Phosphorylation of the Cool-1/β-Pix Protein Serves as a Regulatory Signal for the Migration and Invasive Activity of Src-transformed Cells*

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Previously we showed that Cool-1 (Cloned out of library-1)/β-Pix (Pak-interactive exchange factor) is phosphorylated at a specific tyrosine residue (Tyr-442) in a Src-dependent manner and serves as a dual function guanine nucleotide exchange factor (GEF)/signaling-effector for Cdc42 that is essential for transformation by Src. Here, we show that knocking-down Cool-1 or overexpressing a Cool-1 mutant that contains substitutions within its Dbl homology domain and is defective for GEF activity, inhibits Src-promoted cell migration. Similarly, the expression of a Cool-1 mutant containing a tyrosine to phenylalanine substitution at position 442, making it incapable of being phosphorylated in response to serum, epidermal growth factor (EGF), or Src, also causes a significant inhibition of the migration and invasive activity of cells expressing oncogenic Src. We further demonstrate that the phosphorylation of Cool-1 at Tyr-442 weakens its ability to bind to one of its primary interaction-partners, Cat-1 (Cool-associated tyrosine phosphosubstrate-1)/Git-1 (G protein-coupled receptor kinase-interactor-1), thus making Cat more accessible for binding to paxillin. This enables cells to alternate between states where they contain large numbers of focal complexes (i.e. conditions favoring Cool-1-Cat interactions) versus reduced numbers of focal complexes (conditions favoring Cat-paxillin interactions). Overall, these findings show that the phosphorylation-dephosphorylation cycle of Cool-1 at Tyr-442 can serve as a key regulatory signal for focal complex assembly-disassembly, and consequently, for the migration and invasive activity of Src-transformed cells.

Cell migration is essential for a variety of biological events including embryonic development, cancer cell metastasis, inflammation, wound healing, and the formation of new capillaries during angiogenesis (1–4). This multistep cellular process involves the extension of a protrusion, formation of stable attachments at the leading edge of the protrusion, translocation of the cell body forward, and the release of adhesions and retraction at the rear of the cell (4–7). Adhesion sites known as focal contacts occur at the edges of cells where they mediate the attachment of cells to the extracellular matrix, thereby stabilizing the lamellipodium and contributing to efficient directional cell migration. Rapidly migrating cells, such as leukocytes, generally have few visible focal contacts and thus very small submicroscopic adhesions are likely to be important for their fast migratory capability (3). On the other hand, cells with larger focal adhesions (focal complexes) tend to be more adherent and are typically either nonmigratory or move very slowly (3–4). The mechanisms that regulate the steps responsible for the assembly and disassembly of these adhesion sites in migrating cells are poorly understood.

Rho-family GTPases are molecular switches that control a wide variety of signal transduction pathways in all eukaryotic cells (8–21). They are central regulators of processes required for cell migration including the establishment of polarity, the dynamic events that drive actin and microtubule polymerization, and the turnover of cell adhesions. These actions of the Rho GTPases are tightly controlled by guanine nucleotide exchange factors (GEFs) that stimulate their activation by catalyzing GDP-GTP exchange. The Cool/Pix proteins are members of the Dbl-family of Rho-GEFs (22, 23). They exhibit a variety of functional activities and have been implicated in biological responses ranging from chemoattractant and growth factor-coupled signaling activities to neuronal function and the development of X-linked mental retardation (24–29). We have shown that the Cool/Pix proteins serve in a dual capacity as upstream GEFs for Cdc42 and/or Rac, and as effector proteins for activated Cdc42 (30–33), thus providing them with unique capabilities for regulating cellular activities. This is particularly the case when Cool-1/β-Pix (from here on referred to as Cool-1) is phosphorylated on tyrosine 442 in response to growth factors and/or in a Src/FAK-dependent manner (32). Phosphorylation activates the Cdc42-GEF activity of Cool-1 and promotes the formation of a Cdc42-Cool-1-Cbl complex, which influences the timing for EGF receptor (EGFR) degradation and has important implications for cell growth control, as the phosphorylation of Cool-1 by viral Src (v-Src) is essential for Src-induced cellular transformation (32).

In addition to its ability to influence cell growth, Cool-1 has been implicated in the regulation of cell migration (34–

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1 The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

2 The abbreviations used are: GEF, guanine nucleotide exchange factor; PAK, p21-activated kinase; Cool, cloned out of library; Pix, PAK interactive exchange factor; Cat, Cool-associated tyrosine phosphosubstrate; Git, G protein-coupled receptor kinase-interactor; v-Src, viral sarcoma; Arf, ADP-ribosylation factor; DH, Dbl homology; PH, Pleckstrin homology; HA, hemagglutinin; WT, wild-type; RNAi, RNA interference; siRNA, small interference RNA; EGF, epidermal growth factor.

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Thus far, the molecular mechanism by which Cool-1 plays such a role is poorly understood. However, the members of a family of binding partners for Cool-1, collectively referred to here as Cat (also known as Git, p95APP1, and PKL for paxillin-kinase-linker (38–41)), have been implicated in cell migration and cell adhesion turnover. Cat functions as a GAP for the ADP-ribosylation factor (ARF)-family of proteins, as well as binds to the focal complex protein paxillin (4, 42–46).

In the present study, we have examined the role of Cool-1 in the migration and invasive activity of Src-transformed cells. Various lines of evidence have implicated Src in the development of human cancers, where it not only plays an important role in stimulating cell proliferation, but also in promoting cell migration and invasion (47). Given our earlier findings that showed Cool-1 was required for the transforming actions of Src, it seemed attractive to consider that Cool-1 has an important function in the ability of Src to promote cell motility and invasive activity. Indeed, we show here that Cool-1 contributes to the rapid migration of fibroblasts transformed by v-Src and that this is dependent on the GEF activity of Cool-1 and its ability to activate Cdc42. However, we also show that the phosphorylation of Cool-1 at Tyr-442 has a key role in the migration and invasive activity of these transformed cells. Interestingly, we further demonstrate that the phosphorylation of Cool-1 compromises its ability to bind to Cat, which in turn enables Cat to interact with paxillin, leading to a reduction in the size and numbers of focal complexes. On the other hand, conditions that do not promote the phosphorylation of Cool-1 enhance its ability to bind Cat and result in the formation of larger and increased numbers of focal complexes. Overall, these findings suggest a regulatory mechanism in which the phosphorylation-de-phosphorylation cycle of Cool-1 influences the dynamics of focal complex assembly and disassembly and in doing so, stimulates the migration and invasiveness of Src-transformed cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents, EGF, Lipofectamine, Lipofectamine 2000, Cool-1 siRNAs, and the control siRNA, as well as anti-HA and anti-Myc antibodies, were purchased from Invitrogen. Vinculin and actin antibodies were from Neomarkers. The anti-Cool-1 antibody was obtained from Chemicon and the anti-Git-1(Cat-1) antibody was from Santa Cruz Biotechnology. Anti-PY (4G10) and anti-paxillin antibodies were from Millipore.

**Cell Culture**—NIH 3T3 and v-Src-transformed NIH 3T3 cell lines were grown in DMEM medium containing 10% calf serum (CS). The pcDNA3 constructs encoding the various forms of Cool-1 or Cat-1 were transfected into cells using Lipofectamine, whereas the control and Cool-1 siRNAs were introduced into cells using Lipofectamine 2000. The cells were then either fixed with 3.7% formaldehyde or lysed with cell lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 1 mM NaVO₄, and 1 mM β-glycerol phosphate). Protein concentrations were determined using the Bio-Rad DC protein assay.
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Western Blot Analysis—Equal concentrations of each cell lysate were resolved by SDS-PAGE, and then the proteins were transferred to polyvinylidene difluoride. The filters were incubated with the various primary antibodies diluted in TBST (20 mM Tris, 135 mM NaCl, and 0.02% Tween 20). Primary antibodies were then detected with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) followed by exposure to ECL reagent.

Co-immunoprecipitation Assays—Cell lysates were incubated with primary antibody for 1.5 h on ice followed by mixing with protein G-Sepharose beads (Invitrogen) for 1 h. The beads were washed (3×) with lysis buffer and then resuspended in 5× SDS-PAGE sample buffer. Proteins were eluted by boiling for 5 min, followed by Western blot analysis.

Immunofluorescence—Cells fixed with 3.7% formaldehyde were permeabilized with phosphate-buffered saline containing 0.1% Triton X-100, and then blocked in phosphate-buffered saline containing 7% bovine serum albumin. Following blocking, the cells were incubated with the indicated primary antibodies for 2 h, rinsed with phosphate-buffered saline, and then incubated with either Oregon green- or rhodamine-conjugated secondary antibody (Molecular Probes) for an additional hour. Where indicated, 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. The cells were then washed, mounted, and visualized using either the 40× or 63× objectives on a Zeiss Axioskop fluorescent microscope. Images were captured and processed using IPLAB.

Cell Migration and Invasion Assays—Assays for cell migration and invasion were performed as previously described (48, 49). For migration assays, v-Src-transformed NIH 3T3 cells that had been transfected with control or Cool-1 siRNAs, Cool-1 constructs, or Cat-1 constructs, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.2% calf serum (CS) and seeded at 10,000 cells/well in the upper chamber of a Millicell Culture Plate Insert (Millipore). DMEM medium containing 0.2% CS and 0.1 μg/ml EGF was added to the lower chamber, and the cultures were maintained for 3 h. The cells that accumulated on the lower side of the filter were fixed with methanol, stained with Giemsa stain, and then scored. For invasion assays, the Millicell Culture Plate Insert was pre-coated with Matrigel (BD Biosciences), and the cultures were maintained for 12 h. Both the migration and invasion assays were performed three times, and the results from these experiments were averaged.

Preparation of Membrane Fractions—Cells were subjected to Dounce homogenization in 1 ml of buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 200 mM sucrose, and 1 mM phenylmethylsulfonyl fluoride). The nuclei and cell debris were removed from the homogenate by centrifugation at 900 × g for 10 min at 4 °C. The resulting supernatant was centrifuged at 110,000 × g for 75 min at 4 °C. The membrane pellet was solubilized in buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) for 1 h at 4 °C. Insoluble material was removed by centrifugation at 14,000 × g for 10 min at 4 °C, and 1 μg/ml aprotinin was added to the solubilized membrane samples prior to storage at −80 °C.

RESULTS

Cool-1 Influences the Migration and Invasive Activity of Src-transformed Cells—There have been a number of reports suggesting that the Cool/Pix proteins localize to focal complexes and are important for cell migration (34–37). Because Cool-1 plays a key role in the transformation of NIH 3T3 cells by v-Src (32), we were interested in seeing whether Cool-1 is important for the ability of these transformed cells to migrate and exhibit invasive activity. Fig. 1 shows that knocking-down Cool-1 expression using two different siRNAs caused a 50–60% reduction in the rate of migration of v-Src-transformed cells.

The Cool-1 protein contains tandem Dbl homology (DH) and Pleckstrin homology (PH) domains that are characteristics of GEFs for Cdc42, Rac, and other Rho-family GTPases (22, 23). Previously, we had shown that Cool-1 acts as a Cdc42-specific GEF following its growth factor- and Src/FAK-dependent
phosphorylation at Tyr-442 (32). By changing two conserved leucine residues (Leu-383 and Leu-384) within the DH domain to arginine and serine, respectively, we generated a Cool-1 double-mutant (designated as Cool-1 DHm) that is defective for GEF activity (30, 31, 50). Fig. 2 shows that when we overexpressed Cool-1 DHm in v-Src-transformed cells, their ability to migrate was inhibited, compared with cells that were transfected with control vector. Similarly, the rate of migration of v-Src-transformed cells was reduced upon the overexpression of the dominant-negative Cdc42 T17N mutant that is incapable of undergoing guanine nucleotide exchange (designated as Cdc42 N17 in Fig. 2). Together, these results indicated that Cool-1 is indeed important for Src-transformed cells to achieve their maximal rates of migration (also, see below) and that its ability to activate Cdc42 contributes to this function.

The Phosphorylation of Cool-1 Influences the Migration and Invasive Activity of Src-transformed Cells—Cool-1 is phosphorylated at Tyr-442 when NIH 3T3 cells are treated with serum (see below) or with EGF (32). The EGF-stimulated phosphorylation of Cool-1 in NIH 3T3 cells is transient such that it is maximal within 10 min (32) and fully reversed by 45 min (Fig. 3A). However, in v-Src-transformed cells, the phosphorylation of either endogenous Cool-1 (Fig. 3B) or ectopically expressed Myc-tagged Cool-1 (Fig. 3C) is observed in the absence of added EGF, as well as through 90 min of growth factor treatment, and plays an important role in the transforming activity of v-Src (32). Interestingly, we found that the overexpression of the phosphorylation-defective Cool-1 Y442F mutant resulted in a significant reduction in the migration of v-Src-transformed cells, compared with the lack of an effect upon the overexpression of wild-type Cool-1 (Fig. 4A). On the other hand, the overexpression of the Cool-1 Y442F mutant in control NIH 3T3 cells had little effect on their rate of migration (supplemental Fig. S1), which is slower compared with Src-transformed cells. This suggests that the phosphorylation of Cool-1 is important for the enhanced rate of migration exhibited by transformed cells. The overexpression of Cool-1 Y442F in v-Src-transformed cells also markedly inhibited their invasive activity, whereas again the overexpression of wild-type Cool-1 lacked a significant effect (Fig. 4B).

Because the phosphorylation of Cool-1 at Tyr-442 is necessary to activate its Cdc42-GEF activity (32), the inhibitory effects of the Cool-1 Y442F mutant are consistent with the idea that the activation of Cdc42 contributes to the role played by Cool-1 in the migration and invasive activity of Src-transformed cells. They also suggest that the Cool-1 Y442F mutant is capable of acting as a dominant-negative inhibitor, most likely through its ability to bind and sequester a Cool-1 binding partner that has an important role in v-Src-stimulated cell migration and invasion. A possible clue as to how Cool-1 Y442F might be exerting its inhibitory effects came from the general appearance of v-Src-cells overexpressing the Cool-1 Y442F mutant, as they were flat and more adherent compared with v-Src cells expressing the vector control (supplemental Fig. S2). This suggested that the overexpression of the phosphorylation-defective Cool-1 mutant might be stabilizing focal complexes.

A further examination indicated that this was the case. Treatment of NIH 3T3 cells with EGF led to the loss of focal complexes that was evident within 30 min (Fig. 5A). Similarly, EGF caused cells overexpressing wild-type Cool-1 to round-up and
to lose their focal complexes, whereas NIH 3T3 cells expressing the phosphorylation-defective Cool-1 Y442F mutant were more attached and retained their focal complexes (Fig. 5, B–D).

Cool-1 Influences Cell Migration through Its Interaction with Cat—Given that the Cat/Git proteins, which are primary binding partners of Cool-1, localize to focal complexes and play important roles in cell-spreading and attachment (42–46), we set out to examine whether the phosphorylation of Cool-1 influences its interactions with Cat. We first performed these experiments in NIH 3T3 cells where we could control Cool-1 phosphorylation by comparing serum-starved versus serum- or growth factor-treated cells. Thus, NIH 3T3 cells expressing either Myc-tagged wild-type Cool-1 or the Cool-1 Y442F mutant were first serum-starved and then treated with EGF for 20 min (i.e. to achieve maximum phosphorylation of wild-type Cool-1), at which time the Myc-tagged Cool-1 proteins were immunoprecipitated using an anti-Myc antibody. Co-immunoprecipitated endogenous Cat-1 protein was detected by Western blotting using an anti-Git-1 (Cat-1) antibody. Under conditions where equivalent amounts of wild-type Cool-1 and the Cool-1 Y442F mutant were immunoprecipitated from EGF-treated cells, a greater amount of endogenous Cat-1 was co-immunoprecipitated with Cool-1 Y442F compared with wild-type Cool-1 (Fig. 6A).

When a similar experiment was performed in v-Src-transformed NIH 3T3 cells (i.e. conditions that give rise to the constitutive phosphorylation of Cool-1 at Tyr-442), we again found that a greater amount of endogenous Cat-1 was co-immunoprecipitated with Cool-1 Y442F compared with its wild-type counterpart (Fig. 6A). When a similar experiment was performed in v-Src-transformed NIH 3T3 cells (i.e. conditions that give rise to the constitutive phosphorylation of Cool-1 at Tyr-442), we again found that a greater amount of endogenous Cat-1 was co-immunoprecipitated with Cool-1 Y442F compared with wild-type Cool-1 (Fig. 6B).

Together these findings suggested that the phosphorylation of Cool-1 at Tyr-442 weakens its ability to bind Cat. We then wanted to know whether Cool-1-Cat interactions were necessary for Src-transformed cells to exhibit maximal rates of migration.
far, we have not been able to successfully express sufficient amounts of Cool-1 mutants that are defective for binding to Cat. However, a specific region had been previously delineated in Cat-1/Git-1 to be necessary for the high affinity binding of Cool-1/β-Pix (51), and we have been able to overexpress a Cat-1 double mutant that contains two substitutions within this region (Cat-1(L294A,C295A)) and exhibits a significantly reduced ability to bind to Cool-1 (i.e. referred to as Cat-1 CBD for Cool-1 binding-defective in Fig. 7A). When Cat-1 CBD was expressed in v-Src cells, it significantly reduced their migration, as compared with v-Src cells transfected with control vector (Fig. 7B), as well as inhibited their invasive activity (Fig. 7C).

The Phosphorylation of Cool-1 Influences the Membrane Association of the Focal Complex Protein Paxillin and Its Interactions with Cat—Because the expression of the phosphorylation-defective Cool-1 Y442F mutant stabilized focal complexes, we were interested in seeing whether conditions leading to the phosphorylation of Cool-1 negatively affected the membrane association of focal complex proteins. In fact, we found that the membrane association of the Cat binding partner paxillin was strongly influenced by conditions that promote the phosphorylation of Cool-1. Specifically, under conditions where Cool-1 was phosphorylated in NIH 3T3 cells in a serum-dependent manner (Fig. 8A, top panel), little or no paxillin appeared to be present in the membrane fractions prepared from these cells (Fig. 8A, bottom-left panel). Paxillin was clearly detected in the membrane fractions from serum-starved (Fig. 8A, bottom-left panel), while fibronectin (a membrane marker) was detected in the membrane fractions from both serum-starved and serum-treated cells, whereas IKBα (a cytosolic marker) was only detected in the cytosol (Fig. 8A, bottom-right panel). Similar results were obtained when comparing serum-starved versus EGF-treated cells (supplemental Fig. S4).

We then examined whether the expression of the phosphorylation-defective Cool-1 Y442F mutant in NIH 3T3 cells increased the levels of paxillin detected in the membrane fractions. Serum-treated NIH 3T3 cells expressing the Myc-tagged Cool-1 Y442F mutant showed greater amounts of paxillin in the membrane fractions compared with vector control cells (Fig. 8B, compare lanes 1 and 2 in the top panel). However, the co-expression of the Myc-Cool-1 Y442F mutant...
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Further support for these conclusions came from two additional sets of experiments. Fig. 9A shows that under conditions where paxillin was immunoprecipitated from serum-starved NIH 3T3 cells (lane 3), i.e. conditions that do not lead to Cool-1 phosphorylation, only Cat-1 and not Cool-1 was co-immunoprecipitated with paxillin. The ability of Cat-1 to be co-immunoprecipitated with paxillin was increased in v-Src-transformed cells, i.e. under conditions where Cool-1 is constitutively phosphorylated, while Cool-1 was again missing from the Cat-paxillin complex (Fig. 9A, lane 4). When Cat-1 was immunoprecipitated from serum-starved NIH 3T3 cells, only Cool-1 and not paxillin was co-immunoprecipitated with Cat-1 (Fig. 9A, lane 2). These findings are consistent with the idea that Cat binds to paxillin most effectively under conditions that promote Cool-1 phosphorylation, whereas Cat binds preferentially to Cool-1 under conditions where Cool-1 is not phosphorylated. Moreover, when Cat-1 CBD was expressed in NIH 3T3 cells, i.e. conditions that caused the loss of paxillin from the membrane (Fig. 8B, lane 3), those cells expressing the Cat-1 double mutant lacked detectable focal complexes (as indicated when staining for paxillin or vinculin; see the arrows labeled (a) and (b) in Fig. 9B and supplemental Fig. S5, respectively). Cells that lacked Cat-1 CBD but contained endogenous Cat-1 (indicated by the lighter red staining in the white box in the bottom-left panel of Fig. 9B) showed focal complex staining (see the white box in the top-left panel of Fig. 9B and the arrows labeled (b) in Fig. 9B and supplemental Fig. S5). These results provide yet a further indication that under conditions where Cat binds weakly to Cool-1, it preferentially associates with paxillin and causes its removal from the membrane and a loss of focal complexes.

DISCUSSION

The Cool/Pix proteins have been shown to serve as GEFs for Cdc42 and Rac, as well as downstream target effectors for GTP-bound Cdc42 (28–32). In the case of Cool-1/β-Pix, this dual-function capability, coupled with its ability to be phosphorylated at Tyr-442 in response to EGF and Src/FAK, has important ramifications for cell growth control, and in particular, for Src-induced cellular transformation (32). Interestingly, Cool-1 and one of its primary binding partners, Cat/Git, have also been implicated in focal complex formation and cell adhesion (34–

together with V5-tagged Cat-1 CBD in these cells yielded results similar to the vector control cells (Fig. 8B, lane 1 versus lane 3), or cells expressing wild-type Cool-1 (lane 4), as there was no increase in the membrane association of paxillin. Collectively, these results suggest that the phosphorylation-defective Cool-1 Y442F mutant, which exhibits enhanced binding to Cat, increases the membrane association of paxillin, while Cat-1 CBD circumvents the effects of the phosphorylation-defective Cool-1 mutant and hinders the membrane association of paxillin. Thus, conditions favoring Cool-1-Cat interactions (i.e. when Cool-1 is not phosphorylated) appear to be optimal for the membrane association of paxillin and for stabilizing focal complex assembly, whereas the amount of paxillin detected within the membrane fraction is reduced and accompanied by focal complex disassembly under conditions that weaken the binding of Cool-1 to Cat (i.e. when Cool-1 is phosphorylated), or upon the expression of the Cool binding-defective mutant (Cat-1 CBD).
FIGURE 7. **The Cool-1-Cat interaction is important for the migration and invasive activity of v-Src-transformed NIH 3T3 cells.**

**A.** NIH 3T3 cells were transfected with V5-tagged wild-type Cat-1 or with the V5-tagged, Cool-1 binding-defective Cat-1 double-mutant (Cat-1 CBD). Top panels, Cat-1 was immunoprecipitated with the anti-V5 antibody, and the immunocomplexes were probed with an anti-Cool-1 antibody or with an anti-V5 antibody. Bottom panels, shown are the relative amounts of endogenous Cool-1 and V5-Cat-1 CBD in the whole cell lysates.

**B.** Viral-Src-transformed NIH 3T3 cells were transfected with an empty vector (control) or with V5-tagged Cat-1 CBD. The cells were cultured for 24 h and then scored for their ability to migrate as described in the legend to Fig. 1. Left panel, the results from three experiments were averaged and plotted. The error bars indicate ± S.D. Middle panel, cell lysates were subjected to Western blot analysis to assess the expression of V5-Cat-1 CBD. Right panels, microscopic images were obtained for the migration assays shown on the left. The cells are visualized by GIErMA staining (dark purple). The clear circles represent holes in the filters.

**C.** Viral-Src-transformed NIH 3T3 cells were transfected with empty vector or with V5-tagged Cat-1 CBD. These cells were cultured for 24 h and then scored for invasiveness as described in the legend to Fig. 4B. Top panel, results from three experiments were averaged and plotted. The error bars indicate ± S.D. Bottom panels, microscopic images of the invasion assays are shown. The cells are visualized by GIErMA staining (dark purple).
Given that these processes are fundamental aspects of cell migration and invasiveness, which in turn have important consequences for cancer metastasis, we were interested in seeing whether Cool-1 plays an essential role in the migration and invasive activity of Src-transformed cells. Indeed, we show here that Cool-1 is necessary for v-Src-transformed NIH 3T3 cells to undergo their accelerated rates of cell migration, compared with normal fibroblasts, as well as for their invasive activity.

A potentially important insight regarding how Cool-1 fulfills these functions came from our finding that the expression of the Cool-1 Y442F mutant, which is unable to be phosphorylated in v-Src-expressing cells, inhibited their migration and blocked their invasive activity. We also found that the Cool-1 Y442F mutant stabilized focal complexes, suggesting that its inhibitory actions toward cell migration reflected its ability to block the cycle of focal complex assembly and disassembly. The ability of the Cool-1 Y442F mutant to act as a dominant-nega-

FIGURE 8. Cool-1-Cat interactions lead to the release of paxillin from cellular membranes. A, top panels, NIH 3T3 cells were serum-starved and then were left untreated or stimulated with 10% serum for 30 min. Endogenous Cool-1 was immunoprecipitated and the immunocomplexes were probed with an anti-PY antibody or with an anti-Cool-1 antibody. Bottom-left panel, membrane fractions were prepared from the serum-starved or the serum-stimulated cells as described under “Experimental Procedures” and then Western blotted with an anti-paxillin antibody. Bottom-right panels, the membrane-associated protein fibronectin and the cytosolic protein IKBα were used as controls to assess the quality of the membrane and cytosolic fractions. B, NIH 3T3 cells were transfected with empty vector (control), the Myc-tagged Cool-1 Y442F mutant, the Myc-tagged Cool-1 Y442F and the V5-Cat-1 CBD mutant, or with Myc-tagged wild-type Cool-1. The membrane fractions were prepared and Western blotted with anti-paxillin (top panel), anti-Git-1 (Cat-1) (middle panel), and anti-Myc (bottom panel) antibodies.

FIGURE 9. Conditions leading to the phosphorylation of Cool-1 influences whether Cat associates with Cool-1 or paxillin. A, NIH 3T3 cells were transfected with empty vector (control) (lanes 1–3), or with HA-tagged v-Src (lane 4). Immunoprecipitations were performed with either an anti-Git-1 (Cat-1) or an anti-paxillin antibody as indicated. The co-immunoprecipitated Cool-1, Cat, or paxillin were detected by Western blotting using an anti-Cool-1, an anti-Git-1 (Cat-1), or an anti-paxillin antibody. The bottom panel shows a Western blot for HA-tagged Src from whole cell lysates (WCL) of v-Src-expressing cells. B, NIH 3T3 cells were transfected with V5-Cat-1 CBD. Immunofluorescence was performed on the samples using anti-paxillin antibody to visualize focal complexes (top left) and anti-Git (Cat-1) antibody (bottom left). The lack of focal complexes in cells expressing Cat CBD-1 is indicated by (b). The white box in the top panel shows the presence of focal complexes in a cell lacking Cat CBD-1. The white box in the bottom panel shows the presence of endogenous Cat-1 in the same cell. Right panels represent higher magnification of the boxed areas. Focal complexes are indicated by (b).
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It also provides an intriguing way to directly couple cell migration to cell growth regulation, because a similar phosphorylation-de-phosphorylation cycle of Cool-1 appears to be regulating the timing for EGF receptor-Cbl interactions and EGF receptor degradation (32, 33). In v-Src-transformed cells, the constitutive tyrosine phosphorylation of Cool-1 apparently promotes the rapid turnover of focal complexes, resulting in accelerated migration and increased invasiveness.

The model described in Fig. 10 is consistent with the various findings implicating Cool-1 and Cat in cell migration. It was recently reported that endogenous paxillin was unable to associate with Cool-1-Cat complexes (43), thus suggesting the idea that Cat binds either to Cool-1 or to paxillin but not to both proteins simultaneously. Interestingly, this same group also suggested that knocking-down Cool-1/β-Pix did not increase the formation of Cat-paxillin complexes. According to our model, we might have predicted that decreasing Cool-1 expression would favor the binding of Cat to paxillin in cells. However, it may be that the phosphorylation of Cool-1, which favors its dissociation from Cat, or the knock-down of Cool-1, is not sufficient to promote the interactions between endogenous Cat and paxillin. The phosphorylation of Cat, which also occurs on tyrosine residues and can be stimulated by integrins and by Src/FAK activation (39), and/or the phosphorylation of paxillin (37), might also be required to optimize the binding of Cat to paxillin. Still, we have found that when the Cool-1 binding-defective Cat-1 CBD is expressed in cells, it is able to bind to paxillin and interfere with the cycling between focal complex assembly and disassembly, thus inhibiting cell migration, again consistent with the model presented in Fig. 10.

As alluded to above, we recognize that cell migration is a complex process that involves the coordination of many types of protein-protein interactions, and there remain a number of questions regarding how these various events fit into the regulatory scheme presented here. For example, where does the GEF activity of Cool-1 fit into the picture? We would assume that the phosphorylation of Cool-1 at Tyr-442, by activating its Cdc42-GEF activity, helps to initiate Cdc42-signaling events that in some way influence the cycle of focal complex assembly and disassembly and/or cell migration. What other proteins participate together with paxillin in the formation of focal complexes, and how does the interaction of Cat with paxillin affect its interactions with these proteins? Where does the Arf-GAP activity of Cat come into play and how is the regulatory role of Cool-1 in EGF receptor signaling coupled to its ability to regulate the migration of Src-transformed cells? Whereas a good deal remains to be determined regarding the complex regulatory schemes underlying cell migration, the implication of Cool-1 and its phosphorylation-de-phosphorylation cycle in this process provides us with an important step toward obtaining a fuller understanding of this important biological activity.
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