.

In addition to soluble proteins that initiate EMT, ECM molecules have been shown to induce similar changes in epithelial cells. For example, forced expression of hyaluronan by normal MDCK cells promotes EMT by inducing matrix metalloproteinase production and activating PI3K (Zoltan-Jones et al., 2003). Several laboratories, including our own, have shown that collagen I induces EMT-like changes in various cell types. For example, Tiam1/Rac signaling promotes motility in MDCK cells plated on collagen I but not other substrates, and this activity is regulated by PI3K (Sander et al., 1998). Pancreatic cancer is highly invasive and metastatic (Baumgart et al., 2005) and is characterized by extensive deposition of collagen I (Imamura et al., 1995; Linder et al., 2001; Bachem et al., 2005). Pancreatic cancer cells cultured in collagen I gels form looser aggregates than the same cells cultured in Matrigel (Yamanari et al., 1994) and grow as scattered individual cells on collagen I, in contrast to the small clusters of cells that are seen on non-coated dishes (Armstrong et al., 2004). In addition, pancreatic cancer cells undergo Src-dependent morphological transformation in response to plating on collagen I (Menke et al., 2001) and activate FAK, which then associates with β -catenin to activate β -catenin/LEF1 target genes such as c-myc and cyclin D1 (Koenig et al., 2006).

We recently showed that mouse mammary epithelial cells up-regulate N-cadherin and undergo EMT in response to collagen I through a pathway involving integrins, PI3K, Rac1, and JNK and that the response to collagen I is much like the response of these cells to other EMT-inducing stimuli (Shintani et al., 2006b). Likewise, human lung cancer cells respond to collagen I by increasing production of active TGF β , which then promotes EMT-like changes through canonical Smad signaling (Shintani et al., 2008b). We also showed that human pancreatic cancer cells respond to collagen I by scattering and up-regulat-

ing expression of N-cadherin, and overexpressing N-cadherin promotes tumor growth, invasion, and metastasis in an orthotopic mouse model for pancreatic cancer. In addition, inhibiting N-cadherin up-regulation in response to collagen prevents cell scattering and motility in vitro and limits tumorigenesis. Furthermore, inhibiting N-cadherin function with the N-cadherin antagonist ADH1 also decreases cell motility and limits tumorigenesis (Shintani et al., 2008a). Thus, it is clear that up-regulating N-cadherin expression promotes an aggressive phenotype in pancreatic cancer.

We have previously shown that inhibiting JNK activity in pancreatic cancer cells prevents N-cadherin up-regulation, cell scattering, and cell motility in response to collagen I, whereas inhibiting PI3K signaling does not (Shintani et al., 2006a). In addition, expressing short hairpin RNA (shRNA) against JNK1 prevents N-cadherin expression and limits tumorigenesis in the orthotopic model. Thus, it is clear that signaling downstream of collagen I is complex and plays a significant role in tumor cell behavior, especially in highly fibrotic cancers like pancreatic cancer.

The current study was designed to understand the signals that promote up-regulation of N-cadherin and cell scattering in human pancreatic cancer cells in response to collagen I. We found that initiation of the signal requires two collagen receptors, α 2 β 1 integrin and discoidin domain receptor (DDR) 1 and that each receptor propagates signals through separate pathways that converge to up-regulate N-cadherin expression.

Results

BxPC3 cells scatter in response to collagen I but not fibronectin

From our previous studies, we knew that the interaction of human pancreatic cancer cells with collagen I leads to activation of JNK1, which promotes scattering and N-cadherin up-regulation. To investigate the pathways responsible for these activities, we first examined the interactions with collagen that initiate the signal. We showed that the only integrin collagen receptor expressed by these cells is α 2 β 1 (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200708137/DC1>; and not depicted). This is in agreement with published studies showing that α 2 β 1 is the functional integrin collagen receptor expressed by BxPC3 cells (Grzesiak and Bouvet, 2006). BxPC3 cells expressed very low levels of the fibronectin receptor α 5 β 1 (Fig. S1 A), presenting the possibility that they may respond to collagen but not fibronectin simply because they do not express an appropriate receptor for fibronectin. Thus, we overexpressed α 5 integrin in these cells to form the α 5 β 1 integrin receptor (Fig. 1 B). The surface level of α 5 was roughly equivalent to the level of α 2 (Fig. S1 B). As we have shown in the past, BxPC3 cells scattered when plated on collagen but not when plated on plastic or fibronectin (Fig. 1 A, a–c). Cells overexpressing α 5 integrin continued to scatter on collagen (Fig. 1 A, e) and did not scatter on fibronectin (Fig. 1 A, f) even though they expressed significant levels of α 5 integrin (Fig. 1 B). Fig. 1 C shows that mock-transduced BxPC3 cells plated on collagen had phosphorylated FAK, whereas the level of phosphorylated FAK in parental cells plated on fibronectin

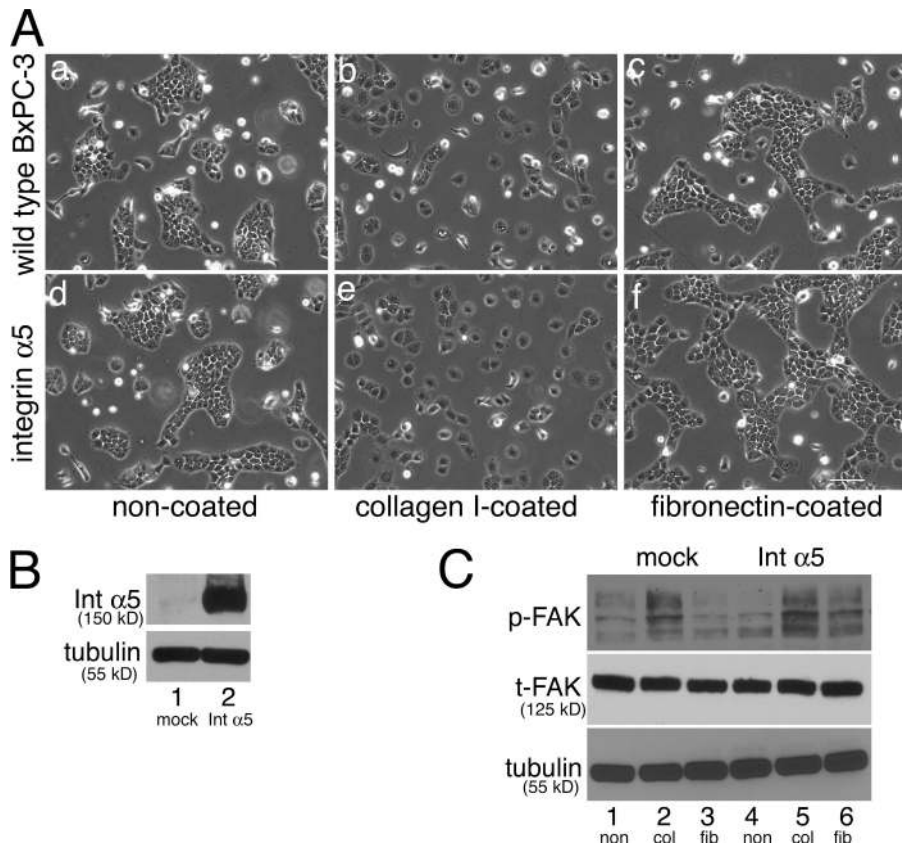


Figure 1. Overexpression of integrin $\alpha 5$ in BxPC3 cells. (A) BxPC3 cells (a–c) or integrin $\alpha 5$ -overexpressing BxPC3 cells (d–f) were cultured on noncoated (a and d), collagen I-coated (b and e), or fibronectin-coated (c and f) dishes. Bar, 100 μ m. (B) 30 μ g of protein from mock or integrin $\alpha 5$ -overexpressing BxPC3 cells (2 d after seeding) was resolved by SDS-PAGE and blotted for integrin $\alpha 5$ (Int $\alpha 5$) or tubulin. (C) 60 μ g of protein from mock or integrin $\alpha 5$ -overexpressing BxPC3 cells (4 h after seeding) was resolved by SDS-PAGE and blotted for phospho-FAK (p-FAK; Y577), total FAK (t-FAK), or tubulin.

was similar to that of cells plated on plastic. When the cells overexpressed $\alpha 5$, they still activated FAK in response to collagen; however, they now also activated FAK in response to fibronectin. Thus, merely activating FAK is not sufficient to promote scattering. We concluded from these experiments that BxPC3 cells obtain specific signals from exposure to collagen I that they do not receive from fibronectin, even though the integrin receptors are activated on either substrate.

Interaction with collagen I activates both FAK and Pyk2

To directly examine the role of integrin $\beta 1$ in the response to collagen I, we used shRNA to knock down its expression (Fig. 2 A, a). Cell scattering in response to collagen I was partially, but not completely, prevented (Fig. 2 B, a–d). In addition, shRNA against integrin $\beta 1$ only partially prevented the up-regulation of N-cadherin in response to collagen I (Fig. 2 A, b). shRNA against integrin $\alpha 2$ had the same effect as shRNA against integrin $\beta 1$, whereas shRNA against integrin $\alpha 5$ had no effect (unpublished data). Using phase microscopy to document a partial response can be subjective. Thus, the criteria we considered were an increase in the number of single cells and a decrease in colony size. In addition, an important and less subjective criterion was a decrease in the up-regulation of N-cadherin in response to collagen I.

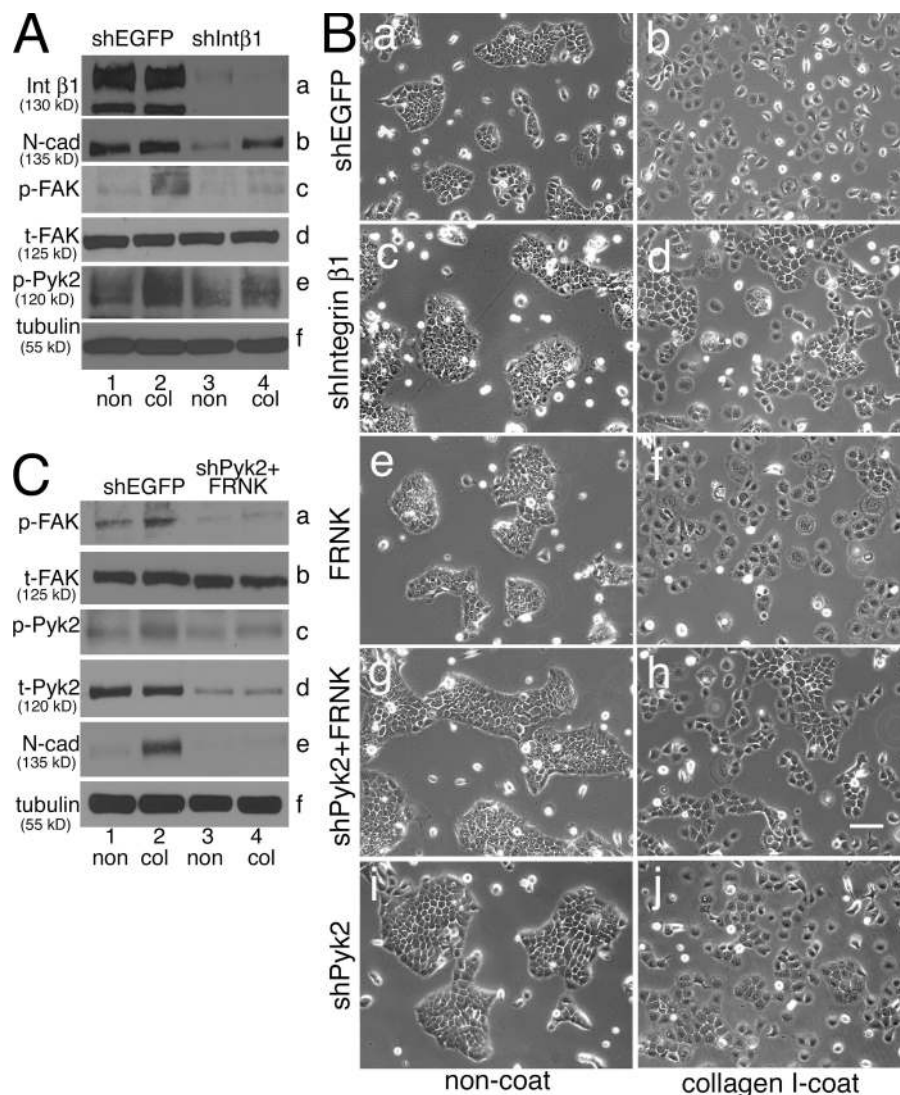
To go one step down the pathway from integrins, we expressed a dominant-negative form of FAK (FRNK). Interestingly, FRNK also showed only a partial inhibition of cell scattering (Fig. 2 B, f). Because BxPC3 cells express the FAK homologue

Pyk2, which can also signal downstream of integrins, and BxPC3 cells plated on collagen I showed increased phosphorylation of Pyk2 (Fig. 2 A, e, lanes 1 and 2), we knocked down its expression in BxPC3 cells expressing FRNK (Fig. 2 C, d, lanes 3 and 4). These cells did not show increased phosphorylation of either FAK or Pyk2 in response to collagen I (Fig. 2 C, a–d). Importantly, cells knocked down for Pyk2 and expressing FRNK did not scatter (Fig. 2 B, h) nor did they up-regulate N-cadherin in response to collagen I (Fig. 2 C, e). To rule out the possibility that Pyk2 alone was sufficient to transmit the signal from collagen I, we knocked it down in parental BxPC3 cells and showed that these cells also had only a partial response to collagen I (Fig. 2 B, i–j). These experiments suggested that activation of integrins in response to collagen I transmits signals through both FAK and Pyk2. To verify this, we compared phosphorylation of FAK and Pyk2 in parental BxPC3 cells plated on collagen I to that of BxPC3 cells knocked down for integrin $\beta 1$. We were surprised to find that knocking down integrin $\beta 1$ prevented phosphorylation of FAK (Fig. 2 A, c, lanes 3 and 4) but had little effect on phosphorylation of Pyk2 (Fig. 2 A, e, lanes 3 and 4).

The response of BxPC3 cells to collagen I requires both integrins and DDR1

The data in Fig. 2 suggested that both FAK and Pyk2 are involved in collagen I-induced changes and that FAK, but not Pyk2, is downstream of integrin activation. Most epithelial tumor cells (including BxPC3) express another collagen receptor, DDR1, which is a not an integrin receptor but rather is a receptor tyrosine kinase that binds to and is activated by

Figure 2. Inhibition of integrin signaling in BxPC3 cells. (A) RIPA extracts (30 μ g of protein) from cells expressing shEGFP (control) or shIntegrin β 1 (shInt β 1) cultured on noncoated or collagen I-coated dishes for 2 d were resolved by SDS-PAGE and blotted for integrin β 1 (a), N-cadherin (b), or tubulin (f). 60 μ g of protein from cells cultured on noncoated or collagen I-coated dishes for 4 h was blotted for phospho-FAK (Y577; c), total FAK (d), or phospho-Pyk2 (Y579/Y580; e). (B) BxPC3 cells expressing shEGFP (a and b), shIntegrin β 1 (c and d), FRNK (e and f), both FRNK and shPyk2 (g and h), or shPyk2 alone (i and j) were cultured on noncoated (a, c, e, and g) or collagen I-coated (b, d, f, and h–j) dishes for 2 d. Bar, 100 μ m. (C) RIPA extracts from cells expressing shEGFP or both shPyk2 and FRNK were blotted for phospho-FAK (Y577; a), total FAK (b), phospho-Pyk2, (Y579/Y580; c), total Pyk2 (d), N-cadherin (e), or tubulin (f).



collagen (Vogel et al., 2000). When BxPC3 cells were cultured on plastic, DDR1 had very low levels of tyrosine phosphorylation (Fig. 3 A). However, when cells were plated on collagen I, DDR1 was phosphorylated similarly to cells treated with soluble collagen I (Fig. 3 A, lanes 3 and 4). Soluble collagen I has been previously shown to activate DDR1 (Vogel et al., 2000). We next showed that knocking down expression of DDR1 using shRNA inhibited collagen-induced cell scattering and partially prevented collagen I-mediated up-regulation of N-cadherin (Fig. 3). As with the experiments described in Fig. 2, a partial response to collagen I is difficult to document with a single low-magnification phase micrograph. Thus, we have provided additional fields of view (Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200708137/DC1>). In addition, we have shown that the small colonies of cells seen on collagen continue to display cell–cell border staining for E-cadherin (Fig. S2 B). These experiments suggested to us that it was the combination of signals from DDR1 and integrin β 1 that produced the full response of BxPC3 cells to collagen I. To test this idea, we knocked down DDR1 and integrin β 1 in the same cells. When these cells were plated on collagen they

did not scatter (Fig. 3 C, f) and did not up-regulate N-cadherin (Fig. 3 D, c).

To ensure we were studying a pathway relevant to pancreatic cancer and not specific to a single pancreatic cancer cell line, we showed that several pancreatic cancer cells respond similarly to collagen I but not to other substrata (Shintani et al., 2006a) and that several pancreatic cancer cell lines express DDR1 (Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200708137/DC1>). We further showed that knocking down DDR1 in Capan-1 cells reduced, but did not eliminate, their response to collagen I (Fig. S3, B and C).

Because it required knocking down both integrin β 1 and DDR1 to completely block the response to collagen I and we saw the same effect only when inhibiting signaling through both FAK and Pyk2, we asked whether Pyk2 was downstream of DDR1 rather than integrin β 1. Knocking down integrin β 1 had little effect on Pyk2 phosphorylation (Fig. 2). Thus, we examined the activation of FAK and Pyk2 in cells knocked down for DDR1. In mock cells FAK and Pyk2 were phosphorylated in response to collagen I as expected (Fig. 4 A). In cells knocked down for DDR1, FAK phosphorylation was similar to that of control cells,

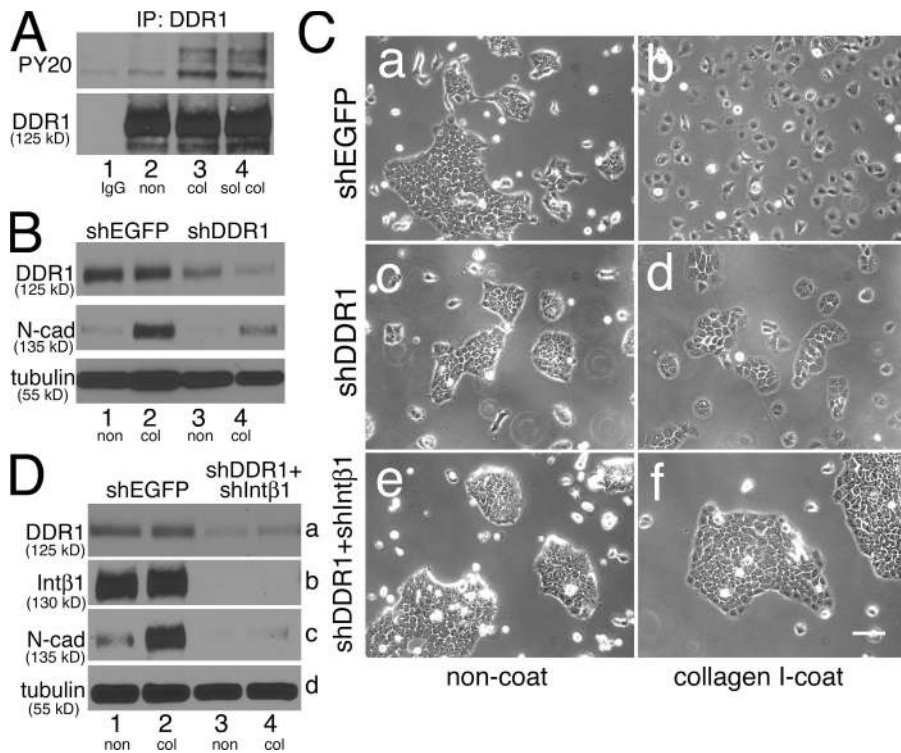


Figure 3. DDR1 is necessary for collagen I-induced changes in BxPC3 cells. (A) BxPC3 cells were extracted in TNE buffer 12 h after plating on noncoated or collagen I-coated dishes. 600 μ g of protein was immunoprecipitated with nonspecific rabbit IgG or anti-DDR1 rabbit pAb and blotted with anti-phosphotyrosine mAb (PY20) or anti-DDR1 pAb. TNE extracts from cells stimulated with soluble collagen type I (sol col; 50 μ g/ml) for 120 min served as a positive control (lane 4). (B) RIPA extracts (30 μ g of protein) from cells expressing shEGFP or shDDR1 and cultured on noncoated or collagen I-coated dishes for 2 d were resolved by SDS-PAGE and blotted for DDR1, N-cadherin, or tubulin. (C) BxPC3 cells expressing shEGFP (a and b), shDDR1 (c and d), or both shDDR1 and shIntegrin β 1 (e and f) were cultured on noncoated (a, c, and e) or collagen I-coated (b, d, and f) dishes for 2 d. Bar, 100 μ m. (D) RIPA extracts (30 μ g of protein) from cells expressing shEGFP or both shDDR1 and shIntegrin β 1 and cultured on noncoated or collagen I-coated dishes for 2 d were blotted for DDR1 (a), integrin β 1 (b), N-cadherin (c), or tubulin (d).

whereas phosphorylation of Pyk2 was attenuated. When we knocked down both DDR1 and integrin β 1, phosphorylation of both FAK and Pyk2 was prevented (Fig. 4 A). Together, these data suggest that BxPC3 cells receive two signals from collagen I, one initiated by integrin β 1 and propagated through FAK and the other initiated by DDR1 and propagated through Pyk2. Although the suggestion that Pyk2 may be downstream of DDR1 is novel, it has been previously reported that Pyk2 can be activated by other receptor tyrosine kinases (Park et al., 2004). Coimmunoprecipitation showed that DDR1 interacts with Pyk2 and that DDR1 is in a complex with Pyk2 but not with FAK (Fig. 4 B). In addition, the amount of Pyk2 that coimmunoprecipitated with DDR1 was slightly higher when cells were plated on collagen than when they were plated on plastic. When we immunoblotted the DDR1 immunoprecipitation reactions for p-Pyk2, we did not see a convincing signal. However, it is likely this antibody would not be able to detect the small amount of Pyk2 present in the immunoprecipitation reaction.

We previously reported that JNK is necessary for collagen-induced changes in BxPC3 cells (Shintani et al., 2006a). Thus, it was important here to determine the effect on JNK activation of knocking down integrin β 1, DDR1, FAK, and Pyk2. Fig. 4 C shows that knocking down individual components of these pathways either had no effect or slightly lowered the activation of JNK but that knocking down integrin β 1 together with DDR1 or expressing dominant-negative FAK together with knocking down Pyk2 completely prevented JNK phosphorylation in response to collagen. Thus, the signal to scatter and up-regulate N-cadherin in response to collagen is multifaceted and requires both integrin β 1 and DDR1 collagen receptors together with their respective downstream partners FAK and Pyk2, which together propagate a signal to JNK.

P130 Crk-associated substrate (CAS) functions as a scaffold to facilitate signaling from collagen I to JNK

The next objective of this study was to determine how the signal gets from FAK/Pyk2 to JNK. Signaling cascades often involve scaffolds, and p130CAS plays a crucial role in controlling integrin-dependent processes (Chodniewicz and Klemke, 2004). In addition, the SH3 domain of p130CAS binds FAK and Pyk2 leading to activation of JNK (Tanaka and Hanafusa, 1998; Blaukat et al., 1999). Thus, we asked if DDR1 was also capable of binding to p130CAS. Coimmunoprecipitation showed that DDR1 was in a complex with p130CAS, and this association was increased when cells were plated on collagen I (Fig. 5, A and B).

p130CAS function is dependent on tyrosine phosphorylation of its substrate domain (SD). It has been previously reported that p130CAS SD fused to an Src kinase domain [Src*/CAS(SD)] acts as a dominant negative by blocking p130CAS-mediated signaling events including JNK activation (Kirsch et al., 2002). The control for this dominant-negative form of p130CAS is SrcKM/CAS(SD), in which the Src component has an inactive kinase domain (K295M). Expression of this latter construct does not promote tyrosine phosphorylation of the p130CAS SD (Kirsch et al., 2002). To ask if p130CAS is necessary for collagen I-dependent signaling, we expressed dominant-negative p130CAS in BxPC3 cells and examined the response to collagen I. Overexpression of Src*/CAS(SD) prevented N-cadherin up-regulation (Fig. 5 C), whereas SrcKM/CAS(SD)-infected cells up-regulated N-cadherin similarly to mock infected cells (Fig. 5 C, top). In addition, cell scattering was prevented by overexpressing Src*/CAS(SD) but not by the inactive SrcKM/CAS(SD) (Fig. 5 D). These data show that p130CAS plays a crucial role in collagen I-mediated cell scattering as well as up-regulation of N-cadherin and,

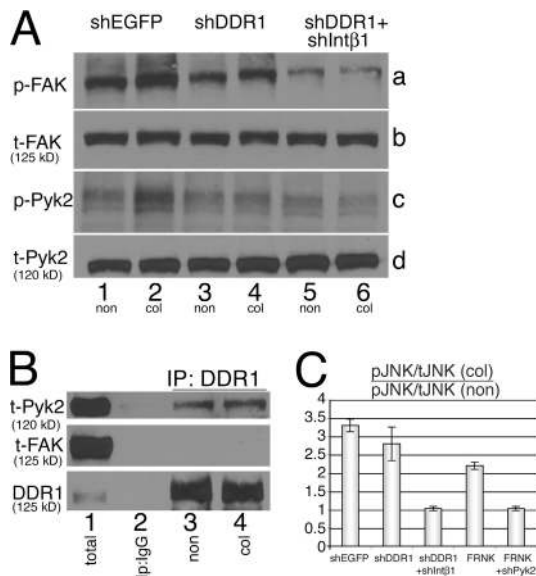


Figure 4. Collagen I signals through DDR1-Pyk2 in BxPC3 cells. (A) RIPA extracts (60 μ g of protein) from cells expressing shEGFP, shDDR1, or both shDDR1 and shIntegrin β 1 and cultured on noncoated or collagen I-coated dishes for 4 h were resolved by SDS-PAGE and blotted for phospho-FAK (Y577; a), total FAK (b), phospho-Pyk2 (Y579/Y580; c), or total Pyk2 (d). (B) 1 mg of protein from BxPC3 cells extracted 4 h after plating on noncoated or collagen I-coated dishes was immunoprecipitated with rabbit IgG or anti-DDR1 rabbit pAb and blotted for total Pyk2, total FAK, or DDR1. (C) RIPA extracts (60 μ g of protein) from cells expressing shEGFP, shDDR1, both shDDR1 and shIntegrin β 1, FRNK, or FRNK together with shPyk2 and cultured on noncoated or collagen I-coated dishes for 4 h were resolved by SDS-PAGE and blotted for phospho-JNK (T183/Y185) or total JNK1. Immunoblots for pJNK were quantified by densitometry using Photoshop and normalized to immunoblots for total JNK1. Shown is the ratio of phospho-JNK/total JNK for cells on collagen I-coated dishes divided by the ratio of phospho-JNK/total JNK for cells on noncoated dishes. The data represent the mean and standard deviation from three independent experiments.

together with data already in the literature, suggest that p130CAS serves as a scaffold for signaling complexes comprised of integrin β 1 and DDR1 in the plasma membrane and FAK and Pyk2 in the cytosol.

Inhibition of Rap1 prevents the response to collagen I

It is common for signals downstream of cell adhesion to be mediated by small GTPases, usually Rac1 (Kooistra et al., 2007). When we expressed dominant-negative Rac1 (Rac1N17) in BxPC3 cells, we saw no effect on the ability of the cells to respond to collagen I (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200708137/DC1>). Likewise, we saw no effect when we expressed dominant-negative Cdc42 (Cdc42N17). Even the Cdc42-Rac interactive binding (CRIB) domain of p21-activated kinase (PAK1), which inhibits both Rac1 and Cdc42 (Arthur et al., 2004), did not prevent scattering or up-regulation of N-cadherin in response to collagen I, ruling out these GTPases (Fig. S4).

It has been previously reported that the Pyk2-p130CAS complex activates JNK through C3G-Rap1 (Blaukat et al., 1999). Because the obvious candidate GTPases Rac1 and Cdc42 did not appear to play a role in collagen I-induced scattering and

N-cadherin up-regulation, we asked if Rap1 might be a relevant GTPase in our system. Rap1 was activated when cells were plated on collagen I (Fig. 6 A) and Rap1GAP prevented cell scattering and N-cadherin up-regulation (Fig. 6, B and C). Because Rap1 plays a role in cell attachment and spreading, the Rap1GAP-expressing cells had a delayed attachment on all substrata (Fig. 6 B). However, it is clear that the cells formed colonies on collagen I rather than remaining as single cells, as did the mock cells. In addition, there was no difference in the localization of E-cadherin in cells expressing Rap1GAP, whether they were plated on non-coated or collagen I-coated coverslips (Fig. S2 B). Furthermore, cells expressing Rap1GAP were prevented from increasing their level of phosphorylated JNK in response to collagen I (Fig. 6 D). These data implicate Rap1 in a pathway from the cell surface through FAK/Pyk2/p130CAS to JNK to mediate cell scattering and up-regulation of N-cadherin in response to collagen I.

The MLK3-MKK7-JNK1-cJun cascade plays a crucial role in the response to collagen I

We recently reported that MAPK kinase (MKK)7/JNK1 signaling is important for the response of BxPC3 cells to collagen I (Shintani et al., 2006a). Thus, in this study we focused on signaling between Rap1 and MKK7. MKKs are activated by MKK kinases (MAP3Ks). Several MAP3Ks have been reported and we prepared shRNAs to knock down many of these, including MEK kinases 1, 2, 3, and 4, TGF β activated kinase 1, apoptosis signal-regulating kinases 1 and 2, mixed lineage kinases (MLKs) 1, 2, and 3, dual leucine zipper kinase, and leucine zipper kinase, using sequences from K. Taira's Laboratory (University of Tokyo, Tokyo, Japan; unpublished data). The only shRNA that prevented cell scattering and N-cadherin up-regulation targeted MLK3 (Fig. 7, A and B). To confirm this, we used a second independent shRNA and obtained identical results. Importantly, knocking down MLK3 reduced JNK activity, as indicated by decreased phosphorylation of a cJun substrate in an in vitro kinase assay (Fig. 7 C).

Thus far, we have characterized a novel signaling pathway initiated by interactions with collagen I that requires both integrin and DDR1, is propagated through FAK and Pyk2, requires the p130CAS scaffold, and requires Rap1 GTPase. The outcome is activation of JNK1, using the upstream kinases MKK7 and MLK3. To confirm that activated JNK1 up-regulates expression of N-cadherin, we expressed constitutively active JNK1 in BxPC3 cells. It has been reported that fusing MKK7 to JNK1 results in constitutive JNK1 activity and activates c-Jun in the absence of any stimulus (Zheng et al., 1999). The JNK1 portion of the fusion protein is phosphorylated on both Thr¹⁸³ and Tyr¹⁸⁵ residues by the MKK7 portion of the fusion protein. Overexpression of MKK7-JNK1 increased N-cadherin expression even when cells were plated on noncoated dishes, whereas a control construct with kinase-dead MKK7 did not, showing that activation of JNK is sufficient to up-regulate N-cadherin (Fig. 8). Interestingly, overexpression of MKK7-JNK1 did not induce cell scattering on noncoated dishes, indicating that although JNK1 activation is necessary for cell scattering, it is not sufficient to induce cell scattering in the absence of a collagen I

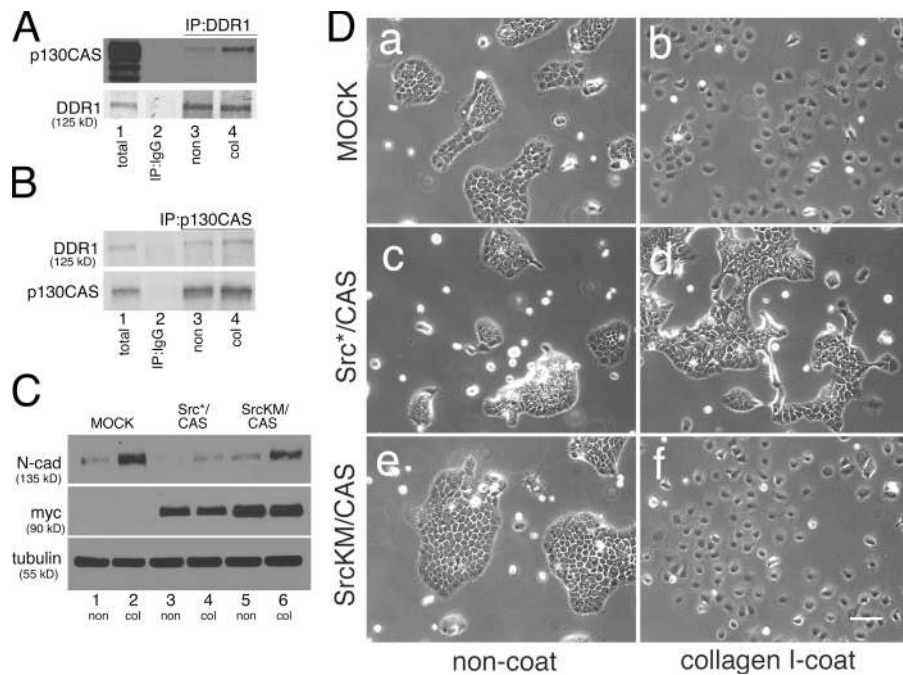


Figure 5. p130CAS plays a key role in collagen I-induced changes in BxPC3 cells. (A) 600 μ g of protein from BxPC3 cells 12 h after plating on noncoated or collagen I-coated dishes was immunoprecipitated with rabbit IgG or anti-DDR1 rabbit pAb and blotted for p130CAS or DDR1. (B) 600 μ g of protein from BxPC3 cells 12 h after plating on noncoated or collagen I-coated dishes was immunoprecipitated with mouse IgG or anti-p130CAS mouse mAb and blotted for DDR1 or p130CAS. (C) BxPC3 cells expressing the neomycin resistance gene (mock), Src*/CAS(SD) (dominant-negative p130CAS), or SrcKM/CAS(SD) (inactive control for dominant-negative p130CAS) were extracted in RIPA buffer 2 d after plating on noncoated or collagen I-coated dishes. 30 μ g of protein was resolved by SDS-PAGE and blotted for N-cadherin, myc tag (to detect p130CAS), or tubulin. (D) Mock BxPC3 cells (a and b), cells expressing Src*/CAS(SD) (c and d), or cells expressing SrcKM/CAS(SD) (e and f) were cultured on noncoated (a, c, and e) or collagen I-coated dishes (b, d, and f) for 2 d. Bar, 100 μ m.

stimulus (Fig. 5 B). These data are consistent with our previous studies (Shintani et al., 2006a) and with data in Fig. S5 (available at <http://www.jcb.org/cgi/content/full/jcb.200708137/DC1>) showing that artificially up-regulating N-cadherin also did not induce cell scattering in the absence of a collagen I signal, indicating that up-regulation of N-cadherin is necessary, but not sufficient, for cell scattering.

The classical downstream target of JNK is cJun; however, JNK does have other cellular targets (Bogoyevitch and Kobe, 2006). To determine if activation of c-Jun is necessary to up-regulate N-cadherin, we overexpressed a dominant-negative form

of cJun (cJun-DN) in BxPC3 cells that not only prevented up-regulation of N-cadherin in response to collagen I but also decreased its expression on noncoated dishes (Fig. 8 C). In addition, overexpression of cJun-DN prevented cell scattering in response to collagen I (Fig. 8 D).

Discussion

Studies from our laboratory and others have shown that various cell types undergo aspects of EMT in response to collagen in the ECM. The Collard laboratory showed that Ras-transformed

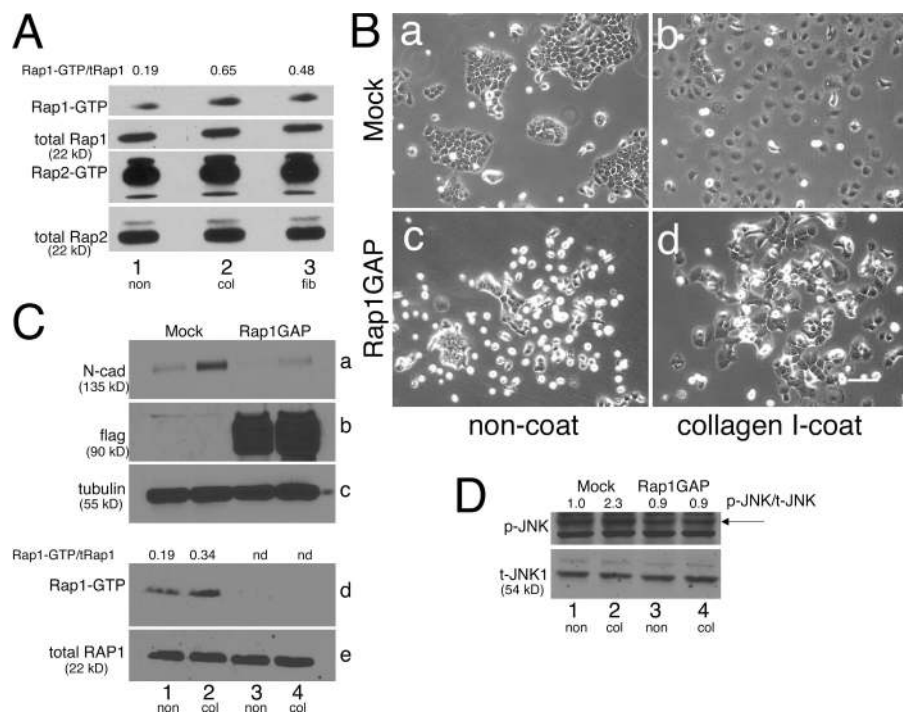
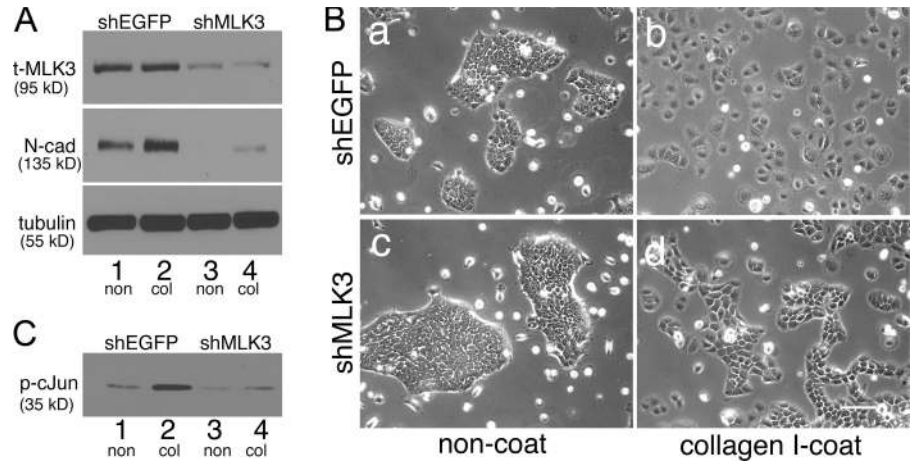


Figure 6. Rap1GAP prevents collagen I-induced changes in BxPC3 cells. (A) 1 mg of protein from BxPC3 cells 4 h after plating on noncoated, collagen I-coated, or fibronectin-coated dishes was incubated with GST-Ral-GDS-coupled beads and resolved by SDS-PAGE. Rap1-GTP, total Rap1, Rap2-GTP, and total Rap2 were detected by Rap1 and Rap2 blots. The ratio of Rap1-GTP/total Rap1 is shown above the gel. (B) Mock BxPC3 cells or cells expressing Rap1GAP were cultured on noncoated (a and c) or collagen I-coated (b and d) dishes for 2 d. Bar, 100 μ m. (C) Mock BxPC3 cells or cells expressing Rap1GAP were extracted and blotted for N-cadherin (a), flag (to detect Rap1GAP; b), or tubulin (c). Rap1 pull-down assays were performed as described in A (d and e). The ratio of Rap1-GTP/total Rap1 is shown (nd, none detected). (D) Mock BxPC3 cells or cells expressing Rap1GAP were extracted and blotted for p-JNK (T183/Y185) and total JNK1. The ratio of p-JNK/t-JNK is shown above the gel. Bands corresponding to p-JNK indicated (arrow).

Figure 7. MLK3 is necessary for collagen I-induced changes in BxPC3 cells. (A) 30 μ g of protein from BxPC3 cells expressing shEGFP or shMLK3 extracted 2 d after plating on noncoated or collagen I-coated dishes was resolved by SDS-PAGE and blotted for total MLK3, N-cadherin, or tubulin. (B) BxPC3 cells expressing shEGFP (a and b) or shMLK3 (c and d) were cultured on noncoated (a and c) or collagen I-coated (b and d) dishes for 2 d. Bar, 100 μ m. (C) In vitro kinase assays were performed using cells expressing shEGFP or shMLK3. Kinase activity was detected using p-cJun pAb (S73).

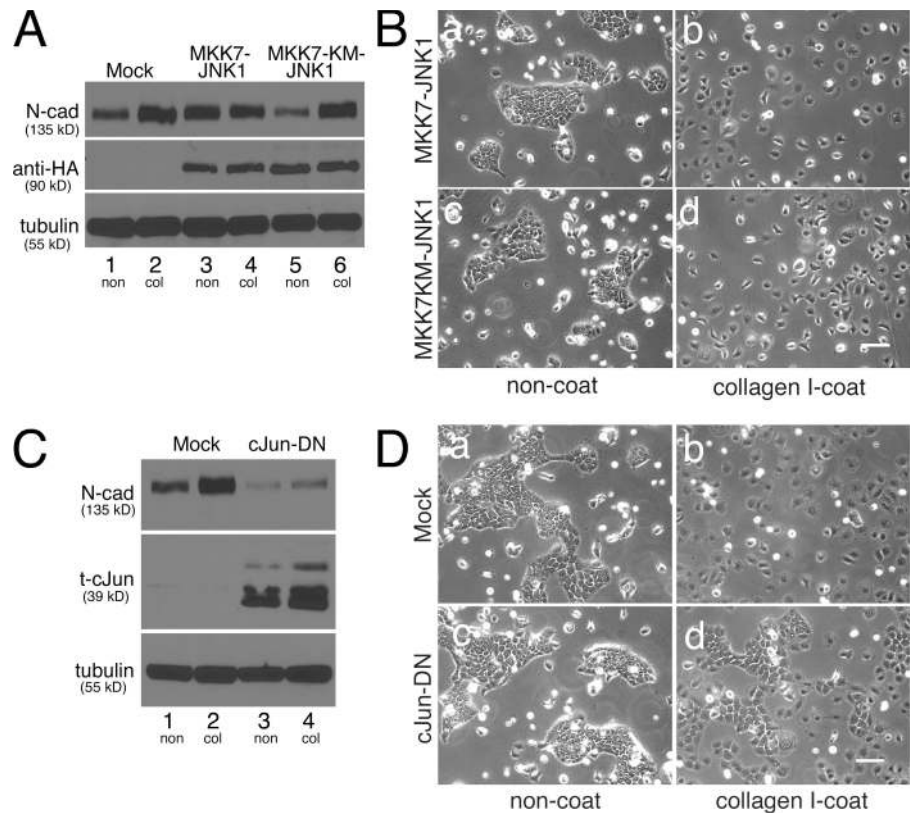


MDCK cells were induced to form E-cadherin-mediated junctions when plated on fibronectin or laminin, but not on collagen, and that the response to collagen was dependent on Tiam1 and Rac (Sander et al., 1998). Our laboratory showed that mouse mammary epithelial cells (NMuMG) respond to collagen by up-regulating N-cadherin and increasing cell motility (Shintani et al., 2006b) and that these changes were dependent on PI3K, Rac, and JNK. In another study, we showed that human lung epithelial cells undergo similar changes in response to collagen, but in this case, collagen induces the cells to release active TGF β 3, which induces EMT via canonical TGF β signaling (Shintani et al., 2008b). We have recently shown that human pancreatic cancer cells also respond to collagen by undergoing EMT-like changes, including up-regulation of

N-cadherin expression and increased metastasis in an animal model for pancreatic cancer (Shintani et al., 2006a). In this case, the cellular changes require JNK activity but not Rac. Thus, the response of cells to collagen varies among different cell types.

Understanding collagen I-induced changes in cellular behavior is critical because many disease states, including chronic kidney disease, lung fibrosis and several cancers, are characterized by extensive deposition of collagen I, which contributes significantly to disease progression (Zeisberg et al., 2001, 2002; Liu, 2004; Keating et al., 2006; Shintani et al., 2006a; Ross et al., 2007). Our laboratory and others have shown that up-regulation of N-cadherin has a profound effect on the ability of tumor cells to invade and metastasize (Nieman et al., 1999; Hazan et al., 2000;

Figure 8. JNK1/cJun activation plays a crucial role in collagen I-mediated changes in BxPC3 cells. (A) Mock BxPC3 cells or cells expressing MKK7-JNK1 (constitutively active JNK1) or MKK7-KM-JNK1 (inactive control) were extracted 2 d after plating on noncoated or collagen I-coated dishes. 30 or 60 μ g of protein was blotted for N-cadherin, HA tag (to detect MKK7/JNK1), or tubulin. (B) BxPC3 cells expressing MKK7-JNK1 (a and b) or MKK7-KM-JNK1 (c and d) were cultured on noncoated (a and c) or collagen I-coated (b and d) dishes for 2 d. Bar, 100 μ m. (C) 30 μ g of protein from mock BxPC3 cells or cells expressing dominant-negative cJun (cJun-DN) were extracted 2 d after plating on noncoated or collagen I-coated dishes, resolved by SDS-PAGE, and blotted for N-cadherin, total cJun, or tubulin. (D) Mock BxPC3 cells (a and b) or cells expressing cJun-DN (c and d) were cultured on noncoated (a and c) or collagen I-coated (b and d) dishes for 2 d. Bar, 100 μ m.



Cavallaro et al., 2002; De Wever et al., 2004; Maeda et al., 2005; Shintani et al., 2006a). Thus, the purpose of the current study was to delineate the signaling pathways that are activated when pancreatic cancer cells are in contact with collagen I to promote N-cadherin up-regulation. Our study had four novel findings. First, the response of pancreatic cancer cells to collagen I requires cooperation between two separate collagen receptors, one an integrin receptor and the other the receptor tyrosine kinase DDR1. Second, the FAK homologue Pyk2 is activated by DDR1 and this activation is essential for the full response of BxPC3 cells to collagen I. This is the first study to report an interaction between DDR1 and Pyk2. Third, p130CAS likely serves as a scaffold for the entire signaling complex. Fourth, we have defined a pathway downstream of the p130CAS signaling module that includes the small GTPase Rap1 rather than the usual suspects, Rac and Cdc42, and signals through MLK3 to MKK7 and ultimately JNK1 to promote up-regulation of N-cadherin (Fig. 9).

Integrins and DDR1 cooperate in collagen I-mediated EMT in BxPC3 cells

Cells can interact with collagen using two different receptor families, the integrins and discoidin domain tyrosine kinases. Integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, and DDR1 are the epithelial cell receptors for collagen I (Hynes, 2002; Vogel et al., 2006). It has been proposed that integrins cooperate with other receptors to amplify or modulate signals, and one type of cooperation is receptor coordination, in which two or more receptors contribute components that are necessary for the downstream event (Miranti and Brugge, 2002). The results of our study suggest that integrin $\beta 1$ and DDR1 coordinately activate JNK to up-regulate N-cadherin expression and promote cell scattering. Knocking down $\beta 1$ integrin expression or DDR1 expression alone each partially inhibited up-regulation of N-cadherin expression in response to collagen I. Likewise, knocking down one receptor or the other partially prevented cell scattering, but it was not until we prevented expression of both receptors that the cells remained in compact epithelial colonies when plated on collagen I. Integrin coordination with receptor tyrosine kinases has been reported. For example, integrin interaction with fibronectin increases the synthesis of PIP2 to enhance the breakdown of PIP2 in response to PDGF, which amplifies downstream signaling by the PDGF receptor (McNamee et al., 1993). In addition, integrins have been reported to coordinate with other adhesion receptors. For example, the formation of focal adhesions in fibroblasts adhering to fibronectin requires the ligation of two separate fibronectin receptors, $\alpha 5\beta 1$ integrin and syndecan-4 (Wilcox-Adelman et al., 2002). The binding sites on fibronectin for integrins and syndecan are separate domains, and cells plated on fragments of fibronectin that contain only the integrin binding site will attach but will not make focal adhesions, nor will they organize actin stress fibers (Saoncella et al., 1999). Thus, coordination between integrins and receptor tyrosine kinases, and coordination between integrins and other ECM receptors have been reported. However, this is the first study to report coordination between integrin collagen receptors and the DDR1 tyrosine kinase collagen receptor.

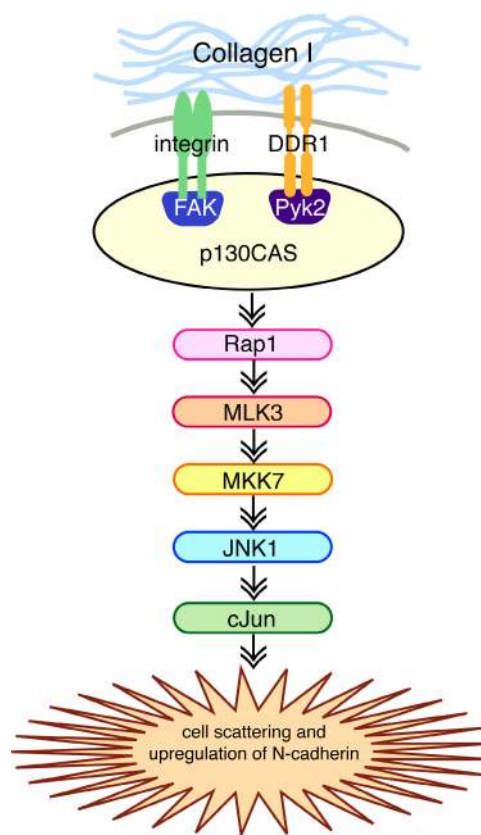


Figure 9. Model of the signaling pathway active in BxPC3 cells from collagen I outside the cell to induction of cell scattering and up-regulation of N-cadherin.

DDR1 is in a complex with Pyk2 and p130CAS

When integrins interact with substrate, they cluster in the membrane and recruit complexes of proteins to form the structure known as a focal adhesion and to transduce signals that promote changes in cellular behavior and phenotype. A central protein in this signaling/structural complex is the nonreceptor tyrosine kinase FAK, which binds to integrin-associated proteins like paxillin and talin (Schaller et al., 1992; Schlaepfer and Hunter, 1998). FAK is autophosphorylated upon integrin clustering, recruiting additional kinases that further phosphorylate FAK to create binding sites for other downstream proteins including the p130CAS scaffold. Pyk2 is structurally homologous to FAK and shares ~45% amino acid identity (Schlaepfer and Hunter, 1998). Although Pyk2 can become associated with integrins upon engagement with ECM proteins, its activation has also been tied to multiple other cellular activities including stress (Orr and Murphy-Ullrich, 2004). In this paper, we show for the first time that Pyk2 is in a complex with the receptor tyrosine kinase DDR1. In addition, we show that activation of Pyk2 is essential for pancreatic cancer cells to undergo the full response to collagen I and that Pyk2 is phosphorylated in pancreatic cancer cells knocked down for integrin $\beta 1$. These data suggest that Pyk2 is activated specifically by its interaction with DDR1.

FAK and Pyk2 share many common signaling partners, including the scaffold protein p130CAS. (Orr and Murphy-Ullrich,

2004). We further show that Pyk2 is associated with DDR1, which is in a complex with p130CAS. p130CAS has been shown to scaffold proteins that activate JNK and ERK, which are upstream activators of transcription factors responsible for regulation of genes involved in cell survival, transformation, migration, and invasion (Defilippi et al., 2006). When we expressed a dominant-negative form of p130CAS in pancreatic cancer cells, up-regulation of N-cadherin expression in response to collagen I was completely prevented, suggesting that p130CAS plays a central role in this response. We showed that integrin or DDR1 binding to collagen each initiated only a partial response to collagen I, suggesting that the two partial responses are integrated by p130CAS to produce the full response. Thus, in this paper we suggest that cooperation between integrin β 1 and DDR1 may be caused by a large complex of proteins interacting with the p130CAS scaffold. This complex includes the receptors integrin and DDR1 together with their immediate downstream effectors, FAK and Pyk2, respectively.

Signaling from p130CAS to up-regulation of N-cadherin expression

Signals downstream of ECM interactions typically involve small GTPases of the Rho family (Kooistra et al., 2007). Surprisingly, when pancreatic cancer cells interacted with collagen I, dominant-negative Rac1, dominant-negative Cdc42, or the CRIB domain of PAK1, which is expected to inhibit the activity of both Rac1 and Cdc42 (Arthur et al., 2004), did not prevent up-regulation of N-cadherin. It has been previously reported that the small GTPase Rap1 can activate JNK downstream of a complex that involves Pyk2 and p130CAS (Blaukat et al., 1999). Indeed, in our system Rap1 was activated when cells were plated on collagen I, and inhibiting Rap1 function prevented cell scattering and N-cadherin up-regulation in response to collagen I. Rap1 can be activated by integrins, receptor tyrosine kinases, G protein-coupled receptors, and other extracellular stimuli, and its activation, like that of other small GTPases, influences numerous cellular processes (Arthur et al., 2004). Of particular interest to our study, Rap1 has been shown to mediate inside/out integrin signaling by controlling integrin affinity for substrate and integrin clustering within the membrane (Caron et al., 2000; Reedquist et al., 2000; Bos, 2005). In addition, Rap1 plays a role in the organization of adherens junctions (Bos, 2005). *Drosophila melanogaster* cells mutant for Rap1 show disorganized adherens junctions and increased invasion into surrounding tissues (Knox and Brown, 2002), which has been confirmed in mammalian cells (Yajnik et al., 2003; Price et al., 2004). These studies place Rap1 in an interesting position to coordinate signaling emanating from cell-cell adhesion systems with those cells obtained from their extracellular environment. A recent study (Zhang et al., 2006) implicated Rap1GAP, a negative regulator of Rap1 function, as a tumor suppressor that is frequently lost during pancreatic cancer progression and showed that over-expressing Rap1GAP limited tumor growth and metastasis in an orthotopic mouse model for pancreatic cancer. These authors did not investigate expression of N-cadherin in their pancreatic cancer model, but it would be interesting to examine cadherin switching in their model.

Signaling from Rap1 to JNK1

In the signaling cascades leading to JNK, there are two members of the MKK family called MKK4 and 7, which are immediately upstream (Guo and Giancotti, 2004). BxPC3 cells lack MKK4 (Wang et al., 2004; Shintani et al., 2006a) and knocking down MKK7 inhibits collagen I-mediated up-regulation of N-cadherin. Upstream of MKKs, several different members of the MAP3K family have been identified. In the current study, we used shRNA targeted to a dozen MAP3Ks, and the only shRNA constructs that prevented cell scattering and N-cadherin up-regulation in response to collagen I were those that targeted MLK3. Thus we are proposing here that MLK3 is directly upstream of MKK7 in our system.

Summary

We have presented data and discussion supporting a pathway that includes both novel and previously validated components (Fig. 9). This paper is the first to report cooperation between the integrin and DDR1 collagen receptors to promote EMT-like changes in a tumor cell. Likewise, it is the first to report DDR1 activating Pyk2. In this paper, we propose that p130CAS serves as a scaffold to bring together a complex of the collagen receptors and their respective downstream effectors, FAK and Pyk2. Another unique feature of this pathway is the involvement of the small GTPase, Rap1. Future studies in our laboratory will be focused on determining if we can interfere with various components of this pathway to prevent pancreatic cancer progression in our orthotopic model for pancreatic cancer.

Materials and methods

Reagents, antibodies, and cultured cells

All reagents were obtained from Sigma-Aldrich or Thermo Fisher Scientific unless otherwise indicated. Mouse mAb against E-cadherin (HECD-1) was a gift from M. Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan). Mouse mAb against N-cadherin (13A9) has been previously described (Johnson et al., 1993). Anti-FAK phosphospecific (Y577) rabbit polyclonal antibody (pAb), anti-Pyk2 phosphospecific (Y579/Y580) rabbit pAb, and anti-JNK phosphospecific (T183/Y185) rabbit pAb were obtained from Invitrogen. Anti-integrin β 1 mouse mAb, anti-FAK mouse mAb, anti-Pyk2 mouse mAb, anti-phosphotyrosine (PY20) mouse mAb, anti-p130CAS mouse mAb, anti-JNK1 mouse mAb, and anti-Rap2 mouse mAb were obtained from BD Biosciences. Anti-DDR1 rabbit pAb (C-20), anti-Rap1 rabbit pAb, and anti-MLK3 rabbit pAb were obtained from Santa Cruz Biotechnology, Inc. Anti-c-Jun rabbit pAb and anti-human integrin α 2 mouse mAb (HAS3) were obtained from Cell Signaling Technology. Anti-integrin α 5 rabbit pAb and anti-human integrin α 5 mouse mAb (SAM-1) were obtained from EMD. Anti-human integrin β 1 mouse mAb (4B4) was obtained from Beckman Coulter. Anti-tubulin mouse mAb was obtained from Developmental Studies Hybridoma Bank. Anti-myc epitope mouse mAb (9E10) was a gift from K. Green (Northwestern University, Chicago, IL).

Human BxPC3 has been previously described (Shintani et al., 2006a). Capan-1, Panc-1, and CFPAC were obtained from American Type Culture Collection. Cells were maintained in DME or RPMI containing 10 or 20% (Capan-1) FBS (Hyclone). Substrate-coated dishes and rat tail collagen I were obtained from BD Biosciences. Serum was reduced to 1% to examine signals primarily from adhesion to ECM.

Detergent extraction, SDS-PAGE, immunoblot, immunoprecipitation, and kinase assays

Monolayers of cultured cells were extracted with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, and 0.2 U/ml aprotinin) for SDS-PAGE or Tris/NP40/EDTA (TNE) buffer (10 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, and 1 mM EDTA) and immunoprecipitated as previously described (Wahl et al., 2003).

Protein was determined using a protein assay kit (Bio-Rad Laboratories). Pull-down assays for Rac1 and Cdc42 were performed as previously described (Johnson et al., 2004). The cDNA encoding Ral-GDS was obtained from American Type Culture Collection (BC059362). AA776-872 was PCR amplified (Herrmann et al., 1996) and ligated into pGEX-2T for production of the GST fusion in Rap pull-down assays. JNK activity was assayed by a KinaseSTAR JNK Activity Screening kit (BioVision). Immunoblots were quantified by densitometry using Photoshop (Adobe).

Flow cytometry

To stain surface integrins, cells were suspended in DME, 1% FBS, and 0.03% sodium azide and incubated for 30 min at 4°C with 10 µg/ml mAbs. After washing, the cells were incubated with FITC-conjugated anti-mouse for 30 min at 4°C and analyzed using a FACSCalibur (BD Biosciences).

Constructs, transfection, and infection

Phoenix cells (Grignani et al., 1998) were transfected using TransIT-LT1 reagent (Mirus) with LZRS-MS-Neo, pSUPER.retro.puro, or pSUPER.retro.neo (OligoEngine) and selected in 1 mg/ml G418 or 2 µg/ml puromycin. Viral expression and cell infection have been previously described (Maeda et al., 2005). Human integrin α5 cDNA was from American Type Culture Collection (BC008786). A Sap1 (blunted) + Nsil fragment was moved into a retroviral vector derived from LZRS (Iretton et al., 2002). FRNK was a gift from T. Parsons (University of Virginia, Charlottesville, VA). N-terminal GFP-tagged Rac1N17 and Cdc42N17 (gifts from K. Hahn, University of North Carolina, Chapel Hill, NC) were moved into LZRS. To prepare the GFP-CRIB construct, the CRIB domain from PAK1 was ligated into monomeric (A206K) pEGFP-C and the GFP fusion construct was moved into a retroviral vector derived from LZRS (Johnson et al., 2004). Src*/CAS(SD) and SrcKM/CAS(SD) (gifts from A. August [Pennsylvania State University, University Park, PA] and K. Kirsch [Boston University School of Medicine, Boston, MA]), MKK7-JNK1 and MKK7KM-JNK1 (gifts from A. Lin, University of Chicago, Chicago, IL), and Flag-tagged Rap1GAP (gift from L. Quilliam, Indiana University School of Medicine, Indianapolis, IN) were moved into a retroviral vector derived from LZRS. The dominant-negative c-Jun (S63A; S73A) was constructed by R. Wisdom (University of California, Davis, Davis, CA; obtained from F. Martin, Conway Institute, Dublin, Ireland) and moved into LZRS.

Target sequences for shRNA constructs were as follows: integrin β1, GCGCATATCTGGAAATTG (Cordes et al., 2006); Pyk2, AGCCGAGTA-AAGTTGGGCA (Yano et al., 2004); DDR1, AGATGGAGTTGAGTTGACC (as suggested by W. Vogel, University of Toronto, Ontario, Canada). Targets sequences for MAP3Ks were kindly provided by S. Matsumoto and M. Miyagishi (University of Tokyo, Tokyo, Japan) and the entire list is available upon request. To knockdown MLK3, we used the following nucleotides inserted into pSUPER.retro: 5'-GATCCCCGATGCCACTT-GACTTCA-GGCGTGTGCTGTCCGCTTGAAGTCGAGTGGCATGCTTTTAA-3' and 5'-AGCTTAAAAAGCA-TGCCACTCGACTTCAAGCGGACAGCACA-CGCCTGAAGTCAAGTGGCAGCAGGG-3'; or 5'-GATCCCCGTTGGGG-CCTGTAGAGCTAGGGTGTGCTGTCCCTGGCTGTGCAGGCTCCCAAC-TTTTAA-3' and 5'-AGCTTAAAAAGTGGAGCCTGCAGAGCCAGGGGA-CAGCACACCC-TAGCTCTACAGGCCCAACGGG-3'.

Image acquisition and manipulation

Cells were fixed with HistoChoice Tissue Fixative (Amresco), processed as previously described (Shintani et al., 2006b), mounted in Vectashield (Vector Laboratories), and examined on a microscope (Axiovert 200M; Carl Zeiss, Inc.) equipped with a digital camera (ORCA-ER; Hamamatsu). Secondary antibodies for immunofluorescence microscopy were labeled with FITC (Jackson ImmunoResearch Laboratories). Images were collected at room temperature and processed using SlideBook software (Intelligent Imaging Innovations) and composite images were composed in Photoshop. Phase micrographs were taken using a 10× 0.25 numerical aperture air objective (A-plan; Carl Zeiss, Inc.). Fluorescence micrographs were taken using a 100× 1.4 numerical aperture oil objective (Plan-Acrochrome; Carl Zeiss, Inc.; Fig. S2 B) or a 40× 1.3 numerical aperture oil objective (Plan-Neo; Carl Zeiss, Inc.; Fig. S4).

Online supplemental material

Fig. S1 shows integrin expression and signaling in BxPC3 cells. Fig. S2 shows characterization of cells knocked down for DDR1 or integrin β1 or expressing FRNK or Rap1GAP. Fig. S3 shows that DDR1 plays a role in collagen I-induced changes in various pancreatic cancer cells. Fig. S4 shows that Rac1 and Cdc42 are not necessary for collagen I-induced changes. Fig. S5 shows that overexpression of N-cadherin does not restore scattering on collagen I in cells knocked down for both DDR1 and

integrin β1. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200708137/DC1>.

We thank Drs. Takeichi, Green, Parsons, Hahn, August, Kirsch, Lin, Quilliam, Wisdom, Martin, Matsumoto, Miyagishi, Y. Kim and Vogel for reagents, constructs and useful information.

This work was supported by National Institutes of Health grants R01-DE12308 (K.R. Johnson) and R01-GM51188 (M.J. Wheelock) and by grant NCI P30 CA36727 to the Eppley Institute.

Submitted: 21 August 2007

Accepted: 25 February 2008

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