Migfilin Interacts with Src and Contributes to Cell-Matrix Adhesion-mediated Survival Signaling*

Jianping Zhao†, Yongjun Zhang‡, Sujay Subbayya Ithychanda‡, Yizeng Tu§, Ka Chen†, Jun Qin§, and Chuanyue Wu†,†

From the †Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261 and the §Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195

Integrin-mediated cell-extracellular matrix (ECM) adhesion is essential for protection of epithelial cells against apoptosis, but the underlying mechanism is incompletely understood. Here we show that migfilin, an integrin-proximal adaptor protein, interacts with Src and contributes to cell-ECM-mediated survival signaling. Loss of cell-ECM adhesion markedly reduces the migfilin level in untransformed epithelial cells and concomitantly induces apoptosis. Overexpression of migfilin substantially desensitizes cell detachment-induced apoptosis. Conversely, depletion of migfilin promotes apoptosis despite the presence of cell-ECM adhesion. At the molecular level migfilin directly interacts with Src, and the migfilin binding surface overlaps with the inhibitory intramolecular interaction sites in Src. Consequently, the binding of migfilin activates Src, resulting in suppression of apoptosis. Our results reveal a novel mechanism by which cell-ECM adhesion regulates Src activation and survival signaling. This migfilin-mediated signaling pathway is dysfunctional in multiple types of carcinoma cells, which likely contributes to aberrant Src activation and anoikis resistance in the cancerous cells.

Integrin-mediated cell-extracellular matrix (ECM)2 adhesion plays an important role in regulation of cell survival. It has been well documented, for example, that loss of cell-ECM adhesion in untransformed epithelial cells results in apoptosis (1, 2), a process that has been termed as anoikis. Although the phenomenon of anoikis has been well documented and its importance in the pathogenesis and progression of human diseases (e.g. cancer) well recognized (3–8), how loss of cell-ECM adhesion triggers anoikis remains to be determined.

Cell-ECM adhesion is mediated by integrins (9) and a selective group of adaptor and catalytic proteins that are recruited to ECM contacts in response to cell-ECM adhesion. Src, which is recruited to and activated in cell-ECM adhesions, plays an important role in mediating cellular responses to cell-ECM adhesion and other extracellular stimuli (10–13). The studies on Src regulation have provided a framework for our understanding of the principle of modular signaling mechanisms. Several lines of evidence suggest that Src is involved in protection against anoikis. First, Src expression or activity is frequently elevated in carcinoma cells that are resistant to anoikis (13–18). Second, inhibition of Src expression or activity increases the susceptibility of carcinoma cells to anoikis (19, 20). Finally, overexpression of activated Src is sufficient to confer anoikis resistance in a variety of epithelial cells (2, 19).

Structural studies have shown that Src folds into an “assembled” or autoinhibited conformation through two intramolecular interactions; one mediated by the Src homology 2 (SH2) domain and phosphorylated Tyr (Tyr-527 in chicken and Tyr-530 in human) at the C-terminal tail and the other mediated by the SH3 domain and the linker region between the SH2 and the kinase domain (21–25). These intramolecular interactions are of low affinities, and therefore, disruption of either the SH2- or the SH3-mediated intramolecular interaction is sufficient to release Src from the inactive conformation, resulting in Src activation (for reviews, see Refs. 13 and 26).

Migfilin was initially identified as a binding protein for Mig-2/kindlin-2 (27), a FERM domain-containing protein that directly interacts with integrin β cytoplasmic domains (28–33). The integrin-binding recruits kindlin-2/Mig-2 to cell-ECM adhesions (29), which in turn recruits migfilin to these sites (27). Migfilin is an adaptor protein consisting of three structurally distinct regions (27, 34). The C-terminal region is composed of three LIM domains that mediate the interaction with kindlin-2/Mig-2 (27). Migfilin N-terminal region, which does not have obvious sequence homology with any other known proteins, possesses a binding site for filamin (27, 35, 36). In between the N-terminal region and the C-terminal LIM region lies a proline-rich domain that contains multiple proline-rich sequences. In this study we show that migfilin functions as an important activator of Src, linking cell-ECM adhesion to Src activation and survival signaling. Furthermore, we provide information on the structural basis by which migfilin interacts with and activates Src. Finally, we show that this migfilin-mediated signaling pathway is dysfunctional in multiple types of carcinoma cells, which likely contributes to aberrant Src activation and anoikis resistance in these cancerous cells.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Other Reagents—Human mammary epithelia cells were obtained from Clonetics and cultured in mammary epithelial growth media provided by the same sup-
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MCF-10A mammary epithelial cells were grown in Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen) supplemented with 5% horse serum, 20 ng/ml epidermal growth factor (BD Biosciences), 10 µg/ml insulin (Sigma), 1 ng/ml cholera toxin (Sigma), and 100 µg/ml hydrocortisone (Sigma). In some experiments the cells were cultured in medium containing 100 µM benzoyloxy-carbonyl-VAL (Molecular Probes) or 10 µM MG132 (American Peptide). SKBR-3 breast carcinoma cells were grown in McCoy’s 5A and 10% fetal bovine serum. HaCat and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum. Antibodies (Abs) recognizing Src, Tyr(P)-416 Src and Tyr(P)-527 Src were from Cell Signaling. Monoclonal anti-migfilin Ab (clone 43) was described (27). Anti-FLAG Ab M2 was from Sigma. Anti-His Ab was from Santa Cruz Biotechnology. Anti-glyceroldehyde 3-phosphate dehydrogenase (GAPDH) Ab was from Novus Biologicals. Fluorescein isothiocyanate-conjugated anti-mouse IgG and Rhodamine RedTX-conjugated anti-rabbit IgG Abs were from Jackson ImmunoResearch Laboratories.

DNA Constructs, RNAi, and Transfection—Vectors encoding FLAG-, GST-, or His-tagged migfilin or Src proteins were generated by inserting migfilin or Src cDNAs into the corresponding (pFLAG-C6, pGEX-5x-1, or pET-15b) vectors. The sequences of the expression vectors were confirmed by DNA sequencing. Migfilin siRNA MF#1 (target sequence 5-AGAAGGGCATCCACAGACATC-3), MF#2 (target sequence 5-AGAAGGGTCATGGCTGTCTGTTT-3), and MF#3 (target sequence 5-GGGCCCTACATCTGCAGACTTAG-3) were obtained from Invitrogen. Cells were transfected with DNA expression vectors or siRNA with Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocols. The knockdown or overexpression of migfilin was confirmed by Western blotting. The densities of migfilin, GAPDH, phospho-Tyr-419 Src, and Src bands were quantified using NIH Scion Image program. To analyze the changes of the levels of migfilin and phospho-Tyr-419 Src, the relative levels (RLs) of migfilin (defined as densities of migfilin bands/densities of GAPDH bands) and those of phospho-Tyr-419 Src (defined as phospho-Tyr-419 Src bands/densities of Src bands) in migfilin knockdown or overexpressing cells were compared with those in control cells (normalized to 1).

Cell Detachment and Apoptosis Assays—Cells were trypsinized, resuspended in complete medium, and plated in culture dishes containing a layer of 0.5% agarose as described (50, 51). The cells were incubated in suspension at 37 °C under a 5% CO2/95% air atmosphere for various lengths of time (as specified in each experiment). At the end of incubation the cells were harvested and analyzed by Western blotting and apoptosis assays. The RLs of migfilin and phospho-Tyr-419 Src in detached cells were calculated as described above and compared with those in attached cells (normalized to 1).

Apoptosis was analyzed using two different assays. In the first assay caspase-3 activities were measured in triplicate using fluorogenic caspase-3 substrate VII (Calbiochem) as described (52). In the second assay DNA fragmentation was analyzed using a Cell Death Detection enzyme-linked immunosorbent assay kit (Roche Applied Science) following the manufacturer’s protocol. The activities of caspase-3 and the levels of DNA fragmentation in detached cells were compared with those of attached cells (normalized to 1). To analyze the effect of wild type or mutant forms of migfilin on anoikis, cells were transfected with vectors encoding different forms of migfilin. One day after DNA transfection, the transfectants were cultured in suspension for 24 h. Anoikis was analyzed using both caspase-3 and DNA fragmentation assays as described above.

Immunofluorescent Staining—MCF-10A cells were plated on fibronectin-coated coverslips and incubated at 37 °C under a 5% CO2/95% air atmosphere overnight. At the end of incubation, the cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and doubly stained with the mouse anti-migfilin and rabbit anti-Tyr(P)-416 Src Abs. The primary Abs were detected with fluorescein isothiocyanate-conjugated anti-mouse IgG, and Rhodamine RedTX-conjugated anti-rabbit IgG Abs.

Immunoprecipitation—MCF-10A cells expressing FLAG-migfilin were lysed with PBS containing 1% Triton X-100, 10 mM NaF, 1 mM NaN3VO4, 0.2 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin, 1 µg/ml aprotinin, and 10 µM leupeptin (the lysis buffer). The cell lysates (500 µg) were incubated with agarose beads conjugated with anti-FLAG Ab M2 (Sigma) at 4 °C for 4 h. The beads were washed 5 times with 1% Triton X-100 in PBS, and immunoprecipitates were analyzed by Western blotting with anti-FLAG and anti-Src Abs.

GST Fusion Protein Pulldown Assays—Escherichia coli (BL21) cells were transformed with pGEX-5x-1 or pET-15b vectors encoding wild type or mutant forms of migfilin or Src. The expression of GST and His fusion proteins was induced with 0.3–0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 4 h. The bacteria were harvested and sonicated in ice-cold lysis buffer (1% Triton X-100 in PBS containing protease inhibitors). The GST and His fusion proteins were isolated using glutathione-Sepharose beads (Thermo Scientific) and nickel-nitriolactiace acid-agarose beads (Qiagen), respectively. For GST-migfilin pulldown studies, MCF10A cells were lysed with the lysis buffer. The cell lysates (500 µg) were pre-cleared with glutathione-Sepharose beads and then incubated with glutathione-Sepharose containing GST-migfilin or GST at 4 °C for 4 h. The precipitates were washed five times with the lysis buffer and analyzed by Western blotting and Coomassie Blue staining. To analyze direct interactions between migfilin and Src proteins, purified His-tagged wild type or mutant forms of migfilin were incubated with GST-tagged wild type or mutant forms of Src in the lysis buffer at 4 °C for 2 h. The GST fusion proteins were precipitated with glutathione-Sepharose beads and washed five times with the lysis buffer. The samples were analyzed by Western blotting with anti-His Ab and Coomassie Blue staining.

NMR Analyses—DNA fragment encoding human Src SH3 domain was cloned in pGex4T vector and expressed in E. coli BL21(D3) using 0.1 mM isopropyl 1-thio-β-D-galactopyranoside at room temperature and purified using glutathione-Sepharose beads. Heteronuclear single-quantum correlation experiments were performed on a Bruker 600 MHz spectrometer equipped with a cryogenic triple resonance probe at 30 °C in 20 mM sodium phosphate buffer (pH 6.8) containing 5 mM NaCl. NMR spectra were processed and analyzed using
NMRPipe (53). NMR signals in the $^{15}$N-labeled spectra were assigned according to Yu et al. (54). Although the original assignment by Yu et al. (54) was at pH 6.0 and our spectra were recorded at pH 6.8, most peaks were readily transferred due to similarity of the spectra.

**Results**

**Loss of Cell-ECM Adhesion Reduces the Level of Migfilin in Anoikis-sensitive Epithelial Cells**—To begin to test whether migfilin plays a role in anoikis signaling, we analyzed the effect of loss of cell-ECM adhesion on migfilin in untransformed human epithelial cells including primary human mammary epithelial cells (HMECs), HaCat keratinocytes, and MCF-10A mammary epithelial cells that are known to be sensitive to anoikis. The results showed that loss of cell-ECM adhesion resulted in substantial reductions of the level of migfilin, whereas the levels of irrelevant proteins such as GAPDH were not changed (Fig. 1, A, D, G, and H). Concomitant to the reduction of migfilin, the activity of caspase-3, a key executioner of apoptosis, was significantly increased (Fig. 1, B, E, and I). Analyses of the level of DNA fragmentation confirmed the increase of apoptosis in detached HMEC (Fig. 1C), HaCat (Fig. 1F), and MCF-10A (Fig. 1J) cells. These experiments reveal a close correlation between loss of cell-ECM adhesion, down-regulation of migfilin, and induction of apoptosis in anoikis-sensitive epithelial cells.

**Down-regulation of Migfilin Is Critical for Induction of Anoikis**—Next, we sought to determine the functional significance of cell detachment-induced down-regulation of migfilin in anoikis signaling using MCF-10A epithelial cells as a model system. We reasoned that the reduction of the migfilin level in response to loss of cell-ECM adhesion could be either a consequence or a cause of caspase-3 activation. To test the former possibility, we treated attached cells as well as detached cells with caspase inhibitor benzylxoycarbonyl-VAD. Loss of cell-ECM adhesion reduced the level of migfilin both in the absence (Fig. 1K, compare lanes 2 and 3 with lane 1) and presence (Fig. 1K, compare lanes 5 and 6 with lane 4) of the caspase inhibitor, suggesting that the down-regulation of migfilin was not caused by the activation of caspase-3. In contrast to treatment with caspase inhibitor, treatment of cells with proteasome inhibitor MG132 effectively prevented cell detachment-induced down-regulation of migfilin (Fig. 1L), suggesting that cell detachment-induced down-regulation of migfilin is likely dependent on proteasomes.

To test the latter possibility (i.e. down-regulation of migfilin is a cause of caspase-3 activation), we depleted migfilin from MCF-10A cells by RNAi. Transfection of MCF-10A cells with migfilin siRNAs substantially reduced (albeit did not completely eliminate) the level of migfilin (Fig. 2A, compare lanes 1, 2, and 4 with lane 3). Importantly, the activity of caspase-3 was significantly increased in response to down-regulation of migfilin (Fig. 2B), with the highest level of caspase-3 activity detected in cells expressing the lowest level of migfilin (Fig. 2A, lane 2). Thus, down-regulation of migfilin is sufficient to promote caspase-3 activation. Consistent with this, the level of DNA fragmentation was also augmented in response to knock-down of migfilin (Fig. 2, C and D). To further test this, we sought to increase the level of migfilin and test whether it reverses cell detachment-induced caspase-3 activation. To do this, we transiently transfected MCF-10A cells with an expression vector encoding FLAG-migfilin (Fig. 2E, lanes 2 and 4) or a FLAG vector lacking migfilin sequence as a control (Fig. 2E, lanes 1 and 3). As expected, the levels of caspase-3 activity (Fig. 2F) and DNA fragmentation (Fig. 2G) were markedly increased in response to loss of cell-ECM adhesion in control transfectants. Forced overexpression of migfilin significantly reduced cell detachment-induced caspase-3 activation (Fig. 2F) and DNA fragmentation (Fig. 2G). Thus, the down-regulation of migfilin is responsible for, at least in part, the increase of apoptosis in response to loss of cell-ECM adhesion.

**Migfilin Regulates Anoikis through Control of Src Activation**—How does down-regulation of migfilin promote apoptosis? Src is known to play an important role in suppression of anoikis in cancer cells (19, 20, 37–40). Concomitant to cell detachment-induced down-regulation of migfilin, the level of activating (i.e. Tyr-419) phosphorylation of Src, but not that of Src protein or phosphorylation of Tyr at Src C-terminal tail (Tyr-530 in human and Tyr-527 in chicken), was substantially reduced (Fig. 3A, compare lanes 2–4 with lane 1) in MCF-10A cells. Down-regulation of Src activation was also observed in other untransformed epithelial cells including HMEC and HaCat cells in which the level of migfilin was reduced in response to loss of cell-ECM adhesion (Fig. 3B, compare lanes 2 and 4 with lanes 1 and 3). To test whether the down-regulation of Src activation contributes to anoikis, we expressed a constitutively active form of chicken Src (Y527F) in MCF-10A cells. Expression of the constitutively active form of Src (Fig. 3C, lane 3), like overexpression of migfilin (Fig. 2, E and F), significantly reduced cell detachment-induced caspase-3 activation (Fig. 3D), suggesting that cell detachment-induced inhibition of Src activation contributes to anoikis signaling.

We next tested whether migfilin functions in anoikis through regulation of Src activation. To do this we first analyzed the effect of increased level of migfilin on Src activation. Overexpression of FLAG-migfilin did not significantly alter Tyr-419 phosphorylation of Src.

**FIGURE 1.** Loss of cell-ECM adhesion reduces the level of migfilin. A–C, HMECs were cultured under attached (lane 1) or detached (lane 2) condition for 48 h. The cells were analyzed by Western blotting (A), caspase-3 (B), and DNA fragmentation (C) assays. Bars in panels B and C are the means ± S.D. from two independent experiments. D–F, HaCat cells were cultured under attached (lane 1) or detached (lane 2) conditions for 24 h. The cells were analyzed by Western blotting (D), caspase-3 (E), and DNA fragmentation (F) assays. Bars in panels E and F are the means ± S.D. from two independent experiments. G–J, MCF-10A cells were cultured under attached or detached conditions for 6, 12, 24, or 48 h and analyzed by Western blotting (G). The relative level of migfilin (H), caspase-3 activity (I), and DNA fragmentation (J) were analyzed as described under “Experimental Procedures.” Bars in panels I and J are the means ± S.D. from two independent experiments. K and L, MCF-10A cells were cultured under attached or detached conditions in media supplemented with or without benzylxoycarbonyl-VAD (Z-VAD) for 24 or 48 h (K) or in media supplemented with or without MG-132 for 24 h (L) and analyzed by Western blotting. The RLS of migfilin protein (means ± S.D. from two independent experiments) in panels A, D, K, and L were calculated as described under “Experimental Procedures.”
phosphorylation of Src in attached cells (Fig. 3E, compare lanes 1 and 2). Noticeably, however, in cells that lost cell-ECM adhesion, overexpression of FLAG-migfilin resulted in a significant increase of Tyr-419 phosphorylation (Fig. 3E, compare lanes 3 and 4). Neither the protein level nor Tyr-530 phosphorylation of Src was altered in response to overexpression of FLAG-migfilin (Fig. 3E, compare lanes 3 and 4). To further analyze the role of migfilin in regulation of Src activation, we depleted migfilin from cells that maintained cell-ECM adhesion. Knockdown of migfilin, like cell detachment-induced reduction of migfilin,
substantially inhibited Tyr-419 phosphorylation but not the protein level or Tyr530 phosphorylation of Src (Fig. 3, compare lanes 2–4 with lane 1). In contrast to a role of migfilin in regulation of Src activation, overexpression of constitutively active Src did not prevent cell detachment-induced reduction of migfilin (Fig. 3, lanes 2 and 3). Taken together, these results suggest that 1) migfilin acts upstream of Src and 2) down-regulation of migfilin induced by cell detachment is responsible for, at least in part, inactivation of Src associated with loss of cell-ECM adhesion.

Migfilin Directly Interacts with Src—How does migfilin regulate Src activation? Migfilin possesses no catalytic activities but instead contains multiple protein-binding motifs (27, 34). Immunofluorescent staining showed that migfilin was co-clustered with Tyr-419-phosphorylated Src in a large number of focal adhesions (Fig. 4, A, B, and C), raising a possibility that migfilin might physically interact with Src. To test this, we immunoprecipitated FLAG-migfilin from cell lysates and found that Src was readily co-immunoprecipitated with FLAG-migfilin (Fig. 4D, lane 2). In control experiments no Src was co-precipitated with anti-FLAG Ab in the absence of FLAG-migfilin (Fig. 4D, lane 1), confirming the specificity of co-immunoprecipitation. To further test the interaction between migfilin and Src, we generated recombinant GST-tagged and His-tagged migfilin fusion proteins. GST-migfilin (Fig. 4E, lane 4), but not GST (Fig. 4E, lane 3), readily pulled down Src from cell lysates. Furthermore, purified His-migfilin bound GST-Src (Fig. 4F, lane 4) but not GST (Fig. 4F, lane 3). Thus, consistent with the co-localization and co-immunoprecipitation of these two proteins, migfilin directly interacts with Src.

Structural Basis of the Migfilin-Src Interaction—To identify the domains of Src that mediate the interaction with migfilin, we generated GST fusion proteins containing individual Src domains including the SH2, the SH3, or the kinase domain. His-migfilin bound strongly to GST-SH3 (Fig. 4G, lane 5) and modestly to GST-SH2 (Fig. 4G, lane 4). By contrast, neither the GST kinase domain (Fig. 4G, lane 6) nor GST alone (Fig. 4G, lane 3) bound His-migfilin. These results suggest that the SH3 domain and to a less extent the SH2 domain, but not the kinase domain, mediate the interaction of Src with migfilin.

We next sought to identify the domain of migfilin that mediates the interaction with Src SH3 domain. To do this...
we generated His-tagged proteins containing various domains of migfilin (Fig. 4H). His-tagged proteins containing the N-terminal and proline-rich regions (Fig. 4I, lane 4) or the proline-rich domain and the LIM region (Fig. 4I, lane 16), like His-migfilin (Fig. 4G, lane 5), bound to Src SH3. Furthermore, His-tagged proline-rich domain alone was able to interact with Src SH3 domain (Fig. 4I, lane 20). By contrast, neither migfilin LIM region (Fig. 4I, lane 8) nor a migfilin protein containing the N-terminal and LIM regions but lacking the proline-rich domain (Fig. 4I, lane 12) bound SH3. Thus, the proline-rich domain of migfilin is both necessary and sufficient for mediating the interaction with Src SH3 domain.

Migfilin proline-rich domain contains two clusters of proline-rich sequences; the first is located at positions 86–112, which we previously found to mediate the interaction with the vasodilator-stimulated phosphoprotein (VASP) EVH1 domain (41), and the second encompasses residues 140–170. Deletion of residues 142–170 (Fig. 4I, lane 24), but not that of residues 84–112 (data not shown), eliminated Src SH3 binding, suggesting that the second cluster of proline-rich sequences located at residues 142–170 mediates Src SH3 binding. Deletion of individual polyproline sequences (e.g. deletion of 140PPP...145 or 167PPP...173) within this region, however, failed to eliminate the interaction with Src SH3 domain (data not shown). The Src SH3 binding activities of different migfilin mutants are summarized in Fig. 4H.

To further analyze the structural basis underlying the interaction between Src SH3 domain and migfilin, we synthesized two migfilin peptides (peptide 1, 139SPPPPPPQAPEGSPVQPGPLR160; peptide 2, 156PGPLRPMEEELPPPPAEPVE175), which collectively encompass the entire region of 142–170. NMR analyses showed that titration of either peptide into the 15N-labeled Src SH3 domain induced selective chemical shift changes of a number of residues (Fig. 5, A and B), indicating that both proline-rich sequences can interact with Src SH3 domain. Noticeably, however, peptide 2 (Fig. 5B) induced many more substantial spectral changes than peptide 1 (Fig. 5A), suggesting that peptide 2 possesses the major binding site for Src SH3. Importantly, the migfilin binding surface on the Src SH3 domain (Fig. 5C) overlaps with the binding surface for Src SH2/kinase linker segment (Fig. 5D), which is known to be crucial for maintaining the inactive conformation of Src.
FIGURE 5. The structural basis and functional significance of migfilin binding to Src SH3 domain. A and B, shown are NMR analyses of migfilin interaction with Src SH3 domain. Two-dimensional $^1$H,$^1$H heteronuclear single-quantum correlation of 0.1 mM $^{15}$N-labeled SH3 domain of human c-Src in the absence (black) or presence (red) of 0.5 mM migfilin peptide 1 (A) or 2 (B) is shown. Residues that have significant chemical shift perturbation are labeled. C, shown is the crystal structure of human c-Src SH3 domain (PDB code 2SRC) with chemical shift changes highlighted (orange). The side chains of the most significantly perturbed residues are shown and point to the same side for recognizing migfilin peptides. D, shown is the crystal structure of the inactive Src (PDB code 2SRC) where the kinase domain and SH3 domain (magenta) are bound by the linker peptide (red) between the SH2 domain and kinase domain. The SH3 domain is in the same view as in panel C, and residues in the SH3 domain perturbed by migfilin peptide 2 binding are in orange, which overlap with the linker peptide binding surface. E–G, MCF-10A cells were transfected with vectors encoding different forms of migfilin or a control vector lacking migfilin sequence as indicated. The cells were cultured under attached or detached conditions for 24 h and analyzed by Western blotting (E), caspase-3 (F), and DNA fragmentation (G) assays. Bars in panels F and G are the means ± S.D. from three independent experiments (*, $p < 0.05$ compared with control under detached conditions).
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**FIGURE 6. Migfilin binding to Src SH2 domain promotes Src activation and inhibits anoikis.** A, shown is a schematic representation of wild type and mutant forms of migfilin and their Src SH2 binding activity. B, His fusion proteins containing various migfilin sequences were incubated with GST-SH2 or GST. The input and pulldown assays were analyzed by Western blotting (WB) with anti-His Ab and Coomassie Blue staining. C–E, MCF-10A cells were transfected with vectors encoding different forms of migfilin or a control vector lacking migfilin sequence as indicated. The cells were cultured under attached or detached conditions for 24 h and analyzed by Western blotting (C), caspase-3 (D), and DNA fragmentation (E) assays. Bars in panels D and E are the means ± S.D. from three independent experiments (*, p < 0.05 compared with control under detached conditions).

**Binding of Migfilin Promotes Src Activation and Protects Epithelial Cells from Anoikis**—To test whether the binding of migfilin to Src SH3 domain promotes Src activation and protects epithelial cells against anoikis, we expressed different forms of migfilin that were either Src SH3-binding competent or defective in MCF-10A cells. Consistent with the results shown earlier, loss of cell-ECM adhesion significantly reduced the activating phosphorylation of Src (Fig. 5E, compare lanes 1 and 2) and increased caspase-3 activity (Fig. 5F) and DNA fragmentation (Fig. 5G) in control cells. Overexpression of a Src SH3-binding migfilin fragment containing the proline-rich domain and the LIM region (FLAG-Pro + LIM), like that of migfilin (Figs. 2, E, F, and G, and 3E), substantially increased the activating phosphorylation of Src (Fig. 5E, lane 3) and concomitantly reduced cell detachment-induced caspase-3 activation (Fig. 5F) and DNA fragmentation (Fig. 5G). Importantly, ablation of the Src SH3 binding site within the proline-rich domain impaired the ability of FLAG-Pro + LIM to promote Src activation (Fig. 5E, lane 4) or to inhibit cell detachment-induced caspase-3 activation (Fig. 5F) and DNA fragmentation (Fig. 5G). These results suggest that the binding of migfilin to Src SH3 domain promotes Src activation and consequently confers resistance to anoikis.

Migfilin interacts with not only Src SH3 domain but also Src SH2 domain, albeit the latter interaction is weaker than the former (Fig. 4G, compare lanes 4 and 5). His-tagged migfilin N-terminal and proline-rich regions (His-N+Pro), which bound strongly to the Src SH3 domain (Fig. 4I, lane 4), bound modestly to the SH2 domain (Fig. 4I, lane 3). His-N+LIM, which contains the N-terminal and LIM regions but lacks the proline-rich domain and, therefore, was unable to interact with Src SH3 domain (Fig. 4I, lane 12), bound GST-tagged Src SH2 domain (Fig. 4I, lane 11) but not GST alone (Fig. 4I, lane 10). By contrast, migfilin fragment containing the LIM region alone failed to bind Src SH2 domain (Fig. 4I, lane 7). These results confirm the specificity of the interaction with Src SH2 domain and suggest that residues in the N-terminal region of migfilin are essential for mediating the SH2 binding.

To pinpoint migfilin residues that are essential for Src SH2 binding, we introduced a series of mutations into the N-terminal region of migfilin (Fig. 6A). Consistent with the results shown earlier (Fig. 4I, lane 11), GST-SH2 (Fig. 6B, lane 3), but not GST (Fig. 6B, lane 5), bound His-N+LIM. Deletion of 12 residues from the N terminus of migfilin abolished SH2 binding (Fig. 6B, lane 4). By contrast, deletion of five residues from the N terminus of migfilin failed to eliminate SH2 binding (Fig. 6B, lane 9). Additionally, substitution mutations at positions 7 and 8 (K7T/R8G) (Fig. 6I), K7T/R8G deletion of five residues from the N terminus of migfilin failed to eliminate SH2 binding (Fig. 6I). These results confirm the specificity of the binding assay. Collectively, these results suggest that residues 7–12 (KRVASS12) located at migfilin N-terminal region are essential for mediating the interaction with Src SH2 domain.

We next tested whether the binding of migfilin to Src SH2 domain can promote Src activation and protect epithelial cells against anoikis. To do this, we expressed FLAG-tagged SH2-binding migfilin protein containing the N-terminal and LIM regions (FLAG-N+LIM) (Fig. 6C, lane 4) and the SH2-binding defective K7T/R8G mutant form of FLAG-N+LIM (Fig. 6C, lane 5), respectively, in MCF-10A cells. In parallel experiments we transfected the cells with a vector encoding FLAG-migfilin (Fig. 6C, lane 3) or a vector lacking migfilin sequence as a negative control (Fig. 6C, lanes 1 and 2). Loss of cell-ECM adhesion resulted in significant decrease of the activating phosphorylation-
binding FLAG-N+LIM, but not that of SH2-binding defective Lys-7—Arg-8 mutant, also reversed Src inactivation (Fig. 6C, compare lanes 4 and 5), caspase-3 activation (Fig. 6D), and DNA fragmentation (Fig. 6E) induced by loss of cell-ECM adhesion, suggesting that the binding of migfilin to Src SH2 domain can promote Src activation and protect epithelial cells against anoikis.

The Migfilin Anoikis Signaling Pathway Is Dysfunctional in Multiple Types of Anoikis-insensitive Carcinoma Cells—Resistance to anoikis is a hallmark of malignant transformation. Our findings that migfilin links cell-ECM adhesion to Src activation and consequently suppresses caspase-3 activation in untransformed epithelial cells prompted us to test whether this pathway is dysfunctional in anoikis-insensitive carcinoma cells. To do this we analyzed the levels of migfilin in SKBR3 breast carcinoma cells, which are known to be insensitive to anoikis (37, 42, 43). In marked contrast to anoikis-sensitive epithelial cells, loss of cell-ECM adhesion failed to reduce the level of migfilin in these carcinoma cells (Fig. 7A, compare lanes 2 and 3 with lane 1). Furthermore, consistent with a critical role of migfilin in regulation of Src and anoikis, loss of cell-ECM adhesion neither reduced the activating phosphorylation of Src (Fig. 7A) nor induced the activation of caspase-3 (Fig. 7B) in the carcinoma cells. To confirm that the failure of down-regulation of migfilin in response to loss of cell-ECM adhesion is responsible for constitutive activation of Src and resistance to anoikis in these carcinoma cells, we depleted migfilin using three different migfilin siRNA. The levels of migfilin were reduced in all three siRNA transfectants (Fig. 7C, compare lanes 1, 3, and 4 with lane 2). Importantly, Tyr-419 phosphorylation but neither Tyr-530 phosphorylation nor the protein level of Src was reduced in all migfilin knockdown cells (Fig. 7C, compare lanes 1, 3, and 4 with lane 2), with the cells in which migfilin was most effectively knocked down exhibiting the lowest level of Tyr-419 phosphorylation (Fig. 7C, lane 3). The activity of caspase-3 was significantly increased in migfilin

FIGURE 7. Dysregulation of the migfilin-Src signaling pathway in anoikis-insensitive SKBR-3 cells. A and B, SKBR-3 cells were cultured under attached or detached conditions for 24 or 48 h. The cells were analyzed by Western blotting (A) and caspase-3 assays (B). Bars indicate the means ± S.D. from two independent experiments. RLs in panel A are the relative levels of phosphor-Tyr-419 Src (means ± S.D. from two independent experiments). C and D, SKBR-3 cells were transfected with control RNA or migfilin siRNAs as indicated. The samples were analyzed by Western blotting (C) and caspase-3 assays (D). Bars indicate the means ± S.D. from three independent experiments (*, p < 0.05 compared with control). RLs in panel C are the relative levels of phosphor-Tyr-419 Src (means ± S.D. from three independent experiments). E and F, SKBR-3 cells were transfected with control RNA or migfilin siRNAs as indicated. The samples were analyzed by Western blotting (E) and DNA fragmentation assays (F). Bars indicate the means ± S.D. of triplicate samples from one experiment.

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knockdown cells, and again, the cells in which migfilin was most effectively knocked down exhibited the highest level of caspase-3 activity (Fig. 7D).

To further test the effect of migfilin knockdown on apoptosis, we depleted migfilin from SKBR3 cells and analyzed the level of DNA fragmentation. The results showed that depletion of migfilin also significantly augmented DNA fragmentation (Fig. 7, E and F), confirming a crucial role of migfilin in protection of SKBR3 cells against apoptosis. Collectively, our results suggest that the level of migfilin is uncoupled from the status of cell-ECM adhesion in SKBR3 breast carcinoma cells, and the dysfunction of this migfilin-mediated signaling pathway is responsible for, at least in part, the insensitivity of the breast carcinoma cells to anoikis.

To test whether migfilin plays a role in control of Src activation and anoikis resistance in other types of carcinoma cells, we analyze the effect of loss of cell-ECM adhesion on migfilin in HeLa cells, which are also known to be insensitive to anoikis (44–46). The level of migfilin in HeLa cells was not reduced 24 h after loss of cell-ECM adhesion (Fig. 8A, compare lanes 1 and 2), and it was even increased after prolonged (48 h) loss of cell-ECM adhesion (Fig. 8A, compare lane 3 with lane 1). Consistent with an important role of migfilin in regulation of Src activation, the level of Tyr-419 phosphorylation in HeLa cells was not reduced 24 h after loss of cell-ECM adhesion (Fig. 8A, compare lanes 1 and 2), and it was even increased after prolonged (48 h) loss of cell-ECM adhesion (Fig. 8A, compare lane 3 with lane 1). Furthermore, consistent with a critical role of migfilin and Src in anoikis signaling, the level of caspase-3 activity was not increased in response to loss of cell-ECM adhesion (Fig. 8B). To confirm that the relative high level of migfilin confers anoikis resistance in these cells, we knocked down migfilin by RNAi. Again, depletion of migfilin substantially inhibited Tyr-419 phosphorylation but not Tyr-530 phosphorylation or the protein level of Src (Fig. 8C, compare lanes 2 and 3 with lane 1) and promoted caspase-3 activation (Fig. 8D). Consistent with the increase of caspase-3 activity, DNA fragmentation was also augmented in response to depletion of migfilin (Fig. 8, E and F). Collectively, these results suggest that migfilin is critically involved in regulation of Src activation and anoikis resistance in multiple types of carcinoma cells.

**DISCUSSION**

Cell-ECM adhesion is an important determinant of epithelial cell survival, but the underlying molecular mechanism is incompletely understood. The studies presented in this paper have shed new light on how cell-ECM adhesion protects epithelial cells from apoptosis. First, our studies reveal that the level of migfilin in untransformed epithelial cells is dramatically reduced in response to loss of cell-ECM adhesion. The reduction of the migfilin level inhibits Src activation and consequently promotes anoikis. Thus, migfilin appears to serve as a sensor of cell-ECM adhesion in the life and death decision-making process in untransformed epithelial cells. Second, we have shown that migfilin directly binds Src. The binding of migfilin to Src is critically involved in Src activation. Thus, migfilin works in concert with Src and translates changes in the status of cell-ECM adhesion into cell survival signals. Finally, we have shown that this cell-ECM adhesion-sensing pathway is defective in multiple types of carcinoma cells and, therefore, provides an explanation as to why the carcinoma cells are insensitive to anoikis.

The studies presented in this paper not only uncover a novel migfilin-Src signaling pathway but also provide the structural basis through which migfilin regulates Src activation. We have found that migfilin binds Src through two distinct sites. The first Src binding site is located at residues 140–173 within the proline-rich domain of migfilin. The polyproline sequences at residues 140–173 recognize the hydrophobic binding surface in Src SH3 domain, which is known to be involved in the inhibitory intramolecular interaction between the SH3 domain and the linker segment (21–25). Thus, the binding of migfilin pro-
line-rich domain to the hydrophobic binding surface in Src SH3 domain could displace the linker segment, resulting in activation of Src. The findings from the structural analyses are supported by our functional studies in which expression of a SH3 binding migfilin fragment (FLAG-Pro+LIM), but not that of a mutant form of FLAG-Pro+LIM (FLAG-Δ142–170) in which the SH3-binding activity is ablated, promoted Src activation. It is worth noting that migfilin proline-rich domain contains two closely spaced SH3 binding sites, one located within residues 158–173 and the other located within residues 140–158. Thus, the interaction of migfilin with Src SH3 domain not only may perturb the inhibitory SH3-linker interaction (thereby promote the transition to an active conformation) but also could bring two Src proteins into close proximity and thereby facilitate trans-autophosphorylation at Tyr-419, a step that is important for full Src activation (for review, see Ref. 10).

The assembled or inactive conformation of Src is stabilized by two inhibitory intramolecular interactions, one mediated by the SH3 domain and the other by the SH2 domain (21–25). Disruption of either SH3- or SH2-mediated interaction is sufficient for inducing Src activation (for reviews, see Refs. 13 and 26). Interestingly, we have found that migfilin binds not only Src SH3 domain but also Src SH2 domain. Furthermore, the binding of migfilin to Src SH2 domain, like that to Src SH3 domain, promotes Src activation. The binding of Src SH2 domain is independent of the proline-rich domain but instead requires the N-terminal region of migfilin. The presence of both SH3 and SH2 binding sites in migfilin may allow it to bind simultaneously both Src domains. In addition, it may allow migfilin to bind and activate multiple Src proteins simultaneously and, therefore, facilitate trans-autophosphorylation at the activation loop. Finally, we previously found that several types of cells and tissues express a splicing variant of migfilin (termed as migfilin(s)) containing the N-terminal and LIM regions but lacking the proline-rich domain (27). The ability of migfilin to activate Src through a proline-rich domain-independent mechanism could provide a mechanism for migfilin(s) to activate Src in these cell types and tissues.

Although migfilin can interact with both SH3 and SH2 domains and can activate Src through either interaction, we note two important differences. First, Src SH3 domain binds migfilin much more strongly than the Src SH2 domain (Fig. 4G), suggesting that the SH3 domain likely represents a major docking site for migfilin. Second, the migfilin N-terminal residues that are involved in interacting with Src SH2 domain (e.g. Lys-7, Arg-8, and Ser-11) are part of the binding surface for filamin repeat 21 (IgFLN21). The migfilin binding surface in IgFLN21 overlaps with the integrin binding surface (35, 36). Thus, the binding of migfilin to Src SH2 domain could potentially be regulated by the interaction between migfilin and filamin, which in turn can be regulated by the interaction between integrins and filamin. Based on the results obtained from this and previous studies, we propose a model through which migfilin links integrin-mediated cell-ECM adhesion to Src activation. In this model migfilin is recruited to cell-ECM adhesions upon integrin-mediated cell-ECM adhesion, where it binds Src and activates Src through one or both of the following mechanisms. First, migfilin proline-rich domain binds Src SH3 domain, which disassociates the linker segment and thereby induces the transition of Src to the active conformation. The SH3 binding sites (located within migfilin residues 140–173) are separated from the binding sites for other focal adhesion proteins such as Mig-2/kindlin-2 (the Mig-2/kindlin-2-binding site is located in the LIM region). VASP (the VASP-binding site is located at migfilin residues 104–109), and filamin (the filamin-binding site is located at migfilin residues 6–17). Thus, the Src SH3-binding site in migfilin is likely readily available in focal adhesions and, therefore, could serve as a predominant site for migfilin-mediated Src activation. Second, migfilin can interact with Src SH2 domain and thereby activate Src. The binding of migfilin to Src SH2 domain, however, could be influenced by multiple factors including local concentration of integrins. In sites where high concentrations of integrins are available, the binding of β integrin cytoplasmic tails to filamin could release migfilin N-terminal residues from IgFLN21, allowing them to participate in the interaction with Src SH2 domain and consequently contribute to Src activation. The migfilin-mediated Src SH2 binding, therefore, may play a role in augmenting Src activation in cell-ECM adhesions where abundant integrins are present. Upon cell detachment, the ECM adhesions and actin filaments are disassembled, resulting in dissociation of migfilin from these structures and reduction of the level of migfilin, which in turn causes Src inactivation. How does loss of cell-ECM adhesion reduce the level of migfilin? Our studies suggest that the reduction of the migfilin level induced by loss of cell-ECM adhesion is likely mediated by proteasomes. In this regard it is worth noting that unbound migfilin N-terminal region is unstructured (35, 36). The binding of migfilin to Src SH2 domain or actin filament-associated proteins such as filamin may help to stabilize the structure of migfilin N-terminal region and thereby prevent proteasome-mediated degradation of migfilin. Although this model is consistent with findings obtained from this and previous studies, including regulation of the cellular level of migfilin by cell-ECM adhesion, cell-ECM adhesion-induced augmentation of Src activation, and the phenomenon of anoikis, future studies are clearly required to further test this model.

In summary, the work presented in this paper has revealed an important role of migfilin in cell-ECM adhesion-mediated regulation of Src activation and protection against anoikis. Furthermore, we have provided structural insights into the mechanism by which migfilin regulates Src activation. It is worth noting that Src can be regulated by multiple proteins including β3 integrins, focal adhesion kinase, and RIL (47–49). The presence of multiple regulators of Src likely reflects the need for precise control of Src activity in responses to diverse extracellular signals. Migfilin conveys the status of cell-ECM adhesion to Src regulation, and this signaling pathway is not redundant to other Src activation mechanisms, as depletion of migfilin substantially impairs Src activation and survival signaling. Given the prominent role of migfilin in Src activation and anoikis resistance, therapeutic intervention targeting the migfilin signaling pathway may provide a novel approach to restore anoikis sensitivity and alleviate cancer progression.
Migfilin Interacts with Src

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