Up-regulation of N-cadherin by Collagen I-activated Discoidin Domain Receptor 1 in Pancreatic Cancer Requires the Adaptor Molecule Shc1*

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Pancreatic ductal adenocarcinomas are highly malignant cancers characterized by extensive invasion into surrounding tissues, metastasis to distant organs, and a limited response to therapy. A main feature of pancreatic ductal adenocarcinomas is desmoplasia, which leads to extensive deposition of collagen I. We have demonstrated that collagen I can induce epithelial-mesenchymal transition (EMT) in pancreatic cancer cells. A hallmark of EMT is an increase in the expression of the mesenchymal cadherin N-cadherin. Previously we showed up-regulation of N-cadherin in pancreatic cancer cells derived from epithelial tissues inappropriately polarized, typically express N-cadherin (10). However, some tumor immunologic tolerance (2, 4). Previously, our laboratory showed that collagen I can induce epithelial-mesenchymal transition (EMT)2-like changes in pancreatic cancer mediated by two collagen receptors, α2β1-integrin and discoidin domain receptor 1 (DDR1). DDR1 is a receptor-tyrosine kinase widely expressed during embryonic development and in many adult tissues and is also highly expressed in many different cancers. In the signaling pathway initiated by collagen, we have shown proline-rich tyrosine kinase 2 (Pyk2) is downstream of DDR1. In this study we found isoform b of DDR1 is responsible for collagen I-induced up-regulation of N-cadherin and tyrosine 513 of DDR1b is necessary. Knocking down Shc1, which binds to tyrosine 513 of DDR1b via its PTB (phosphotyrosine binding) domain, eliminates the up-regulation of N-cadherin. The signaling does not require a functional SH2 domain or the tyrosine residues commonly phosphorylated in Shc1 but is mediated by the interaction between a short segment of the central domain of Shc1 and the proline-rich region of Pyk2. Taken together, these data illustrate DDR1b, but not DDR1α, mediates collagen I-induced N-cadherin up-regulation, and Shc1 is involved in this process by coupling to both DDR1 and Pyk2.

Pancreatic cancer is currently the fourth leading cause of cancer-related mortality in the United States with a 5-year survival rate of ~6% (American Cancer Society, 2014). This unfortunate outcome is mainly due to the aggressive behavior of this cancer and the lack of symptoms early in the disease. One of the major features of pancreatic cancer is that during the early stages an extensive stromal reaction occurs called desmoplasia. A dense stroma forms, which consists of myofibroblasts (pancreatic stellate cells), abundant extracellular matrix, and different types of inflammatory cells including macrophages, mast cells, lymphocytes, and plasma cells (1, 2). The extracellular matrix components include collagens, fibronectin, hyaluronic acid, and proteoglycans, whereas the major part is type I collagen (3, 4).

The atypical stroma plays many roles in pancreatic cancer. It changes the normal architecture of pancreatic tissue and induces an abnormal configuration of blood and lymphatic vessels resulting in a hypovascular and hypoxic microenvironment. It also presents a barrier to drug delivery and promotes tumor immunologic tolerance (2, 4). Previously, our laboratory showed that collagen I can induce epithelial-mesenchymal transition (EMT)2-like changes in pancreatic cancer mediated by two collagen receptors, α2β1-integrin and discoidin domain receptor 1 (DDR1) (5). EMT is a biologic process that enables polarized epithelial cells to attain a mesenchymal cell phenotype, thus enhancing migratory capacity and invasiveness, so cells can migrate away from the original epithelial layer (6). EMT plays an important role in tumor progression and metastasis in many solid tumors including pancreatic cancer (7).

During EMT, a process called cadherin switching often happens (8, 9). Most normal epithelial tissues in which cells are polarized express the epithelial adhesion molecule E-cadherin, whereas mesenchymal cells, which are more motile and less polarized, typically express N-cadherin (10). However, some cancer cells derived from epithelial tissues inappropriately express N-cadherin and lose E-cadherin function. Our labora-

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2 The abbreviations used are: EMT, epithelial-mesenchymal transition; DDR1, discoidin domain receptor 1; RTK, receptor-tyrosine kinase; Shc1, Src homology and collagen homology 1; SH2, Src homology 2; (m)EGFP, (monomeric) EGFP; Pyk2, proline-rich tyrosine kinase 2; FAT, focal adhesion targeting; PTB, phosphotyrosine binding; JM, juxtamembrane.
tory has shown that N-cadherin expression by tumor cells promotes cell invasion and metastasis regardless of their E-cadherin expression (11). We found in pancreatic cancer cells, collagen I can mediate up-regulation of N-cadherin and a cell scattering morphological change through cooperative signals from α2β1-integrin and DDR1, thus promoting metastasis (5, 12).

DDR1 is type I transmembrane receptor-tyrosine kinase (RTK) activated by collagen, and it has a structure similar to many members of the RTK family (13). However, it is unique among RTKs because it is activated by binding to collagen through the N-terminal discoidin domain and by the unusually slow kinetics of activation (14). The discoidin domain is followed by an extracellular (JM) membrane region, then a single transmembrane (TM) domain and a large cytosolic JM domain. A catalytic kinase domain follows the cytosolic JM domain and, at the very end, a short C-terminal tail.

Transcripts from the DDR1 gene are reported to be alternatively spliced to generate five DDR1 protein isoforms (a–e). The isoforms termed DDR1a and DDR1b are the most widely distributed, whereas the others are much less common, and in addition isoforms d and e are predicted to lack kinase activity (15). DDR1a lacks a 37-residue segment present in the cytosolic juxtamembrane region of DDR1b. DDR1b thus contains two tyrosines (Tyr-513 and Tyr-520) missing in DDR1a. After phosphorylation, these tyrosines are reported to bind proteins such as Src homology and collagen homology 1 (Shc1) and c-src tyrosine kinase (Csk), respectively (14, 16, 17).

The current study was designed to identify the early steps in the signaling pathway that are initiated downstream of DDR1 when pancreatic cancer cells are exposed to collagen. Our experiments identified which isoform of DDR1 is responsible for collagen I-induced up-regulation of N-cadherin and also identified the proteins that interact with DDR1 to initiate the signaling pathway and the regions of the proteins necessary for signaling.

Results

**DDR1b Is More Potent Than DDR1a in Mediating Collagen I-induced Up-regulation of N-cadherin in Pancreatic Cancer Cells**—We examined the expression of DDR1 and N-cadherin in tissues from human pancreatic cancer patients, comparing cancerous with uninvolved tissues (Fig. 1). The immunohistochemistry showed that both DDR1 and N-cadherin are up-regulated in primary pancreatic cancer tissues as well as in liver metastases. Moreover, we performed correlation analysis in both primary and metastatic sites and found that expression levels of DDR1 and N-cadherin in the cancerous tissues are highly correlated (Fig. 1c).

We have shown that collagen I can activate DDR1 and induce the up-regulation of N-cadherin (5). As noted above, DDR1a and DDR1b are the two major isoforms of DDR1. We first determined which isoforms are expressed in several pancreatic cancer cell lines by performing RT-PCR with primers flanking the alternatively spliced exon to semiquantitatively measure the mRNA levels of DDR1a and DDR1b. We found that both DDR1a and DDR1b are expressed in BxPC-3, T3M-4, and L3.6pl cells, and the expression levels of transcripts encoding DDR1a and DDR1b are similar (Fig. 2a). We then set out to test if both of them could mediate collagen I-induced up-regulation of N-cadherin. We used L3.6pl cells to dissect the details of the DDR1 signaling pathway because we found the response of these cells to collagen is primarily mediated by DDR1 arm of the pathway.

We stably knocked down DDR1 in L3.6pl cells with a lentiviral shRNA targeting the 3′-UTR. DDR1 knockdown cells as well as control cells were plated on collagen I-coated dishes (Fig. 2b). We found that after DDR1 knockdown, collagen I failed to induce N-cadherin up-regulation when compared with the control. We then introduced either DDR1a-mEGFP or DDR1b-mEGFP in the DDR1 knockdown cell lines (Fig. 2b). We found that up-regulation of N-cadherin in response to collagen I was fully rescued in cells expressing DDR1b but not in cells expressing DDR1a.

Protein kinases such as DDR1a and DDR1b have residues termed gatekeepers that limit access to the ATP-binding pocket (18). In DDR1a and DDR1b, the gatekeeper residues are Thr-664 and Thr-701, respectively. When the gatekeeper residues are mutated to Ala or Gly, the mutant kinases have larger ATP binding pockets than wild-type DDR1. As a result, the mutant enzymes ought to be more sensitive to protein kinase inhibitors such as 1NM-PP1 (19). This approach allows the use of inhibitors such as 1NM-PP1 to mimic the effects of a specific DDR1 inhibitor. We mutated the gatekeeper residues in DDR1a and DDR1b to either Gly or Ala and expressed the mutants fused to monomeric EGFP (mEGFP) in PSN1 cells, a human pancreatic cancer cell line lacking endogenous DDR1 (Fig. 3a). The mutants with Gly in the gatekeeper position were no longer active (results not shown), whereas those with Ala in the gatekeeper position were activated by collagen.

The collagen I-induced tyrosine phosphorylation of DDR1a-mEGFP (T664A) and DDR1b-mEGFP (T701A) was inhibited by 1NM-PP1. Importantly, the collagen-dependent phosphorylation of wild-type DDR1a and DDR1b was not affected by the drug (Fig. 3a). We then performed a Boyden chamber assay with Matrigel-coated filters to test the effects of DDR1 with gatekeeper mutation on invasion of L3.6pl cells with endogenous DDR1 knocked down. We found DDR1b, but not DDR1a promoted cell invasion, and this invasion was inhibited by 1NM-PP1 (Fig. 3b). We plated the L3.6pl cells expressing the gatekeeper mutants on collagen I and found that in the presence of 1NM-PP1 DDR1b-mEGFP (T701A) failed to rescue the up-regulation of N-cadherin (Fig. 3d). The data show the kinase activity of DDR1b, but not DDR1a, is necessary to mediate collagen I-induced up-regulation of N-cadherin and to increase cell invasiveness.

Recently, specific inhibitors of DDR1 have been developed (20, 21). The specific inhibitor 7rh (22) inhibited collagen I-dependent up-regulation of N-cadherin (Fig. 3f), consistent with the results we obtained with 1NM-PP1 and the gatekeeper mutant form of DDR1b (Fig. 3d). As mentioned above, in pancreatic cancer cell lines we have shown collagen can signal through both integrins and DDR1 (5). L3.6pl cells respond to collagen almost exclusively through the DDR1 arm of this signaling pathway, whereas the inhibition seen with 7rh in
T3M-4 was partial, presumably due to signaling through integrins (Fig. 3f).

Tyrosine 513 of DDR1b Plays a Crucial Role in Collagen I-induced Up-regulation of N-cadherin—As we discussed earlier, due to alternative splicing, DDR1b has an extra 37-residue segment in the cytosolic juxtamembrane region, which includes two tyrosines (Tyr-513 and Tyr-520) that are lacking in DDR1a. Because DDR1 is a tyrosine kinase, we focused on these tyrosines and constructed the DDR1b-mEGFP Y513F, Y520F, and Y513F/Y520F (YFYF) mutants. The mutants as well as wild-type DDR1b-mEGFP were expressed in L3.6pl cells with endogenous DDR1 knocked down (Fig. 4a). We found that up-regulation of N-cadherin was fully restored only when Tyr-513 was intact.

Previously we showed that activation of JNK1 is necessary for collagen I-dependent up-regulation of N-cadherin (5). Activation of JNK1 is indicated by the phosphorylation of Thr-183 and Tyr-185 (23). In knockdown cells, we found JNK1 was activated in response to collagen I when the knockdown cells expressed DDR1b-mEGFP but not when they expressed DDR1b-mEGFP Y513F (Fig. 4c). This shows that Tyr-513 of DDR1b is critical for collagen I-induced signaling to JNK1 and the resulting up-regulation of N-cadherin.

Shc1 Interacts with Tyr-513 of DDR1b through Its Phosphotyrosine Binding (PTB) Domain, and the Interaction Is Necessary for the Up-regulation of N-cadherin—Because Tyr-513 is responsible for the collagen-I activated signaling pathway, we sought to determine which protein interacts with this residue to mediate the downstream signaling. Tyr-513 of human DDR1 is in the sequence NPAY. Many vertebrate species including Xenopus and Zebrafish have the sequence NPAY embedded in homologous sequences that are encoded by orthologous exons (not shown).

Shc1 is a prototypical signaling adaptor with three isoforms, p66, p52, and p46, named according to relative molecular weight (24). All three isoforms of Shc1 have a C-terminal Src homology 2 (SH2) domain, which can bind to phosphotyrosine, as well as an N-terminal PTB domain, which also binds phos-
phototyrosine residues including phospho-Tyr-513 of DDR1b (14, 17, 25). The two domains are connected by a central colla-
gen homology 1 (CH1) region containing consensus tyrosine residues that can be phosphorylated and serve as recognition motifs for other signaling effectors (24). The three isoforms share the same PTB-CH1-SH2 region and are only different at their N termini (see Fig. 6a). p46Shc1 and p52Shc1 are gener-
ated from the same transcripts due to translation initiation at different start codons, whereas p66Shc1 is encoded by trans-
cripts initiated from a different promoter (26).

To determine if Shc1 interacts with DDR1b in pancreatic cancer cells, we performed co-immunoprecipitation assays. Only DDR1b-mEGFP was co-immunoprecipitated by antibod-
ies to Shc1, and the interaction was collagen-dependent. Mutating Tyr-513 largely prevented the co-immunoprecipita-
tion (Fig. 5a). This suggested there was a direct collagen-depen-
don interaction between Tyr-513 of DDR1b and Shc1.

Point mutations have been identified that inactivate the PTB and SH2 domains of Shc1 (24). Using the amino acid numbers from mouse p52Shc1 (NP_035498.2), R175Q inactivates the PTB domain, and R397K inactivates the SH2 domain (27). Phosphorylation of Tyr-239/Tyr-240 and Tyr-313 of Shc1 is important in binding the SH2 domain of another adaptor protein Grb2 (28). We reintroduced 3× FLAG-tagged wild-type or mutated mouse p52Shc1 (R175Q, R397K, Y313F (mutant 1F), Y239F/Y240F (mutant 2F), Y239F/Y240F/Y313F (mutant 3F)) into L3.6pl cells with endogenous Shc1 knocked down and found that only the PTB domain of Shc1 is necessary for collagen I-dependent up-regulation of N-cadherin. Neither inacti-
vation of the SH2 domain nor mutation of the tyrosine residues prevented up-regulation of N-cadherin (Fig. 6b). Because Tyr-
513 is within the Shc1 PTB domain consensus binding motif LLSNPAY (29), these results further support that up-regulation of N-cadherin induced by collagen I is mediated by interaction between Tyr-513 of DDR1b and the PTB domain of Shc1.

In the Collagen I-induced Up-regulation of N-cadherin Shc1 Interacts with Focal Adhesion Kinase-related Protein-tyrosine Kinase (Pyk2)—In the collagen I-induced up-regulation of N-cadherin signaling pathway, we have shown that Pyk2 is downstream of DDR1 (5). The structure of Pyk2 includes an N-terminal protein 4.1, ezrin, radixin, moesin (FERM) domain, a central kinase domain, two proline-rich motifs (PR1, PR2), and a C-terminal focal adhesion targeting (FAT) domain (see Fig. 9a) (30, 31). Using endogenous proteins in L3.6pl cells, we observed collagen I-dependent association between DDR1 and Pyk2 (Fig. 7a, lanes 1 and 8). Using L3.6pl cells with endogenous DDR1 knocked down, we showed that Tyr-513 of DDR1b is
necessary for the collagen I-dependent co-immunoprecipitation of DDR1b and Pyk2 (Fig. 7a).

To determine if Shc1 provides the connection between endogenous DDR1 and Pyk2, we performed co-immunoprecipitations with an antibody to Shc1 using lysates from L3.6pl cells. We found the interaction between endogenous Shc1 and Pyk2 was detectable even in the absence of collagen, but exposure to collagen dramatically enhanced the interaction (Fig. 7d, lanes 1 and 8). In addition, Pyk2 that co-immunoprecipitated with Shc1 after exposure of cells to collagen I was activated as...
shown by an increased signal with anti-phospho-Tyr-402 antibodies. We also observed that Tyr-513 of DDR1b is necessary for the strong association between Shc1 and Pyk2 (Fig. 7d).

When they were transiently expressed in Phoenix cells (a derivative of HEK293T; Ref. 32), Shc1 and Pyk2 co-immunoprecipitated with one another. We took advantage of this to more carefully characterize the interaction between Shc1 and Pyk2. We transiently expressed the three different isoforms of mouse Shc1 together with wild-type rat Pyk2 (NP_059014, 1009 amino acids, with a C-terminal myc tag) or the Pyk2 mutants Y402F and K457A (kinase dead; Ref. 33) in Phoenix cells (Fig. 7b). Phosphorylation at Tyr-402 of Pyk2 provides a binding site for the SH2 domain of Src-family tyrosine kinases. The recruitment of Src-family kinases leads to phosphorylation of Tyr-579 and Tyr-580 within the activation loop, which increases Pyk2 kinase activity (34–36).

We found that all three Shc1 isoforms interact with Pyk2 (Fig. 7b), which is consistent with our earlier finding that all three isoforms of Shc1 rescue collagen-dependent up-regulation of N-cadherin (Fig. 5e). The interaction of all three isoforms of Shc1 with Pyk2 was partially dependent on Tyr-402 but strongly dependent on the kinase activity of Pyk2 (Fig. 7b). We also tested a rat Pyk2 construct with L176A/Q177A mutations in the FERM domain that disrupt the interaction of Pyk2 with calmodulin (37) and found Shc1 interacted with this mutant (not shown) suggesting that calcium signaling does not play a major role in the collagen-dependent interaction between Shc1 and Pyk2.
We transiently expressed Pyk2 and 3× FLAG-tagged wild-type or mutated mouse p52Shc (R175Q, R397K, Y313F, Y239F/Y240F, Y239F/Y240F/Y313F) in Phoenix cells and performed co-immunoprecipitations (Fig. 7f). We found that all the mutated forms of p52Shc interacted with Pyk2. Thus we ruled out the possibilities that Pyk2 interacts with the PTB or SH2 domains of Shc1. In addition, the interaction between Pyk2 and the 3F mutant of Shc1 strongly suggests the interaction between Shc1 and Pyk2 is not mediated by Grb2 as Grb2 is known to couple to one or more of these sites. We do not know why kinase-dead Pyk2 failed to bind Shc1, especially because further mapping (see below) showed the kinase domain itself was not needed for the interaction between Shc1 and Pyk2. We also observed a significant signal for phosphorylated Tyr-402 when Phoenix cells expressed the kinase-dead mutant. Even though it is commonly thought to

FIGURE 5. DDR1b up-regulates N-cadherin through Shc1. a, L3.6pl cells were infected with viral shRNA constructs targeting EGFP (lanes 1 and 8) or DDR1 (lanes 2–7 and 9–14). The DDR1 knockdown cells were then infected with viral constructs encoding various forms of DDR1 fused to mEGFP (lanes 3–7 and 10–14). Endogenous Shc1 was immunoprecipitated (IP), and the immunoprecipitates were blotted with antibodies to DDR1 or Shc1. The amount of DDR1 in the lysates is shown in the bottom panel. In some experiments, lysates of cells expressing DDR1-mEGFP fusion proteins had a protein migrating at the position of endogenous DDR1. However, these proteins only show collagen-dependent association with Shc1 if the full-length fusion protein does, suggesting they are not endogenous DDR1. An antibody against GFP did not recognize these bands but detected bands at the size expected for mEGFP (data not shown). These data are consistent with cleavage of the mEGFP tag from the fusion proteins. b, Shc1 was knocked down in L3.6pl cells with four different shRNAs targeting the 3′-UTR (shShc1 991, 997, 208, 434). shRNA targeting EGFP was used as a control. The three major protein isoforms of Shc1 (p66, p52, and p46, see Fig. 6a) were detected. c, cells expressing shRNA targeting EGFP as well as Shc1 knockdown cells were cultured on uncoated (w/o col I) or collagen-coated plates (col I) for 18 h. Lysates were run on SDS gels and blotted for N-cadherin or β-tubulin. Cells expressing shRNAs 208 and 434 were selected for further experiments. d, expression levels of N-cadherin in all the lanes of c were normalized to β-tubulin. Pairwise comparisons between the lane of L3.6pl with shRNA targeting EGFP on uncoated plates and other lanes were performed using Student’s t test. Data are presented as the mean ± S.D. (n = 3). The asterisks reflect significance (***, p < 0.001). e, cells expressing shRNA constructs targeting Shc1 (lanes 2–9 and lanes 11–18) were stably infected with viral constructs encoding one of the isoforms of Shc1: p46Shc1 (lanes 4, 7, 13, and 16), p52Shc1 (lanes 5, 8, 14, and 17), or p66Shc1 (lanes 6, 9, 15, and 18). Cells were cultured on uncoated (w/o col I) or collagen-coated plates (col I) for 18 h. Cell extracts were blotted with antibodies for N-cadherin or β-tubulin. All three isoforms of Shc1 rescued collagen I-dependent up-regulation of N-cadherin. f, expression levels of N-cadherin in all the lanes of e were normalized to β-tubulin. Pairwise comparisons between lane 1 and other lanes were performed using Student’s t test. Data are presented as the mean ± S.D. (n = 3). The asterisks reflect significance (***, p < 0.001).
be an autophosphorylation site, these data suggest endogenous tyrosine kinases can phosphorylate Tyr-402 in the kinase-dead construct. One candidate is endogenous Pyk2, although Phoenix cells express relatively low levels of endogenous Pyk2 (not shown). The signal with anti-phosphoTyr-402 seen in cells expressing the Y402F mutant is probably due to endogenous Pyk2 expressed by Phoenix cells. Other likely candidates are the Src-family kinases (36).

A Short Sequence in the Central Domain of Shc1 Is Necessary for the Interaction with the Proline-rich Region of Pyk2—Because neither the PTB nor SH2 domains of Shc1 are needed for its interaction with Pyk2, we made a series of truncated mouse p46Shc1 constructs (Fig. 8a). We expressed Pyk2 and mouse 2×HA-tagged wild-type or truncated p46Shc1 (T1, T2, T3, T4, T5, T6, T7) in Phoenix cells and performed co-immunoprecipitations with an antibody against the HA tag (Fig. 8b). We found that neither T1 nor T3 interacted with Pyk2, but T7, as well as the longer constructs, did. This indicates that interaction of mouse Shc1 with Pyk2 requires the sequence RPTLP-SAQMS. Because the sequences of mouse and human Shc1 differ in this region (Fig. 8a), we prepared truncated human Shc1 constructs and found the same region of human Shc1 was also required for the interaction between human Shc1 and Pyk2 (not shown).

We then transiently expressed different Pyk2 constructs together with wild-type p46Shc1 in Phoenix cells to identify which region of Pyk2 interacts with Shc1 (Fig. 9a). Co-immunoprecipitation was performed, and we found that amino acids 700–840 were necessary and sufficient for Pyk2 to interact with Shc1 (Fig. 9b). This region contains 42 amino acids (739–780) encoded by an alternatively spliced exon. The shorter Pyk2 isoform thus encodes 967 amino acids (38, 39). We inserted a mouse EST (AI153373.1) encoding amino acids 635–967 of the shorter isoform of Pyk2 (NP_766086.2) into pEGFP-C2. Transient expression of this construct with p52Shc1 showed the two proteins still interacted (not shown). Thus, Shc1 will interact with either isoform of Pyk2.

We tested if the interaction between Shc1 and Pyk2 is direct. We inserted a fragment encoding amino acids 700–840 of rat Pyk2 into pGST-Parallel and truncations T3 and T5 of mouse p46Shc1 into pMPB-Parallel (40) and purified the fusion proteins from Escherichia coli. We incubated the two fusion proteins together but found no evidence for a direct interaction (Fig. 10a). Using transient transfections, we then co-expressed Pyk2, p52Shc1, and either Grb2, CrkII, or Nck1 in Phoenix cells to determine if one of these scaffolds could mediate the interaction between Shc1 and Pyk2. We found no evidence that one of these scaffolds serves to link Shc1 with Pyk2 (Fig. 10b).

Overall, the interaction between Pyk2 and Shc1 requires a portion of the proline-rich region of Pyk2 and a small region in the central linker of Shc1 that has not previously been identified as interacting with a partner protein. The interaction between Shc1 and Pyk2 is not mediated by any of the common adaptors that function downstream of receptor-tyrosine kinases.

Discussion

Despite progress made in cancer biology and the development of new therapeutic strategies, the prognosis of patients with pancreatic cancer remains poor. Because EMT is important in the progression of pancreatic cancer, combining standard chemotherapy with inhibitors of EMT may hold some promise (41). Pancreatic cancer is characterized by extensive
deposition of collagen I, which can initiate EMT through activating DDR1 (5). Thus, targeting collagen I-induced EMT through inhibiting DDR1 may be a therapeutic option in pancreatic cancer.

To determine if the kinase activity of DDR1 was needed for collagen-dependent up-regulation of N-cadherin, we used the approach pioneered by Shokat and Velleca (19). We found when its gatekeeper residue was mutated to Ala, DDR1 retained collagen-dependent kinase activity, and 1NM-PP1 inhibited the collagen-dependent activation of mutant but not wild-type DDR1 (Fig. 3a). Specifically inhibiting the kinase activity of the gatekeeper-mutant form of DDR1b with 1NM-PP1 inhibited collagen-I induced N-cadherin up-regulation in pancreatic cancer cells (Fig. 3f). We have shown that signaling through both β1-integrins and DDR1 contributes to collagen-dependent up-regulation of

FIGURE 7. DDR1 and Shc1 interact with Pyk2, and collagen-dependent signaling via DDR1 strengthens the interaction. a, L3.6pl cells were infected with viral constructs encoding shRNAs targeting either EGFP (lanes 1 and 8) or DDR1 (lanes 2–7 and 9–14). The DDR1 knockdown cells were then stably infected with viruses encoding various DDR1 constructs (lanes 3–7 and 10–14). Cells were cultured on uncoated (w/o col I) or collagen-coated plates (col I) for 18 h. DDR1 was immunoprecipitated (IP), and the immunoprecipitates were blotted with antibodies to Pyk2 or DDR1. Pyk2 exhibits collagen-dependent association with DDR1 that requires Tyr-513. b, p46Shc1 (lanes 1–3), p52Shc1 (lanes 4–6), or p66Shc1 (lanes 7–9) as well as either wild-type Pyk2 (lanes 1, 4, and 7), Pyk2 Y402F (lanes 2, 5, and 8), or Pyk2 KA (kinase dead, lanes 3, 6, and 9) were transiently expressed in Phoenix cells. Immunoprecipitations were performed with an antibody against Shc1, and the immunoprecipitates were blotted with antibodies to Pyk2 or Shc1. As shown in the bottom two panels, cell lysates were blotted for total Pyk2 and phospho-Pyk2 (Tyr-402). c, levels of Pyk2 being pulled down in all the lanes of b were normalized to the levels of the Pyk2 inputs. Pairwise comparisons between lane 1 and lanes 2 or 3, lane 4, and lanes 5 or 6 and between lane 7 and lanes 8 or 9 were performed using Student’s t test. Data are presented as the mean ± S.D. (n = 3). The asterisks reflect significance (***, p < 0.001; **, p < 0.01). d, L3.6pl cells expressing the same constructs as a were cultured on uncoated (w/o col I) or collagen-coated plates (col I) for 18 h. Shc1 was immunoprecipitated, and the immunoprecipitates were blotted with antibodies to Pyk2 or phospho-Pyk2 (Tyr-402). The amount of endogenous Pyk2 in the lysates is shown in the bottom panel. The collagen-dependent association of Shc1 with Pyk2 requires Tyr-513 of DDR1b. e, levels of Pyk2 or phospho-Pyk2 being pulled-down in all the lanes of d were normalized to the levels of the Pyk2 inputs. Pairwise comparisons between lane 1 and other lanes were performed using Student’s t test. Data are presented as the mean ± S.D. (n = 3). The asterisks reflect significance (**, p < 0.01; *, p < 0.05). f, wild-type Pyk2 (lanes 1–7) as well as various p52Shc1 constructs tagged with 3X FLAG (lanes 1–6) were transiently expressed in Phoenix cells. Immunoprecipitations were performed with antibody against FLAG, and the immunoprecipitates were blotted with antibodies to Pyk2 or Shc1. The amount of total Pyk2 in the lysates is shown in the bottom panel. None of the mutations altered the association of Shc1 with Pyk2.
Thus, inhibiting only the DDR1 arm of the pathway would typically be expected to only partially block the up-regulation of N-cadherin.

Knocking down DDR1 in lung cancer cells reduced cell survival, homing, and colonization in bone metastasis (42). In K-Ras-mutant lung cancers, Ambrogio et al. (43) showed that combining 7rh to inhibit DDR1 with a Notch inhibitor was effective. In addition Ali-Rahmani et al. (44) have shown inhibiting DDR1 with 7rh in combination with an immunotoxin caused shrinkage of tumor xenografts. Aguilera et al. (45) used the KIC mouse model of pancreatic cancer to argue for the importance of collagen-dependent signaling through DDR1 in their studies of anti-VEGF therapy. In our studies we showed a strong correlation exists between the expression of DDR1 and N-cadherin in primary and metastatic pancreatic cancer tissues (Fig. 1). However, the roles played by signaling through DDR1 in the progression of pancreatic cancer are not well characterized. In future studies it will be important to determine how effective specific inhibitors of DDR1 such as 7rh are in treating mouse models of pancreatic cancer. Such studies have been initiated with the KIC model (46) and are planned for the KPC model (47).

In studies with pancreatic cancer-derived cell lines, we previously found that activating DDR1 initiates a pathway leading to the up-regulate N-cadherin and that Pyk2 is a downstream effector (5). In the experiments described here, we extended this work and found that up-regulation of N-cadherin was due to activation of the specific isoform DDR1b (Fig. 2). After DDR1b was activated by collagen, Shc1 via its PTB domain was recruited to tyrosine 513 of DDR1b, leading to the up-regulation of N-cadherin (Figs. 4–6). Our data suggest Shc1 is associated with Pyk2 even before the activation of DDR1, perhaps because of signaling through other receptor-tyrosine kinases. However, the activation of DDR1 significantly increased the association between Shc1, Pyk2, and DDR1 resulting in the activation of Pyk2 (Fig. 7).

**FIGURE 8.** A short segment of the central domain of Shc1 is necessary for the interaction with Pyk2. a, shown are schematic representations of the HA-tagged mouse p46Shc1 constructs (p46Shc1, T1–T7). Numbers on the right represent the last amino acid of the truncated protein using the amino acid numbers of mouse p52Shc1 (NP_035498.2). Portions of the sequences of mouse and human Shc1 are aligned, and the boundaries of truncations T3, T6, and T7 are shown. The amino acids necessary for human (not shown) and mouse Shc1 to interact with Pyk2 are underlined. b, wild-type Pyk2 (lanes 1–13) as well as various p46Shc1 constructs tagged with 2X HA (lanes 1–3, 5–8, and 10–12) were transiently expressed in Phoenix cells. Immunoprecipitations were performed with antibody against HA, and the immunoprecipitates (IP) were blotted with antibodies to Pyk2 or HA. The amount of total Pyk2 in the lysates is shown in the bottom panel. c, levels of Pyk2 being pulled down in all the lanes of b were normalized to the levels of the Pyk2 inputs. Pairwise comparisons were performed using Student’s t test where lanes were compared with the lanes not expressing p46Shc1. Data are presented as the mean ± S.D. (n = 3). The asterisks reflect significance (**, p < 0.01; *, p < 0.05).
Neither the SH2 domain nor any of the well characterized tyrosine phosphorylation sites of Shc1 are necessary for its interaction with Pyk2 or for the collagen-dependent up-regulation of N-cadherin (Figs. 6b and 7f). Although all the isoforms of Shc1 still interact with the Tyr-402 mutant of Pyk2, the interactions are strongly reduced with the kinase dead K457A mutant (Fig. 7b). This is somewhat surprising as the region of Pyk2 that interacts with Shc1 is in between the kinase and FAT domains. This region of Pyk2 has two proline-rich sequences but does not itself contain a recognizable domain.
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Faure et al. (48) mapped several activities such as nuclear accumulation of Pyk2 to the same region we identified. However, they found serine phosphorylation in the alternatively spliced region played a role (48). Thus in their studies, the shorter isoform of Pyk2 lacked the activities, whereas we found the shorter isoform of Pyk2 still interacts with Shc1.

Our data suggest inhibiting Pyk2 may prevent the DDR1-dependent up-regulation of N-cadherin. Pyk2 knock-out mice are viable and fertile (49), whereas knocking out FAK results in embryonic lethality (50), suggesting the selective inhibition of Pyk2 may have therapeutic advantages. In this regard it is notable that recently inhibitors with strong selectivity for Pyk2 over FAK have been identified (51).

Using fusion proteins purified from *E. coli*, we showed the interaction between Shc1 and Pyk2 is probably not direct (Fig. 10a). The short stretch of amino acids we identified in mouse and human Shc1 has a PXXP sequence (Fig. 8), suggesting an adaptor with two or more SH3 domains (52) could bridge Shc1 with the proline-rich sequences in Pyk2. We tested several candidate adaptors (Grb2, CrkII, or Nck1), but none enhanced the association of Shc1 with Pyk2 (Fig. 10b). In this context it is worth noting the PXXP motif in human and mouse Shc1 is not conserved in some mammalian species, plus the context of the PXXP in mouse and human Shc1 does not conform well with known consensus SH3 binding sequences (53). The details of how Shc1 interacts with Pyk2 are the subject of ongoing studies.

It is important to note the region we identified in Shc1 is not conserved in Shc2, Shc3, or Shc4 (not shown), suggesting other family members cannot substitute for Shc1.

In summary, this paper is the first to report that the b isoform of DDR1 mediates collagen-dependent up-regulation of N-cadherin in pancreatic cancer and the adaptor protein Shc1 is necessary in this process. Using an orthotopic model, we have previously shown a cyclic peptide N-cadherin antagonist suppresses pancreatic cancer progression (54). Because it may be advantageous to inhibit the up-regulation of N-cadherin rather than inhibit N-cadherin itself, we will pursue using the DDR1 kinase inhibitors in an orthotopic model of pancreatic cancer to determine if inhibiting the kinase activity of DDR1 provides any therapeutic benefit.

**Experimental Procedures**

**Reagents, Antibodies, and Cultured Cells**—The hybridoma making a mouse monoclonal antibody (mAb) against E-cadherin (HECD-1; hybridoma supernatant, Western blot dilution 1:100) was a gift from Dr. M. Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan), and the mouse mAb against N-cadherin (13A9; hybridoma supernatant, no dilution for immunohistochemistry, Western blot dilution 1:100) has been previously described (55). The specificities of HECD-1 and 13A9 have been previously demonstrated (56). Anti-DDR1 rabbit antisera (C-20; immunohistochemistry dilution 1:100, immunoprecipitation dilution 1:100, Western blot dilution 1:1000) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). The specificity of C-20 was demonstrated by our knockdown and re-expression studies shown, for example, in Fig. 2. With C-20, we sometimes observed nonspecific bands, and these are pointed out in the figures. Anti-Shc1 mouse mAb (PG-797; immunoprecipitation dilution 1:100, Western blot dilution 1:1000) was obtained from Santa Cruz Biotechnology, Inc. Anti-Shc1 rabbit antisera (2432; Western blot dilution 1:1000) was obtained from Cell Signaling Technology (Danvers, MA). The specificity of the two anti-Shc1 preparations was shown by immunoprecipitating with the rabbit antisera and blotted with the mouse mAb and also by knocking down Shc1 (Fig. 5). Anti-JNK1 mouse mAb (2C6; Western blot dilution 1:1000) and anti-JNK phosphospecific (Thr-183/Tyr-185) rabbit antisera (9251; Western blot dilution 1:1000) were obtained from Cell Signaling Technology. The specificity of the JNK1 and phospho-JNK antibodies has been shown by knockdown studies (57, 58) and treating cells with anisomycin (Fig. 4). Anti-Pyk2 mouse mAb (5E2; Western blot dilution 1:1000) and anti-Pyk2 phosphospecific (Tyr-402) rabbit antisera (3291; Western blot dilution 1:1000) were obtained from Cell Signaling Technology. The specificity of 5E2 and 3291 was demonstrated by expressing exogenous wild-type and Y402F Pyk2 in Phoenix cells, which express low endogenous levels of Pyk2 (Fig. 7 and not shown). Anti-GFP rabbit antisera (632376; Western blot dilution 1:200) was obtained from Clontech Laboratories (Mountain View, CA). The specificity of this antisera is shown in Fig. 9, where cells were made to express mEGFP fusions of varying size. Anti-HA rabbit mAb (C29F4; Western blot dilution 1:1000) was obtained from Cell Signaling Technology and anti-HA mouse mAb (16B12; immunoprecipitation dilution 1:100) from Covance Research Products Inc. (Princeton, NJ). The specificity of the anti-HA antibodies is shown in Fig. 8, where HA-tagged proteins were immunoprecipitated with the mouse mAb, and the immunoprecipitates were blotted with the rabbit mAb. Anti-phosphotyrosine mouse mAb (PY20; Western blot dilution 1:1000) was obtained from BD Biosciences. The specificity of PY20 for phosphotyrosine has been demonstrated (59). Anti-FLAG mouse mAb (M2; immunoprecipitation dilution 1:100) was obtained from Sigma. Anti-tubulin mouse mAb E7 (Western blot dilution 1:1000) (developed by Drs. M. McCutcheon and S. Carroll) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA).

The identity of all the cell lines used in these experiments was verified by the University of Arizona Genetics Core, and each cell line was shown to be free from mycoplasma using a PCR kit (PK-CA91-1048 from Promocell (Heidelberg, Germany)). L3.6pl (60) was a gift from Drs. R. Singh and M. Ouellette (University of Nebraska Medical Center, Omaha, NE). PSN-1 was a gift from Dr. P. Tofilon (NCI, National Institutes of Health, Bethesda, MD). T3M-4 was a gift from Dr. T. Hollingsworth (University of Nebraska Medical Center). BxPC-3 was obtained from American Type Culture Collection. L3.6pl and T3M-4 were maintained in DMEM, and BxPC-3 and PSN-1 were maintained in RPMI 1640 containing 10% FBS, respectively, in an atmosphere of 5% CO2 at 37 °C. Collagen I-coated dishes and rat tail collagen I were obtained from BD Biosciences. The synthesis of the DDR1-specific inhibitor 7rh has been described (22).

**Detergent Extraction, SDS-PAGE, Immunoblot, and Immunoprecipitation**—Monolayers of cultured cells were extracted with Tris/Nonidet P-40/EDTA buffer (10 mM Tris-
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HCl, pH 8.0, 0.5% Nonidet P-40, and 1 mM EDTA) for SDS-PAGE or immunoprecipitation as previously described (61). Protein was determined using a protein assay kit (Thermo Scientific, Waltham, MA). Antibodies were added to the indicated volume of cell extract and gently mixed overnight at 4 °C. Anti-mouse or anti-rabbit IgG affinity gel (MP Biomedicals, Santa Ana, CA) was washed with Tris/Nonidet P-40/EDTA buffer once. The cell extract–antibody mix was added to the IgG affinity gel and mixed at 4 °C for 2 h. Immune complexes were washed once with radioimmunoprecipitation assay buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Nonidet P-40) and 2 times with TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). After the final wash, the packed beads were resuspended in 50 μl of 2× Laemmli sample buffer and boiled for 5 min, and the proteins were resolved by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes and blocked in 5% nonfat dry milk or 5% bovine serum albumin in TBST for 1 h. Blocking solution was removed, and primary antibody in TBST was added followed by incubation for 1 h. Membranes were washed for 15 min followed by 2 × 5 min in TBST. Membranes were incubated with horseradish peroxidase-conjugated antiserum or rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:5,000 for 1 h. The secondary antibody was removed by washing 15 min followed by 2 × 5 min in TBST. Immunoreactive bands were detected using SuperSignal West Pico substrate (Thermo Scientific).

Constructs, Transfection, and Infection—Phoenix cells (32) were transfected using TransIT-LT1 reagent (Mirus, Madison, WI). Production of viral supernatants and infection of target cells have been described previously (9). 200 μg/ml hygromycin, 4 μg/ml puromycin, or 1 mg/ml G418 was used for selection of the target cells.

The human DDR1a and DDR1b cDNAs were gifts from Dr. Teizo Yoshimura (NCI, National Institutes of Health) (62). The DDR1a and DDR1b cDNAs were sequenced and shown to encode proteins identical to NP_001945.3 and NP_00054699.2, respectively. For stable expression in cells a mEGFP (A206K) tag was added to the C terminus of each isoform, eliminating the 3′ UTRs. The tagged constructs were inserted into pLZRS-MS-Hygro or pLZRS-MS-Neo for stable expression. Gatekeeper mutants were generated by introducing a T664A mutation in the region of phosphomimetic residues using QuikChange Lightning Multisite-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and were tagged at their N termini with EGFP (48).

Restriction fragments encoding portions of rat Pyk2 and mouse p46Shc1 were cloned into pGST-Parallel or pMBP-Parallel vectors (40). The fusion proteins were expressed in Rosetta2 cells (Novagen, Madison, WI) and purified on glutathione agarose or cross-linked amylose beads.

The human FLAG-tagged CrkII was a gift from Dr. Bruce Mayer (University of Connecticut Health Center, Farmington, CT; Accession BC001718.1) (65). We inserted the tagged cDNA into pLZRS-MS-Neo. GST-Grb2 was purchased from Addgene (Plasmid #46442; Grb2 accession NM_002086.4) (66). We inserted the tagged cDNA into pcDNA3.1. The human 3× HA-tagged Nck1 cDNA was a gift from Dr. Wei Li (University of Southern California, Los Angeles, CA; accession NM_006153.3) (67). We inserted the tagged cDNA into pcDNA3.1.

The shRNA constructs were as follows: DDR1 (TRCN 000121082, Sigma); Shc1 (shShc1 991, TRCN0000332991; shShc1 997, TRCN0000295997; shShc1 208, TRCN0000040208; shShc1 434, TRCN000010434, Sigma). All the shRNAs were in the vector pLKO.1-Puro. For a negative control, we used pLKO.1-puro carrying a sequence targeting EGFP (a gift from Dr. Angie Rizzino, University of Nebraska Medical Center).

Conventional Reverse Transcription (RT)-PCR—Total RNA was extracted with the RNeasy Plus Mini kit (Qiagen, Alameda, CA) and analyzed by RT-PCR using a Titanium One-Step RT-PCR kit (Clontech). Primers used for PCR were the sense human p52Shc1 cDNA was purchased from GE Pharmacon (Lafayette, CO; Clone ID: 4541216, accession number BC014158.2). Mouse 2× HA p46Shc1 (amino acids 46–469 of NP_035498.2), mouse 2× HA p52Shc1 (NP_035498.2), mouse 3× FLAG p52Shc1 wild type, R397K, R175Q, Y313F, Y239F/Y240F, and Y239F/Y240F/Y313F mutants were gifts from Dr. P. Siegel (McGill University, Montreal, Canada) and were all inserted into pMSCV-Blunt vectors (27). We moved human p66Shc1, human p52Shc1, and mouse p46Shc1 into pLZRS-MS-Neo for stable expression. All p46Shc1 truncation mutants (T1 through T7) were PCR products using mouse 2× HA p46Shc1 in pMSCV-Blunt as a template. The primers used were the common sense primer 5′-CTCTTTTCTCTGACCGCGG-3′ and the antisense primers T1 (5′-GAATTCCTACCCTG-CTATGGGGGGTGAC-3′), T2 (5′-GAATTCTCAGTGGACACCGGCTCCG-3′), T3 (5′-GAATTCTCAAGCAGCCTTTCCCGAG-3′), T4 (5′-GAATTCTCAGTCTAGATCTCAGATGTGTTG-3′), T5 (5′-GAATTCTCCAGTCTGGCCTGAGG-3′), T6 (5′-GAATTCTCAATATGCTGCCCTA- TAGGC-3′) T7 (5′-GAATTCTCAGCAGCATTGGGCATAG-3′). All the truncation mutants as well as all the constructs we generated via PCR were completely sequenced and shown to encode the desired AA sequences with no unintended changes.

Rat Pyk2 (NP_059014.2, C-terminally myc tagged) and the mutants Y402F and K457A in pcDNA3 were gifts from Dr. Lee Graves (University of North Carolina at Chapel Hill, Chapel Hill, USA) (64). The truncation mutants of rat Pyk2 (NP_059014.2) were gifts from Dr. Jean-Antoine Girault (Institut du Fer à Moulin, INSERM, Paris, France) and were tagged at their N termini with EGFP (48).

The shRNA constructs were as follows: DDR1 (TRCN 000121082, Sigma); Shc1 (shShc1 991, TRCN0000332991; shShc1 997, TRCN0000295997; shShc1 208, TRCN0000040208; shShc1 434, TRCN000010434, Sigma). All the shRNAs were in the vector pLKO.1-Puro. For a negative control, we used pLKO.1-puro carrying a sequence targeting EGFP (a gift from Dr. Angie Rizzino, University of Nebraska Medical Center).

Conventional Reverse Transcription (RT)-PCR—Total RNA was extracted with the RNeasy Plus Mini kit (Qiagen, Alameda, CA) and analyzed by RT-PCR using a Titanium One-Step RT-PCR kit (Clontech). Primers used for PCR were the sense
primers, 5′-GAGCTGACGGTTAACCCTCTGTGC-3′, and the antisense primer, 5′-CAATGTACGCTCGGCATAATTG-3′. The conditions for PCR were as follows: 94°C for 45 s, 60°C for 30 s, and 72°C for 90 s for 15, 25, or 35 cycles. PCR products were analyzed by electrophoresis on 1.5% agarose gels.

**Immunohistochemistry**—Tissue microarray slides were obtained from Rapid Autopsy Program at the University of Nebraska Medical Center (68). Slides were deparaffinized with xylene, rehydrated with an alcohol gradient, and washed with water. Antigen retrieval was performed with an alkaline citrate buffer and microwave treatment. Peroxidase activity was quenched by 3% hydrogen peroxide. Slides were blocked with 5% bovine serum albumin and incubated with primary and secondary antibodies. 3,3′-Diaminobenzidine substrate was added and followed by hematoxylin counterstaining. Slides were evaluated and annotated by a pathologist (A. J. Lazenby). The score scale ranged from 0 to 3, with 0 being no staining or <10% cancer cells stained, 0.5 being faint to weak staining in >10% cancer cells, 1 being weak staining in >10% cancer cells, 1.5 being weak to moderate staining in >10% cancer cells, 2 being moderate staining in >10% cancer cells, 2.5 being moderate to strong staining in >10% cancer cells, 3 being strong staining in >10% cancer cells, and 3.5 being very strong staining in >10% cancer cells. Spearman correlation analysis was then performed.

**Cell Invasion Assay**—Cells (1.5 × 10⁵) were plated in the upper chamber of BD BioCoat Matrigel Inserts (24-well plates, pore size = 8 μm; BD Biosciences). Culture medium containing 0.5% FBS was added to the upper chambers, and medium containing 10% FBS was added to the bottom chamber. 1NM-PP1 (Calbiochem) was added to both upper and bottom chambers. Cells were incubated on the membranes for 18 h, and motility was quantified as described (69).

**Author Contributions**—H. H., R. A. S., J. S., N. C., and K. R. J. designed and/or performed the experiments. H. H. and A. J. L. evaluated the stained tissue slides. K. D. provided the inhibitor of DDR1. M. J. W. provided resources. H. H. and K. R. J. wrote the manuscript.

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