PINCH-1 Regulates the ERK-Bim Pathway and Contributes to Apoptosis Resistance in Cancer Cells

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Resistance to apoptosis is a hallmark of cancer cells. We report here that PINCH-1, a cytoplasmic component of cell-extracellular matrix adhesions, is required for protection of multiple types of cancer cells from apoptosis. Furthermore, using HT-1080 fibrosarcoma cells as a model system, we have investigated the signaling pathway through which PINCH-1 contributes to apoptosis resistance. Loss of PINCH-1 markedly increases the level of Bim and promotes Bim translocation to mitochondria, resulting in activation of the intrinsic apoptosis pathway. Depletion of Bim completely blocked apoptosis induced by the loss of PINCH-1. Thus, PINCH-1 contributes to apoptosis resistance through suppression of Bim. Mechanistically, PINCH-1 suppresses Bim not only transcriptionally but also post-transcriptionally. PINCH-1 promotes activating phosphorylation of Src family kinase and ERK1/2. Consistent with this, ERK1/2-mediated Ser69 phosphorylation of Bim, a key signal for turnover of Bim, is suppressed by the removal of PINCH-1. Our results demonstrate a strong dependence of multiple types of apoptosis-resistant cancer cells on PINCH-1 and provide new insights into the molecular mechanism by which cancer cells are protected from apoptosis.

Apoptosis is a fundamental cellular process that is essential for normal development in embryo and tissue homeostasis in adult. Resistance to apoptosis is crucial for pathogenesis and progression of many common human diseases, including cancer (1). It has been shown, for example, that the metastatic potential of human cancer cells is strictly linked to increased resistance to apoptosis (2). Experimental modulation of key antiapoptotic or proapoptotic factors alters metastatic efficiency (reviewed in Ref. 3). Thus, apoptosis provides a major barrier to metastasis (1, 3). Overcoming resistance of cancer cells to apoptosis, therefore, represents both a challenge and an exciting opportunity in cancer biology and therapy. To achieve this goal, it is essential to identify proteins that protect cancer cells from apoptosis and determine signaling pathways through which they protect cancer cells from apoptosis.

Studies over the past 2 decades suggest that apoptosis is mediated primarily by two distinct signaling pathways, namely the extrinsic (or the death receptor) and the intrinsic (or the mitochondrial) pathways (reviewed in Refs. 4–6). The extrinsic pathway is mediated by the FAS receptor (CD95) or members of the tumor necrosis factor receptor superfamily. The intrinsic pathway, on the other hand, involves multiple Bcl-2 family proteins (e.g. proapoptotic proteins, such as Bim and Bax, and pro-survival proteins, such as Bcl-2) (7–9), which impinge on mitochondrial integrity. The two apoptosis pathways converge on common “executioner” caspases (e.g. caspase-3) that are responsible for execution of apoptosis (10). Although the extrinsic and intrinsic apoptosis pathways have been well described, how cancer cells evade the activation of the apoptosis pathways remains elusive.

The activation of the apoptosis pathways is controlled at least in part by integrin-mediated cell-extracellular matrix (ECM) adhesion (reviewed in Refs. 11–14). Thus, components of the integrin signaling pathway are attractive targets for therapeutic control of tumor growth and metastasis. At the molecular level, cell-ECM adhesion is mediated by a complex protein network consisting of integrins and proteins that are directly or indirectly associated with integrins (15–22), many of which are so-called focal adhesion (FA) proteins due to their ability to cluster at FAs. How integrins and associated proteins regulate apoptosis, however, is incompletely understood. PINCH-1 and Mig-2 (mitogen-inducible gene-2, also known as kindlin-2) are integrin-proximal scaffolding proteins that are essential for integrin-mediated cell-ECM adhesion, cytoskeletal organization, and embryonic development (23–31). Although the role of PINCH-1 and Mig-2 in FA and cytoskeletal organization has been well established, their functions in apoptosis remain to be determined. Several lines of evidence suggest that PINCH-1 probably functions in the suppression of apoptosis. First, depletion of PINCH-1 from human HeLa cervical carcinoma cells promotes apoptosis (32). Second, an increase of apoptotic endodermal cells was observed in PINCH-1 null embryoid bodies (33). Third, excessive apoptosis was detected in PINCH-1 null mouse embryos (34). Finally, knock-out of PINCH-1 in embryonic neural crest cells, which share many characteristics with metastatic cancer cells, induces apoptosis (35). An increase of apoptosis, however, was not reported in PINCH-1-deficient ventricular cardiomyocytes (34). These studies raise...
an interesting possibility that PINCH-1 functions in protection of certain types of cells (e.g., cancer cells and neural crest cells that must survive a frequently changing microenvironment) from apoptosis. The role of Mig-2 in apoptosis had not been determined.

The current study focuses on the role of PINCH-1 in protection of cancer cells from apoptosis. The goals of this study are 1) to test whether PINCH-1-mediated protection against apoptosis depends on PINCH-1 clustering at FAs, 2) to determine whether PINCH-1 contributes to apoptosis resistance in multiple types of human cancer cells, and 3) perhaps most importantly, to identify the pathway that mediates the effect of PINCH-1 in apoptotic signaling. We report here the findings.

**EXPERIMENTAL PROCEDURES**

**Cells, Abs, and Other Reagents**—Human HeLa cervical carcinoma cells, HT-1080 fibrosarcoma cells, Calu-6 lung carcinoma cells, MDA-231 breast carcinoma cells, nontumorigenic MCF-10A mammary epithelial cells, and KRO and HCT-116 colon carcinoma cells were from ATCC. PC-3 prostate carcinoma cells were from Shoukat Dedhar (British Columbia Cancer Agency, Canada). Hep G2 hepatocellular carcinoma cells were from George Michalopoulos (University of Pittsburgh). Caco-2 colon cancer cells were from Dr. Craig C. Garner (Stanford University). Bax, p53, PUMA, or Smac knock-out HCT-116 cells were previously described (29, 32, 42). A validated Bim-specific siRNA was from Santa Cruz Biotechnology. Cells were transfected with siRNA, Mig-2 siRNA, and control RNA using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocols. In some experiments, cells were first transfected with an expression vector encoding a PINCH-1 transcript that is resistant to the siRNA, following the manufacturer's protocols. In some experiments, cells were first transfected with an expression vector encoding a PINCH-1 transcript that is resistant to the siRNA, following the manufacturer’s protocols. In some experiments, cells were first transfected with an expression vector encoding a PINCH-1 transcript that is resistant to the siRNA, following the manufacturer’s protocols. In some experiments, cells were first transfected with an expression vector encoding a PINCH-1 transcript that is resistant to the siRNA, following the manufacturer’s protocols.

**Isolation of Mitochondrial and Cytosolic Fractions**—The cells were plated on fibronectin-coated coverslips. After incubation overnight at 37 °C, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline, and stained with rabbit anti-PINCH-1 Ab and mouse anti-Mig-2 Ab. After rinsing, the primary Abs were detected with Rhodamine RedTX-conjugated anti-rabbit IgG Ab and fluorescein isothiocyanate-conjugated anti-mouse IgG, respectively.

**Immunofluorescent Staining**—The cells were plated on fibronectin-coated coverslips. After incubation overnight at 37 °C, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline, and stained with rabbit anti-PINCH-1 Ab and mouse anti-Mig-2 Ab. After rinsing, the primary Abs were detected with Rhodamine RedTX-conjugated anti-rabbit IgG Ab and fluorescein isothiocyanate-conjugated anti-mouse IgG, respectively.

**Apoptosis**—Cells (as specified in each experiment) were cultured as monolayers in cell culture plates (Greiner Bio-One) and transfected with PINCH-1 siRNA or control RNA as described above. Two days after siRNA transfection, apoptosis was analyzed using a fluorogenic caspase-3 substrate VII (Calbiochem), following the manufacturer’s protocol. All samples were analyzed in triplicates. The means ± S.D. from PINCH-1-, Mig-2-, and/or Bim-deficient cells were compared with that of the control cells (normalized to 1).

**Real Time PCR—Poly(A)+ RNA was isolated using the Oligotex Direct mRNA minikit (Qiagen). First-strand cDNA was prepared from mRNA with the SuperScript™ III first strand synthesis system for reverse transcription-PCR (Invitrogen). The amounts of Bim transcripts in PINCH-1 knockdown and control cells were analyzed by real time PCR using the SYBR green method. The primers used were 5’-TCCTCCCTTGCCAGGCCCT-3’ (forward) and 5’-CTCGAGTTTCAGCCTGGC-3’ (reverse). The samples were analyzed in triplicates using the Mx3000P™ real time PCR system and software (Stratagene). Samples were compared using glyceraldehyde-3-phosphate dehydrogenase as a normalizer gene. †Fold increase was calculated using the relative Ct method.

**FLAG-BimEL Construct, Transfection, and Immunoprecipitation**—cDNA encoding the BimEL was generated by PCR from a human lung cDNA library (Clontech) using 5’-ctgctcgagtcaatgcattctccacacc-3’ and 5’-ctgctcgagtcaagctgacaccctcc-3’ as primers and inserted into the pFLAG-CMV-6c vector (Sigma). HT-1080 cells were transfected with the FLAG-BimEL vector using Lipofectamine 2000. Overexpression of FLAG-BimEL induced dramatic increase of apoptosis (data not shown). To prevent cell death, the transfectants were cultured in medium containing 20 μM benzoyloxyacarbonyl-Valu-fluoromethyl ketone.

**Small Interfering RNA (siRNA) Transfection—PINCH-1 siRNA, Mig-2 siRNA, and control RNA were previously described (29, 32, 42). A validated Bim-specific siRNA was from Santa Cruz Biotechnology. Cells were transfected with siRNA (as specified) or the control RNA using Lipofectamine 2000 (Invitrogen), following the manufacturer’s protocols. In some experiments, cells were first transfected with an expression vector encoding a PINCH-1 transcript that is resistant to the siRNA (42) or a DNA vector lacking PINCH-1 coding sequence as a control. One day after DNA transfection, the cells were transfected with PINCH-1 siRNA. Cells were analyzed 2 days after siRNA transfection unless otherwise specified.

For treatment with histone deacetylase inhibitor, HT-1080 cells were transfected with PINCH-1 siRNA and control RNA as described above. Thirty hours after RNA transfection, the cells were treated with 2 mM sodium butyrate and cultured for an additional 20 h. The cells were then harvested and analyzed by Western blot.

**Isolation of Mitochondrial and Cytosolic Fractions—Mitochondrial and mitochondrial-free cytosolic fractions were pre-
pared as described (44). Briefly, cells were suspended in 5 mM Tris buffer (pH 7.4) containing 5 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 1 mM di-thiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. The cell suspension was passed through a 22-gauge needle 20 times and centrifuged at 700 × g for 5 min at 4 °C to remove nuclei. The supernatants were centrifuged at 12,000 × g for 15 min at 4 °C. The pellets (mitochondrial fractions) and supernatants (cytosolic fractions) were collected and analyzed by Western blot with Abs for markers of mitochondrial fractions (prohibitin) and non-mitochondrial fractions (tubulin).

RESULTS

PINCH-1 and Mig-2 Are Mutually Dependent for Their FA Clustering—PINCH-1 can be clustered into large supramolecular structures at FAs that are detectable by fluorescence microscopy (referred as FA clustering herein), which is important for regulation of several integrin-mediated processes, including cell-ECM adhesion, contractility, migration, and mechanotransduction (45). To determine whether PINCH-1-mediated protection against apoptosis depends on FA clustering of PINCH-1, we first sought to develop a method through which we could disrupt FA clustering but not the level of PINCH-1. Toward this end, we tested whether Mig-2, a widely distributed and evolutionally conserved cytoplasmic component of FAs, is required for FA clustering of PINCH-1. HT-1080 fibrosarcoma cells, which express abundant PINCH-1 and Mig-2 (Fig. 1, A and B, lane 1), were used. Double immunofluorescent staining with a rabbit anti-PINCH Ab and a mouse anti-Mig-2 Ab showed that HT-1080 cells formed robust FAs in which PINCH-1 (Fig. 1 D) and Mig-2 (Fig. 1 E) were co-clustered. Transfection of the cells with an siRNA that targets Mig-2 dramatically reduced the level of Mig-2 (Fig. 1E) and Mig-2 knockdown cells were double stained with rabbit anti-PINCH-1 (D, F, and H) and mouse anti-Mig-2 (E, G, and I) Abs. Bar in E, 5 μm. J, caspase-3 activities in the control, PINCH-1 knockdown, and Mig-2 knockdown cells were analyzed as described under “Experimental Procedures.” K, lysates (20 μg/lane) of HT-1080 cells transfected with the control (lane 1), PINCH-1 (lane 2), or Mig-2 (lane 3) siRNA were analyzed by Western blot with anti-Bim Ab. Note that the level of Bim was dramatically increased in response to loss of PINCH-1 but not loss of Mig-2. Bim was detected in some (e.g. Figs. 5H and 6C) but not all experiments (e.g. K of this figure) due to its lower abundance. In experiments in which Bim was detected, the level of Bim was also increased in response to loss of PINCH-1 (see Figs. 5H and 6C).
(Fig. 1A, lane 3). Equal protein loading was confirmed by probing the same samples with an anti-actin Ab (Fig. 1C, lanes 1 and 3). Despite the presence of a normal level of PINCH-1 (Fig. 1A, lane 3), FA clustering of PINCH-1 was diminished in Mig-2 knockdown cells (Fig. 1, compare F with D). These results suggest that Mig-2 is required for proper FA clustering of PINCH-1, albeit it is not required for the expression and maintenance of the cellular level of PINCH-1.

We next tested the reciprocal (i.e. whether PINCH-1 is required for FA clustering of Mig-2). To do this, we transfected the cells with a PINCH-1 siRNA, which we had previously shown specifically targets the PINCH-1 transcript (32). Transfection with the PINCH-1 siRNA markedly reduced the level of PINCH-1 (Fig. 1A, compare lane 2 with lane 1). In a control experiment, equal loading was confirmed by probing the same samples with an anti-actin Ab (Fig. 1C, lanes 1 and 2). A similar level of Mig-2 was detected in both the PINCH-1-deficient cells and the control cells (Fig. 1B, compare lanes 1 and 2), indicating that loss of PINCH-1 did not adversely affect the level of Mig-2. Immunofluorescent staining showed that the clusters of Mig-2 were much smaller and less well organized in PINCH-1 knockdown cells (Fig. 1, compare I with E). Thus, depletion of PINCH-1 also compromises FA clustering of Mig-2. Collectively, these results suggest that PINCH-1 and Mig-2 are mutually dependent for their FA clustering. Loss of one protein adversely affects FA clustering of the other protein.

PINCH-1, but Not Mig-2, Is Essential for Protection of HT-1080 Fibrosarcoma Cells from Apoptosis—PINCH-1 is required for the survival of HeLa cervical carcinoma cells (32, 42). It was not known, however, whether PINCH-1 plays a similar role in protection of other cancer cells against apoptosis. To begin to test this, we analyzed apoptosis in response to depletion of PINCH-1 in HT-1080 cells. Depletion of PINCH-1 from HT-1080 cells markedly increased apoptosis (Fig. 1J). Importantly, knockdown of Mig-2, which diminished FA clustering of PINCH-1 (Fig. 1F), did not dramatically increase apoptosis (Fig. 1J). These results suggest that PINCH-1 is required for protection of HT-1080 cells against apoptosis, and it does so through a mechanism that is independent of Mig-2 (and consequently independent of FA clustering of PINCH-1).

PINCH-1 Is Required for Protection of Multiple Types of Human Cancer Cells from Apoptosis—To investigate the role of PINCH-1 in the survival of other types of cancer cells, we depleted PINCH-1 from human colon carcinoma (HCT-116, RKO, and Caco-2), breast carcinoma (MDA-231), prostate carcinoma (PC-3), hepatocellular carcinoma (Hep G2), lung carcinoma (Calu-6), and nontumorigenic mammary epithelial (MCF-10A) cells. HeLa and HT-1080 cells were used as positive controls. Transfection of the cells with PINCH-1 siRNA efficiently depleted PINCH-1 in all cell types that were tested (Fig. 2, A, C, E, G, I, and K, and Supplemental Fig. 1, A, C, E, and G). Depletion of PINCH-1 markedly increased apoptosis in HeLa
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FIGURE 3. Knock-out of Bax inhibits apoptosis induced by the loss of PINCH-1. HCT-116 (A and B) and HCT-116 cells lacking Bax (C and D) were transfected with the control or the PINCH-1 siRNA. A and C, the control transfectants (lane 1), PINCH-1 siRNA transfectants (lane 2), and parental cells (lane 3) were analyzed by Western blot with anti-PINCH-1 Ab (25 μg of lysates/lane). B and D, caspase-3 activities in the control transfectants, PINCH-1 siRNA transfectants, and parental cells were analyzed as described under “Experimental Procedures.”

FIGURE 4. PINCH-1 regulates Bim in multiple types of cancer cells. HCT-116 (lanes 1 and 2), MDA-231 (lanes 3 and 4), HT-1080 (lanes 5 and 6), PC3 (lanes 7 and 8), and HeLa (lanes 9 and 10) cells were transfected with the control (lanes 1, 3, 5, 7, and 9) or PINCH-1 (lanes 2, 4, 6, 8, and 10) siRNA. Cell lysates (25 μg/lane) were analyzed by Western blot with anti-PINCH-1, anti-Bim, anti-Bax, or anti-Bcl-2 Abs as indicated.

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We next sought to determine the downstream signaling effectors involved in PINCH-1-mediated regulation of apoptosis. A reverse genetic approach was employed in these studies. p53, PUMA, Bax, and Smac are apoptotic proteins involved in the killing of cancer cells in response to various external apoptotic stimuli. Thus, we first tested whether they are involved. A panel of HCT-116 cells, in which the expression of each of the proteins (p53, PUMA, Bax, or Smac) was ablated (36–39), were used in these studies. HCT-116 cells that lack p53, PUMA, Bax, or Smac and parental HCT-116 cells as a control, were transfected with PINCH-1 siRNA or a control RNA. Depletion of PINCH-1 in the PINCH-1 siRNA transfectants (Figs. 3, A and C, and Supplemental Fig. 2, A, C, and E, lane 2) but not the control transfectants (Fig. 3, A and C, and Supplemental Fig. 2, A, C, and E, lane 1) or the parental cells (Fig. 3, A and C, and Supplemental Fig. 2, A, C, and E, lane 3) was confirmed by Western blot. As expected, depletion of PINCH-1 markedly increased caspase-3 activity in HCT-116 cells (Fig. 3B). Loss of Bax reduced the increase of caspase-3 activity by more than 70% (Fig. 3D), suggesting a prominent role of Bax in this process. The increase of apoptosis was also reduced (albeit more modestly) in the absence of p53 (Supplemental Figs. 2B). No reduction of apoptosis was observed in the absence of PUMA (Supplemental Fig. 2D) or Smac (Supplemental Fig. 2F). These results suggest that Bax and to a lesser extent p53, but not PUMA or Smac, are involved in PINCH-1-mediated regulation of apoptosis.

PINCH-1 Regulates Bim but Not Bax Expression—The finding that the PINCH-1-mediated regulation of apoptosis is largely dependent on Bax prompted us to test whether the level of Bax is altered in response to loss of PINCH-1. Probing the PINCH-1-deficient and control HCT-116 cells with an anti-Bax Ab showed that the level of Bax was not significantly altered in response to loss of PINCH-1 (Fig. 4, compare lanes 1 and 2). To further test this, we analyzed the effect of PINCH-1 on the level of Bax in another nine human cancer cell lines, including both the PINCH-1-dependent (MDA-231, HT-1080, PC-3, HeLa, Calu-6, Hep G2, and RKO) cells and PINCH-1-independent (Caco-2 and MCF-10A) cells. No dramatic alterations of the Bax level were observed in response to loss of PINCH-1 in all of the cells that were analyzed (Fig. 4, lanes 3–10 and Supplemental Fig. 3). Thus, although Bax plays a role in PINCH-1-mediated regulation of apoptosis, it is not a direct target of PINCH-1 signaling.

We next sought to identify the signaling target that is regulated by PINCH-1. Because Bax is crucially involved in PINCH-1 regulation of apoptosis, we reasoned that this signaling target probably lies upstream of Bax. BH3-only proteins, including Bim, Bcl-2, and PUMA are known to function upstream of Bax. A role of PUMA was ruled out by our reverse genetic studies (Supplemental Fig. 2, C and D). Bcl-2 was not detected in PC3 (Fig. 4, lanes 7 and 8), Calu-6 (Supplemental Fig. 3, lanes 1 and 2), and Hep G2 (Supplemental Fig. 3, lanes 3 and 4) cells, either in the presence or absence of PINCH-1. In other cell types, the level of Bcl-2 was either not changed (e.g. in HeLa and Caco-2 cells) or modestly reduced (e.g. in HCT-116, HT-1080, and RKO) in response to loss of PINCH-1 (Fig. 4 and Supplemental Fig. 3). Notably, loss of PINCH-1 substantially increased the Bim level in many (e.g. MDA-231, HT-1080, PC-3, HeLa, Calu, and Hep G2), albeit not all (e.g. HCT-116 and
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Bim is essential for PINCH-1 regulation of apoptosis. A–C, expression of PINCH-1 reverses the increase of Bim expression and apoptosis induced by the loss of PINCH-1. HT-1080 cells were transfected with the control DNA vector and control RNA (lane 1), the control DNA vector and PINCH-1 siRNA (lane 2), and the “siRNA-resistant” PINCH-1 expression DNA vector and PINCH-1 siRNA (lane 3), respectively. The lysates (25 μg/lane) were analyzed by Western blot with anti-PINCH-1 (A) and anti-Bim (B) Abs. Caspase-3 activities (C) in the control transfectants, the PINCH-1-deficient transfectants, and PINCH-1-expressing transfectants were analyzed as described under “Experimental Procedures.” D–I, depletion of Bim inhibits apoptosis induced by the loss of PINCH-1. HT-1080 (D–F) and HeLa (G–I) cells were transfected with the control RNA (lane 1), PINCH-1 siRNA (lane 2), Bim siRNA (lane 3), and a mixture of PINCH-1 siRNA and Bim siRNA (lane 4). The lysates (25 μg/lane) were analyzed by Western blot with anti-PINCH-1 (D and G) and anti-Bim (E and H) Abs, respectively. Caspase-3 activities (F and I) in the control, PINCH-1 knockdown, Bim knockdown, and the PINCH-1 and Bim double knockdown cells were analyzed as described under “Experimental Procedures.”

RKO), types of cancer cells whose survival is dependent on PINCH-1 (Fig. 4 and Supplemental Fig. 3). No significant increase of the Bim level was observed in cells (MCF-10A and Caco-2) whose survival is independent of PINCH-1 (Supplemental Fig. 3, lanes 7–10). Based on these results, we hypothesized that in at least some types of cancer cells, Bim functions as a key apoptotic signaling mediator in response to loss of PINCH-1.

Bim Functions as a Key Signaling Effector in PINCH-1-mediated Regulation of Apoptosis—We next sought to test this hypothesis. HT-1080 cells, in which the Bim level was markedly increased in response to loss of PINCH-1 (Fig. 4, lanes 5 and 6), were used as a model system. Our hypothesis that Bim functions as a key signaling effector of PINCH-1 has three predictions. First, loss of Mig-2, which alters FA clustering of PINCH-1 but does not promote apoptosis, should not increase the Bim level. Consistent with this, Western blotting analyses showed that the Bim level in the Mig-2 knockdown cells (Fig. 1K, lane 3), unlike that in the PINCH-1 knockdown cells (Fig. 1K, lane 2), was not dramatically increased.

The second prediction of our hypothesis is that re-expression of PINCH-1 in the PINCH-1-deficient cells, which suppresses apoptosis induced by the loss of PINCH-1 (42), should reverse the increase of the Bim level. We tested this experimentally. Re-expression of PINCH-1 in the PINCH-1 knockdown cells (Fig. 5A, lane 3) indeed reversed the increase of Bim induced by the loss of PINCH-1 (Fig. 5B, lane 3). In control experiments, re-expression of PINCH-1 also suppressed the increase of apoptosis induced by the loss of PINCH-1 (Fig. 5C).

The third and perhaps the most important prediction is that apoptosis induced by the loss of PINCH-1 should be blocked by depletion of Bim. To test this, we depleted Bim in HT-1080 cells by RNA interference. Knockdown of PINCH-1 (Fig. 5D, lane 2) dramatically increased the level of Bim (Fig. 5E, lane 2) and caspase-3 activity (Fig. 5F). Depletion of Bim (Fig. 5E, lanes 3 and 4) completely blocked the increase of caspase-3 activity induced by the PINCH-1 knockdown (Fig. 5F). To further test this, we depleted PINCH-1 (Fig. 5G, lane 2), Bim (Fig. 5H, lane 3), or both PINCH-1 and Bim (Figs. 5, G and H, lane 4) from HeLa cells. Again, knockdown of Bim completely blocked the increase of caspase-3 activity induced by the loss of PINCH-1 (Fig. 5I). Collectively, these results provide strong evidence for a critical role of Bim in PINCH-1-mediated regulation of apoptosis.

PINCH-1 Regulates Bim Expression Both Transcriptionally and Post-transcriptionally—Next, we investigated how PINCH-1 regulates the Bim level. Real time reverse transcription-PCR analyses showed that the Bim mRNA level was significantly increased in response to loss of PINCH-1 (Fig. 6A), suggesting that PINCH-1 suppresses Bim expression, at least in part, at the transcriptional level. Up-regulation of Bim transcription is a major mechanism for cancer cell apoptosis induced by histone deacetylase inhibitors. Treatment of HT-1080 cells with histone deacetylase inhibitor sodium butyrate, like depletion of PINCH-1 (Fig. 6B, lane 3), significantly increased the level of Bim (Fig. 6C, lanes 2 and 3). Interestingly, depletion of PINCH-1 from histone deacetylase inhibitor-treated cells (Fig. 6B, lane 4) resulted in a further increase of the Bim level (Fig. 6C, compare lane 4 with lane 2 or 3). These results suggest that PINCH-1 regulates Bim expression not only transcriptionally but also post-transcriptionally. To test this, we transfected the cells with a FLAG-Bim expression vector that is controlled under a different promoter (the cytomegalovirus promoter). One day after FLAG-Bim DNA transfection,
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FIGURE 6. Transcriptional regulation of Bim expression by PINCH-1. A, real time PCR analyses of Bim mRNA levels were performed as described under “Experimental Procedures.” The Bim mRNA levels in the PINCH-1 knockdown HT-1080 cells were compared with those of the control cells (normalized to 1). Bars, means ± S.D. from three experiments. B and C, the control (lanes 1 and 2) and PINCH-1 knockdown HT-1080 cells (lanes 3 and 4) were treated with histone deacetylase inhibitor sodium butyrate. The cells treated with sodium butyrate (lanes 2 and 4) or untreated cells (lanes 1 and 3) were analyzed by Western blot (25 μg of lysates/lane) with anti-PINCH-1 (B) and anti-Bim (C) Abs.

half of the transfectants were transfected with the PINCH-1 siRNA, and the other half were transfected with a control RNA. Depletion of PINCH-1 in the PINCH-1 siRNA transfectants (Fig. 7A, lane 2) but not the control RNA transfectants (Fig. 7A, lane 1) was confirmed by Western blot. Importantly, the level of FLAG-Bim in the PINCH-1-deficient cells was substantially higher than that in the control cells (Fig. 7B, compare lanes 1 and 2). Thus, consistent with a role of PINCH-1 in post-transcriptional regulation of Bim, loss of PINCH-1 increases not only the levels of endogenous Bim mRNA (Fig. 6A) but also the level of exogenously expressed FLAG-tagged Bim protein (Fig. 7B).

PINCH-1 Regulates ERK1/2 and Ser69 Phosphorylation of BimEL—ERK1/2-mediated phosphorylation of BimEL at Ser69 is an important signal for Bim turnover (reviewed in Ref. 46). We therefore tested whether Ser69 phosphorylation is altered in response to loss of PINCH-1. To do this, we immunoprecipitated FLAG-BimEL from PINCH-1 knockdown and control cells, respectively. Consistent with the result of Western blotting (Fig. 7B), a much larger amount of FLAG-BimEL was immunoprecipitated from PINCH-1 knockdown cells (Fig. 7D, compare lane 2 with lane 1). Despite the much larger amount of FLAG-BimEL, the amount of Ser69-phosphorylated BimEL was not increased (Fig. 7E, lanes 1 and 2), suggesting that loss of PINCH-1 reduces the efficiency of phosphorylation of BimEL at Ser69.

Phosphorylation of BimEL at Ser69 is catalyzed by ERK1/2 (reviewed in Ref. 46). The finding that loss of PINCH-1 reduces the efficiency of Ser69 phosphorylation prompted us to test whether depletion of PINCH-1 impairs ERK1/2 activation. Although the levels of ERK1/2 proteins were largely unchanged (Fig. 7F, lanes 1 and 2), the levels of ERK1/2 that were phosphorylated at the TEY sites, which is essential for ERK activation, were substantially reduced in response to loss of PINCH-1 (Fig. 7G, lane 2). Equal loading was confirmed by probing the same membrane with anti-tubulin Ab (Fig. 7G). It has been well documented that SFK plays an important role in regulation of ERK1/2 (47, 48). Notably, loss of PINCH-1 substantially reduced autophosphorylation of Tyr (Tyr418) of human c-Src at the activation loop (Fig. 7H), which is known to be crucial for SFK activation. The level of SFK was not reduced in response to loss of PINCH-1 (Fig. 7I). Equal loading was confirmed by probing the same samples with anti-glyceraldehyde-3-phosphate
Bim (Fig. 8C, compare lanes 1 and 2), but not that of cytosolic Bim (Fig. 8C, compare lanes 3 and 4), was markedly increased in PINCH-1-deficient cells. These results suggest that loss of PINCH-1 not only increases the cellular level of Bim but also promotes Bim translocation to the mitochondria. In contrast to the increase of mitochondrial Bim level, loss of PINCH-1 reduces the level of mitochondrial Bcl-2 (Fig. 8D, compare lanes 1 and 2).

Depletion of Bim Reverses the Reduction of the Mitochondrial Bcl-2 Level Induced by the Loss of PINCH-1—We next tested whether Bim mediates the effect of PINCH-1 on Bcl-2. To do this, we suppressed the expression of Bim (Fig. 8E, lane 3) in PINCH-1 knockdown cells. As expected, knockdown of PINCH-1 in HT-1080 cells dramatically increased the level of mitochondrial Bim (Fig. 8E, lane 2) and concomitantly reduced the level of mitochondrial Bcl-2 (Fig. 8F, lane 2). Importantly, knockdown of Bim (Fig. 8, E and F, lane 3) reversed the reduction of the mitochondrial Bcl-2 level induced by the loss of PINCH-1. These results suggest that PINCH-1 regulates the level of mitochondrial Bcl-2 through Bim, providing additional evidence supporting a central role of Bim in PINCH-1–mediated protection against apoptosis in these cells.

**DISCUSSION**

Cancer cells, in particular metastatic cancer cells, are insensitive to apoptosis induced by alterations in the microenvironment, which allows them to survive drastic changes in microenvironment during metastasis. Identification of proteins and signaling pathways that confer apoptosis resistance is an important topic in cancer cell biology and therapy. We previously found that depletion of PINCH-1 induces apoptosis in HeLa cells (32). The results presented in this paper demonstrate that PINCH-1 is also required for protection of many other types of cancer cells, including HT-1080 fibrosarcoma cells, MDA-231 breast carcinoma cells, PC-3 prostate carcinoma cells, Hep G2 hepatocellular carcinoma cells, Calu-6 lung carcinoma cells, and RKO and HCT-116 colon cancer cells, from apoptosis. Remarkably, PINCH-1 is not required for the survival of nontumorigenic MCF-10A cells and weak tumorigenic Caco-2 cells. Based on these studies, we propose that PINCH-1 functions as a key apoptosis suppressor in multiple types of cancer cells, particularly in certain aggressive cancer cells. These results suggest a model in which PINCH-1 is actively involved in suppression of stress-induced apoptosis signals in cancer cells. Removal or PINCH-1 renders cancer cells sensitive to stress-induced apoptosis signals, resulting in a marked increase of apoptosis.

Our studies on the apoptosis signaling pathway activated by the loss of PINCH-1 lend strong support to this model. We have found that the level of Bim is significantly increased in response to loss of PINCH-1 in several types of PINCH-1-dependent cancer cells. Furthermore, depletion of Bim completely blocks the increase of apoptosis induced by the loss of PINCH-1. Thus, Bim appears to serve as a key converging point in PINCH-1 regulation of apoptosis (Fig. 9).

How does PINCH-1 regulate the Bim apoptosis pathway? The results presented in this paper suggest that PINCH-1 regulates Bim at multiple levels. First, loss of PINCH-1 increases dehydrogenase Ab (Fig. 7). Thus, consistent with inhibition of Ser69 phosphorylation of Bim EL, depletion of PINCH-1 reduces the activating phosphorylation of SFK and ERK1/2.

**Loss of PINCH-1 Promotes Bim Translocation to Mitochondria and Reduces Mitochondrial Bcl-2—**Mitochondria are the primary functional site of Bim-mediated apoptosis. To further analyze the mechanism whereby PINCH-1 regulates apoptosis, we isolated mitochondrial fractions and mitochondria-free cytosolic fractions from the cells. Prohibitin, a marker of mitochondria, was readily detected in the mitochondrial fractions (Fig. 8A, lanes 1 and 2) but not the cytosolic fractions (Fig. 8A, lanes 3 and 4). Probing the same samples with an anti-tubulin Ab showed that, as expected, tubulin was present in the cytosolic fractions (Fig. 8B, lanes 3 and 4) but not the mitochondrial fractions (Fig. 8B, lanes 1 and 2), confirming that the mitochondrial fractions and mitochondria-free cytosolic fractions were effectively separated. Importantly, the level of mitochondrial...
PINCH-1 Regulates the ERK-Bim Pathway

FIGURE 9. A model of PINCH-1-mediated protection of cancer cells from apoptosis. The results presented in this paper suggest that PINCH-1 protects cancer cells from apoptosis through suppression of Bim. PINCH-1 inhibits bim transcription. In addition, PINCH-1 promotes ERK activation, which in turn increases phosphorylation of Bim at Ser69, resulting in increased degradation and inactivation of Bim, and consequently confers resistance of cancer cells to apoptosis (see “Discussion”).

the mRNA level of Bim, suggesting that PINCH-1 suppresses Bim expression, at least in part, at the transcription level. Second, PINCH-1 regulates the Bim level post-transcriptionally, since the level of FLAG-Bim, whose expression was controlled by a different promoter, was also increased in response to depletion of PINCH-1. Finally, loss of PINCH-1 increases the Bim level in the mitochondrial fraction but not that of the tubulin-rich cytosolic fraction, suggesting that PINCH-1 also plays a role in regulation of mitochondrial translocation of Bim. The fact that PINCH-1 regulates Bim not only transcriptionally but also post-transcriptionally is consistent with our previous studies, in which we found that loss of PINCH-1 inhibited Akt activation (32). Akt, through direct phosphorylation of transcription factor FOXO3a, regulates bim transcription (49, 50). Although inhibition of Akt probably contributes to apoptosis induced by the loss of PINCH-1, expression of a constitutively activated Akt was insufficient for suppression of apoptosis induced by the loss of PINCH-1 (32). Thus, we previously proposed that PINCH-1 functions in apoptosis by regulating not only Akt but also a second signaling pathway, albeit its identity was unknown at that time (32). We have now obtained evidence showing that loss of PINCH-1 impairs activating phosphorylation of ERK1/2 (Fig. 7, F and G). Furthermore, Ser69 phosphorylation of BimEL, which is known to be catalyzed by ERK1/2 (46, 51–54), is reduced in the absence of PINCH-1 (Fig. 7, D and E). Because ERK1/2-mediated Ser69 phosphorylation of BimEL is a key signal for BimEL turnover (46) and activation (55) and forced depletion of Bim blocks apoptosis induced by the loss of PINCH-1 (Fig. 5), our results strongly suggest that the ERK1/2-Bim pathway represents a key pathway through which PINCH-1 regulates apoptosis. Collectively, the results from this and previous studies suggest that PINCH-1 functions in protection against apoptosis through at least two mechanisms. First, it facilitates Akt activation and hence regulates the targets of Akt (e.g. FOXO3a and bim transcription). Second, it promotes ERK1/2 activation and consequently ERK1/2-dependent Ser69 phosphorylation and degradation of BimEL. How does PINCH-1 regulate ERK1/2 activation? We have found that PINCH-1 promotes activating Tyr18 phosphorylation of SFK, which is known to play an important role in regulation of the Ras-Raf-ERK pathway (47, 48). SFK activity can be regulated by direct interactions with certain integrin (e.g. β3) cytoplasmic tails or integrin proximal proteins (e.g. focal adhesion kinase) (reviewed in Refs. 56–60). Thus, it is conceivable that PINCH-1 could participate in SFK activation through regulation of signaling events mediated by integrins or integrin-associated proteins. Clearly, future studies are required to test this model.

In summary, the studies presented in this paper demonstrate a strong dependence of several types of cancer cells on PINCH-1. Furthermore, our studies shed new light on the molecular mechanism by which the cancer cells are protected from apoptosis. We show that PINCH-1 functions in the activating phosphorylation of SFK and ERK1/2, which are known to function in oncogenic transformation. Removal of PINCH-1 from these cancer cells inhibits SFK and ERK1/2 activation and promotes Bim accumulation, resulting in activation of a Bim-dependent apoptosis pathway. Therapeutic approaches targeting PINCH-1, therefore, may help to eliminate cancer cells and consequently alleviate tumor growth and metastatic progression.

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