Cdc42 and Ras Cooperate to Mediate Cellular Transformation by Intersectin-L*

Received for publication, December 21, 2004, and in revised form, March 3, 2005 Published, JBC Papers in Press, April 11, 2005, DOI 10.1074/jbc.M414375200

Jian-Bin Wang‡, Wen Jin Wu‡, and Richard A. Cerione‡§¶ From the Departments of §Molecular Medicine, Veterinary Medical Center and ¶Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca, New York 14853

Cdc42, a Ras-related GTP-binding protein, has been implicated in the regulation of the actin cytoskeleton, membrane trafficking, cell-cycle progression, and malignant transformation. We have shown previously that a Cdc42 mutant (Cdc42(F28L)), capable of spontaneously exchanging GDP for GTP (referred to as “fast-cycling”), transformed NIH 3T3 cells because of its ability to interfere with epidermal growth factor receptor (EGFR)-Cbl interactions and EGFR down-regulation. To further examine the link between the hyperactivation of Cdc42 and its ability to alter EGFR signaling and thereby cause cellular transformation, we examined the effects of expressing different forms of the Cdc42-specific guanine nucleotide exchange factor, intersectin-L, in fibroblasts. Full-length intersectin-L exhibited little ability to stimulate nucleotide exchange on Cdc42, whereas a truncated version that contained five Src homology 3 (SH3) domains, the Db and pleckstrin homology domains (DH and PH domains, respectively), and a C2 domain (designated as SH3A-C2) showed modest guanine nucleotide exchange factor activity, whereas a form containing just the DH, PH, and C2 domains (DH-C2) strongly activated Cdc42. However, DH-C2 showed little ability to stimulate growth in low serum or colony formation in soft agar, whereas SH3A-C2 gave rise to a much stronger stimulation of cell growth in low serum and was highly effective in stimulating colony formation. Moreover, although SH3A-C2 strongly transformed fibroblasts, it differed from the actions of the Cdc42(F28L) mutant, as SH3A-C2 showed little ability to alter EGFR levels or the lifetime of EGFR-Cbl interactions, thereby causing EGFRs to accumulate and exhibit sustained colony formation. However, Cdc42 as well as other Rho-family proteins, in the control of normal cell growth and, when aberrantly regulated, in tumorigenesis and metastasis. Perhaps foremost among the lines of evidence linking these GTP-binding proteins to cell proliferation was the initial discovery that members of the Db family of guanine nucleotide exchange factors (GEFs), either when point-mutated or truncated, were capable of potently transforming mouse fibroblasts (5, 6). Mutations in Cdc42 which mimic the functional activity of Db proteins and cause the constitutive exchange of GDP for GTP (called “fast-cycling” mutants) are transforming (7–9). Both Rac1 and Cdc42 have also been linked to cell invasiveness (10–12), and another Rho family member, RhoC, has been strongly implicated in metastasis (13). Moreover, the activation of Cdc42, as well as Rac1 and RhoA, is essential for Ras-induced malignant transformation (14–16).

We have recently obtained some important insights into the mechanistic basis by which fast-cycling mutants of Cdc42 transform fibroblasts (17). Somewhat surprisingly, we discovered that cells expressing Cdc42(F28L) exhibited significantly higher levels of epidermal growth factor receptors (EGFRs) compared with control fibroblasts and showed a markedly extended lifetime for EGFR-coupled signaling. These observations demonstrated the ability of activated Cdc42 to negatively regulate the interactions of the EGFR with one of its binding partners, the adaptor protein c-Cbl. Various lines of evidence have shown that the degradation of EGFRs occurs via a cascade of ubiquitination enzymes that culminates in the E3 ligase activity catalyzed by c-Cbl (18–21). Activated forms of Cdc42, by associating with the p55Cool-1/β-Pix protein, a Cbl-binding partner, are able to sequester c-Cbl away from the EGFR. This prevents the EGFR from phosphorylating Cbl, which has been suggested to activate its E3 ligase activity (19). Because the fast-cycling Cdc42(F28L) mutant is constitutively active, it is able to persistently inhibit EGFR-Cbl interactions, thereby leading to a significant reduction in receptor degradation. This, in turn, causes EGFRs to accumulate and exhibit sustained signaling, resulting in cellular transformation.

These findings raised the question of whether the negative regulation of EGFR degradation was a general feature of

* This work was supported by National Institutes of Health Grant GM47458. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. Tel.: 607-253-3888; Fax: 607-253-3659; E-mail: rac1@cornell.edu.

1 The abbreviations used are: GEF, guanine nucleotide exchange factor; EGF, epidermal growth factor; EGF, epidermal growth factor receptor; E3, ubiquitin-protein isopeptide ligase; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; SH, Src homology; EH, Eps15 homology; DH, Db homology; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; HEK, human embryonic kidney; GST, glutathione S-transferase; PBD, p21-binding domain; RBD, Rho-binding domain; WASP, Wiscott-Aldrich syndrome protein; N-WASP, neuronal-enriched WASP; GTPγS, guanosine 5′-3-O-(thiotriphosphate) or guanosine 5′-O-(3-thiotriphosphate).
Intersectin Uses Cdc42 and Ras to Transform Cells

Cdc42-mediated cell growth control and cellular transformation. We were especially interested in seeing whether activated DbI family GEFs that were specific for Cdc42 led to the same effects on EGFR lifetime and signaling as the fast-cycling Cdc42/F28L mutant. To address this question, we examined the cellular effects of different forms of intersectin-L, a Cdc42-specific GEF (22). We thought that intersectin-L would be especially interesting to study in the context of possible effects on EGFR degradation because a shorter splice variant form of the protein, designated intersectin-S, has been implicated in endocytosis (23). In addition, the overexpression of intersectin-S has been shown to exhibit weak transforming activity, perhaps in part because of a disruption of the normal endocytosis of growth factor receptors and because of a reported ability to input into the Ras-signaling pathway via an interaction with the Ras-GEF, Sos (Son-of-sevenless) (24). Here, in an attempt to directly correlate the activation of Cdc42 by intersectin-L with cellular transformation, we have compared the abilities of different versions of intersectin-L to act as Cdc42-GEFs. However, unexpectedly, we found that the most potent transforming activity was induced by an intersectin-L construct that only moderately activated Cdc42, whereas a form of intersectin-L that more strongly activated Cdc42 was less effective in transforming fibroblasts. Maximal transformation by intersectin-L required its ability to activate both Cdc42 and Ras. Unlike the case for fast-cycling Cdc42/F28L, transformation by intersectin-L was not the result of significant changes in EGFR levels or signaling to ERK but, rather, required the combined effects of Ras and Cdc42 to signal to the c-Jun NH2-terminal kinase (JNK) and the phosphoinositide 3-kinase (PI3K). Overall, these results provide an interesting example of how Cdc42 can cooperate with Ras to elicit cellular transformation through signaling pathways that are distinct from those used by fast-cycling Cdc42 mutants.

MATERIALS AND METHODS

Reagents—EGF and the inhibitors for PI3K (LY294002), JNK (SP600125), and ERK (PD98059), were all purchased from Calbiochem. Polyclonal antibody to Sos1 and monoclonal antibody to H-Ras were purchased from Santa Cruz Biotechnology. Monoclonal antibodies to HA and Myc were obtained from Covance. Phospho-Akt (Ser-473), phospho-stress-activated protein kinase/JNK (Thr-183/Tyr-185), and phospho-p44/p42 MAPK (Thr-202/Tyr-204) antibodies were purchased from Cell Signaling Technology.

Primary Cell Lines—Mouse intersectin-L was a gift from Dr. Sean E. Egan (The University of Toronto), and the plasmid encoding intersectin-L that more strongly activated Cdc42 was less effective in transforming fibroblasts. Maximal transformation by intersectin-L functions as a specific GEF for Cdc42 (22). As a first attempt to directly correlate the activation of Cdc42 by intersectin-L with cellular transformation, we have compared the abilities of different versions of intersectin-L to act as Cdc42-GEFs. However, unexpectedly, we found that the most potent transforming activity was induced by an intersectin-L construct that only moderately activated Cdc42, whereas a form of intersectin-L that more strongly activated Cdc42 was less effective in transforming fibroblasts. Maximal transformation by intersectin-L required its ability to activate both Cdc42 and Ras. Unlike the case for fast-cycling Cdc42/F28L, transformation by intersectin-L was not the result of significant changes in EGFR levels or signaling to ERK but, rather, required the combined effects of Ras and Cdc42 to signal to the c-Jun NH2-terminal kinase (JNK) and the phosphoinositide 3-kinase (PI3K). Overall, these results provide an interesting example of how Cdc42 can cooperate with Ras to elicit cellular transformation through signaling pathways that are distinct from those used by fast-cycling Cdc42 mutants.

RESULTS

Comparisons of the Abilities of Different Forms of Intersectin-L to Activate Cdc42—It was reported previously that intersectin-L functions as a specific GEF for Cdc42 (22). As a first step toward developing a correlation between the extent of Cdc42 activation and its ability to transform NIH 3T3 fibroblasts, we assayed the Cdc42-GEF activities of different forms of intersectin-L. In particular, the GEF activity of full-length intersectin-L, which contains two EH domains, five SH3 domains, and Oregon Green-conjugated goat anti-mouse antibody was used to detect Myc-tagged SH3A-C2 and Myc-SH3A-C2 (E1237A). [3H]GDP Dissociation Assays—COS-7 cells were transfected with cDNAs encoding Myc-tagged intersectin-L, SH3A-C2, SH3A-C2 (E1237A), and DH-C2. Forty-eight hours after transfection, cells were trypsinized and counted with a hemocytometer. For the soft agar assays, 104 cells stably expressing the different constructs were mixed with DMEM supplemented with 10% calf serum and 0.3% agarose. Cells were plated on top of a solidified layer of DMEM supplemented with 0.5% agarose and 10% calf serum. Cells were fed weekly by adding 1 ml of DMEM supplemented with 10% calf serum and 0.3% agarose. After 2 weeks, colonies larger than 50 μm were scored.

Immunofluorescence Microscopy—NIH 3T3 cells were plated on dual chamber microscope slides (Nalge Nunc) in DMEM supplemented with 10% calf serum for 24 h and then transfected with 1 μg of plasmid per 100 μl of medium. Cells were fixed with 3.7% formaldehyde plus 0.2% Triton X-100 in phosphate-buffered saline. Rhodamine phalloidin was used to detect actin, and Oregon Green-conjugated goat anti-mouse antibody was used to detect Myc-tagged SH3A-C2 and Myc-SH3A-C2 (E1237A). Equivalent amounts of protein (80 μg) of Cdc42 preloaded with [3H]GDP in 140 μl of reaction buffer at room temperature, and aliquots (23 μl) were diluted into 1.5 ml of ice-cold termination buffer (20 mM Tris-HCl, 10 mM MgCl2, and 100 mM NaCl, pH 7.4) at various time points. The percent [3H]GDP remaining—
which is composed of the five SH3 domains and everything downstream, and a still shorter form that simply contains the DH, PH, and C2 domains (DH-C2). These different intersectin-L constructs were expressed in COS-7 cells as Myc-tagged proteins, immunoprecipitated with anti-Myc antibody (Fig. 2A, bottom panel), and then assayed for their abilities to stimulate the exchange of \(^{3}H\)GDP for GTP/\(^{3}H\)So in Escherichia coli recombinant Cdc42, as monitored by the dissociation of \(^{3}H\)GDP from the GTP-binding protein. The results presented in Fig. 2A (top panel) show that the DH-C2 construct exhibited the strongest GEF activity, whereas SH3A-C2 showed moderate activity, and full-length intersectin-L (designated Myc-Intersectin in Fig. 2A, top panel, and Myc-ITSN in Fig. 2A, bottom panel) was completely inactive. These results are consistent with the idea that full-length intersectin-L is susceptible to an autoinhibition, perhaps because the SH3 domains fold over and block access to the DH domain (25).

We next examined whether the different forms of intersectin-L showed the same relative abilities to activate Cdc42 in cells. We performed this examination using an assay that takes advantage of the binding of activated Cdc42 to the Cdc42/Rac target, PAK3, fused to GST (GST-PBD). The different forms of Myc-tagged intersectin-L were expressed together with Myc-tagged (wild-type) Cdc42. Cell lysates were incubated with GST-PBD and precipitated with agarose beads as described under “Materials and Methods.” Western blotting with anti-Myc antibody was performed to detect GST-bound Cdc42 (top panel) that co-precipitated with GST-PBD (second panel). Total cell lysates were blotted with anti-Myc antibody to compare the relative expression of Cdc42 (third panel) and the different intersectin-L constructs (bottom panel). WCL, whole cell lysate. ITSN, full-length intersectin-L; IP, immunoprecipitate. C, actin cytoskeletal staining in NIH 3T3 cells transfected with the indicated constructs. Actin was detected using rhodamine phalloidin (red), whereas the indicated intersectin-L constructs were detected using anti-Myc antibody (green).
cell lysates. Similar to the results obtained from the in vitro nucleotide exchange assays (Fig. 2), the DH-C2 construct was the most effective at stimulating Cdc42 activation in cells. In fact, the extent of Cdc42 activation promoted by DH-C2 yielded a signal in the PBD assay that was comparable with the positive control obtained from cells expressing constitutively active, GTPase-defective Myc-Cdc42(Q61L). The SH3A-C2 construct gave rise to a weaker cellular activation of Cdc42, whereas full-length intersectin-L was again ineffective at activating Cdc42.

When we compared the abilities of the different forms of intersectin-L to stimulate the formation of microspikes in NIH 3T3 cells, we were not able to distinguish between SH3A-C2 and DH-C2, as both constructs induced this phenotype in the majority of cells examined. Fig. 2C (upper left panel) shows a representative example for cells expressing SH3A-C2. Expression of full-length intersectin-L did not induce these cellular changes (not shown). This suggests that although Cdc42 is less effectively activated by SH3A-C2 compared with DH-C2, the SH3A-C2 construct is able to stimulate sufficient activation of the endogenous Cdc42, such that complete microspike formation is observed.

For the case of the founding member of the Dbl family (i.e. oncogenic Dbl), it has been shown that changing glutamic acid 502 to an alanine yields a Dbl protein that is still capable of binding to Cdc42 but is unable to stimulate nucleotide exchange (26). Sequence alignments indicate that glutamic acid 1237 within the DH domain of intersectin-L corresponds to glutamic acid 502 in oncogenic Dbl. When we changed glutamic acid 1237 in the intersectin-L proteins to alanine, we completely eliminated their ability to exhibit Cdc42-GEF activity. This is demonstrated for SH3A-C2 both in the in vitro nucleotide exchange assay (Fig. 2A) and when monitoring microspike formation (Fig. 2C, upper right panel). As expected (and as will be discussed further below), mutating conserved tryptophan residues to serines within the SH3 domains (e.g. SH3A and SH3C, designated SH3A-C2(WS)) had no effect on Cdc42-GEF activity (see Fig. 2A).

Comparisons of the Abilities of Different Forms of Intersectin-L to Induce Cellular Transformation—Given the distinct capabilities exhibited by the different forms of intersectin-L to promote the activation of Cdc42, we were interested in whether they showed the same relative abilities to induce the transformation of fibroblasts. Cell lines stably expressing each of the intersectin-L constructs were generated and analyzed for their ability to grow under different conditions (Western blots of the different constructs are shown in Fig. 3A, lower panel; H and L refer to cell lines that exhibit higher and lower levels, respectively, of the stable expression of the particular protein of interest). The upper panel in Fig. 3A shows the results of cell growth assays per-
formed in low serum. As reported previously, NIH 3T3 cells expressing the constitutively active, fast-cycling Cdc42(F28L) mutant were extremely effective at growing in low serum (7), whereas control fibroblasts were unable to grow under these conditions. Fig. 3B demonstrates the striking difference in morphology between cells expressing Cdc42(F28L) versus the vector control in low serum conditions. However, surprisingly, we found that cells expressing SH3A-C2 were significantly more effective in their ability to grow in low serum and exhibited a more pronounced transformed appearance compared with cells expressing DH-C2, despite the fact that the latter construct was much better at stimulating Cdc42 activation.

Similar results were obtained when examining the relative ability of the intersectin-L constructs to enable fibroblasts to increase their saturation density and to form colonies in soft agar (Fig. 3, C–E). In each case, SH3A-C2 elicited a stronger transforming phenotype than DH-C2, with the extent of transformation being dependent on the relative levels of expression of SH3A-C2. However, interestingly, SH3A-C2 was even more effective than the Cdc42(F28L) mutant in stimulating colony formation in soft agar (Fig. 3D). The latter assay has been regarded typically as the most reliable indicator of malignant transformation. Given that SH3A-C2 was only moderately effective at stimulating Cdc42 activation in cells (i.e., compared with DH-C2), this then raised the question of how it was able to potently transform fibroblasts.

What Are the Downstream Signals Necessary for SH3A-C2-induced Cellular Transformation?—We had shown previously that an essential aspect of Cdc42(F28L) -induced cellular transformation is the sustained signaling by EGF-Rs because of activated Cdc42 interfering with EGF-R-Cbl interactions (17). However, whereas the stable expression of Cdc42(F28L) in NIH 3T3 cells resulted in EGF-stimulated ERK activation that was still detected after 2 h (Fig. 4A, top panel), cells expressing SH3A-C2 showed the transient profile for EGF-stimulated ERK activation typical of control cells, such that the stimulation of ERK activity was no longer detectable within 15–30 min of treatment with the growth factor (Fig. 4A, middle panel). Moreover, although Cdc42(F28L)-induced cellular transformation, as assayed by the ability of fibroblasts to grow in low serum, was completely blocked by inhibiting ERK activation with the MEK inhibitor PD98059, SH3A-C2-stimulated growth in low serum was unaffected by treatment with PD98059 (Fig. 4B). These results clearly demonstrated that transformation by SH3A-C2 proceeded through a mechanism distinct from that responsible for cellular transformation by the fast-cycling Cdc42 mutant.

We then examined the potential involvement of other downstream signaling activities. As shown in Fig. 5A (top panel), both Cdc42(F28L) and SH3A-C2 augmented EGF-stimulated PI3K activity, as assayed by the phosphorylation of Akt. Fig. 5B shows that Cdc42(F28L)-induced transformation was completely blocked when cells were treated with the JNK inhibitor, LY294002. Likewise, the ability of SH3A-C2 to promote cell growth under these conditions required PI3K activation.

Both Cdc42(F28L) and SH3A-C2 also enhanced the EGF-stimulation of JNK 1 and 2 (Fig. 5A, middle panel). However, it was interesting that although Cdc42(F28L)-induced transformation was not dependent on JNK activation, SH3A-C2-mediated transformation was completely blocked when cells were treated with the JNK inhibitor, SP600125 (Fig. 5C). Thus, these results further indicate that distinct signaling pathways contribute to the ability of constitutively active Cdc42, versus the intersectin-L construct SH3A-C2, to transform cells.

SH3A-C2 Promotes the Activation of Ras—It has been reported that the short form of intersectin (intersectin-S), which lacks the DH and PH domains and is incapable of acting as a Cdc42-GEF, is able to bind through one of its SH3 domains (SH3A) to the Ras-GEF, Sos (24, 26). Thus, we were interested in determining whether intersectin-L was capable of forming a complex with Sos and promoting the activation of Ras. Indeed, the results presented in Fig. 6A (top panel) demonstrate that endogenous Sos1 can be co-immunoprecipitated with Myc-tagged SH3A-C2 from cells. We then examined the ability of Sos to bind to a double point-mutated SH3A-C2 construct, in which the conserved tryptophan residues in the first and third SH3 domains (i.e., Trp-777 in SH3A and Trp-1031 in SH3C) were changed to serines (this construct was designated SH3A-C2(WS)). SH3 domains A and C were mutated because these regions have been most often implicated in intersectin-binding interactions (25, 27). As shown in Fig. 6A, SH3A-C2(WS) was completely ineffective in binding to Sos1, but it was still able to bind to N-WASP (neuronal-enriched wisconsin Synapse protein) (Fig. 6B, top panel), a Cdc42-specific target that has also been shown to interact with intersectin-L through its SH3 domains and to promote its Cdc42-GEF activity (22). As already indicated in Fig. 2A, the SH3A-C2(WS) mutant was also capable of stimulating the \[^{3}H\]GDP-GTP\^S exchange activity of Cdc42 to the same extent as SH3A-C2.

We then examined whether SH3A-C2 was capable of stimulating Ras activation in cells, as this might contribute to its transformation activity. Ras activation was detected by using a GST pull-down assay where the limit-binding domain from the Ras-target Raf was fused to GST (GST-RBD) (28). Fig. 6C (top panel) shows that the treatment of cells expressing HA-tagged (wild-type) Ras with EGF promoted the cellular activation of HA-Ras (compare lanes 3 and 4). Cells expressing SH3A-C2 were also able to effectively stimulate the activation of HA-Ras, even in the absence of EGF treatment (Fig. 6C, lane 1), as were cells expressing the SH3A-C2(E1237A) mutant (lane 5), which is defective for Cdc42-GEF activity. However, SH3A-C2(WS)
was completely ineffective (Fig. 6C, compare lanes 1 and 2). These results then raised the possibility that the ability of SH3A-C2 to induce cellular transformation relied on its ability to activate Ras as well as Cdc42.

Both Ras and Cdc42 Are Necessary for SH3A-C2-induced Cellular Transformation—The results presented in Fig. 7 show that mutations blocking either Ras or Cdc42 activation are sufficient to inhibit completely SH3A-C2-induced cellular transformation. For example, both the Cdc42-GEF-defective mutant SH3A-C2(E1237A) and the double point mutant SH3A-C2(WS), which is unable to bind Sos and stimulate Ras activation, were ineffective in stimulating the growth of NIH 3T3 fibroblasts in low serum (Fig. 7A) and did not induce a transformed morphology (Fig. 7B). Likewise, these mutants were unable to increase saturation density (Fig. 7C) or allow fibroblasts to form colonies in soft agar (Fig. 7D and E).

Given the indications that SH3A-C2 needed to activate both Ras and Cdc42 to transform cells, it was of interest to examine which of these GTP-binding proteins was responsible for signaling to JNK and PI3K, as both are essential for transformation (10–13). To further probe the role of Cdc42 in cell growth regulation, we examined the cellular consequences of expressing a Cdc42 mutant that was capable of constitutive GDP-GTP exchange but also was able to hydrolyze GTP back to GDP (this was referred to as a fast-cycling mutant). Unlike dominant-active, GTPase-defective forms of Cdc42, which we have found to mainly inhibit cell growth, fast-cycling Cdc42 mutants induce the transformation of NIH 3T3 fibroblasts and are particularly effective in stimulating the formation of colonies in soft agar (7–9). Recently, we uncovered an important clue regarding the underlying mechanisms responsible for these Cdc42-induced transformation phenotypes (17); namely, the constitutive activation of Cdc42 results in the negative regulation of EGFR interactions with the E3 ligase, Cbl, and thereby blocks receptor ubiquitination and degradation. The resulting accumulation of EGFRs gives rise to excessive mitogenic signaling and cellular transformation.

Given these findings, we might predict that the aberrant expression or regulation of Cdc42-GEFs would also induce cellular transformation by altering the normal down-regulation of EGFRs. We were particularly interested in examining such a possibility using the Cdc42-specific GEF, intersectin-L, because a shorter version of this protein, intersectin-S, is implicated in endocytic events and has some capability for transforming fibroblasts (23, 29). We suspected that the deregulation of the Cdc42-GEF activity of intersectin-L would result in the hyperactivation of Cdc42, as well as the potential disruption of EGFR endocytosis, and could thus be especially potent in stimulating cellular transformation. Although indeed it turned out that a truncated version of intersectin-L lacking the EH domains (SH3A-C2) was very effective at transforming fibroblasts, as indicated by its ability to stimulate colony for-

**DISCUSSION**

A number of reports have implicated members of the Rho subfamily of the Ras-related GTP-binding proteins in malignant transformation (10–13). To further probe the role of Cdc42 in cell growth regulation, we examined the cellular consequences of expressing a Cdc42 mutant that was capable of constitutive GDP-GTP exchange but also was able to hydrolyze GTP back to GDP (this was referred to as a fast-cycling mutant). Unlike dominant-active, GTPase-defective forms of Cdc42, which we have found to mainly inhibit cell growth, fast-cycling Cdc42 mutants induce the transformation of NIH 3T3 fibroblasts and are particularly effective in stimulating the formation of colonies in soft agar (7–9). Recently, we uncovered an important clue regarding the underlying mechanisms responsible for these Cdc42-induced transformation phenotypes (17); namely, the constitutive activation of Cdc42 results in the negative regulation of EGFR interactions with the E3 ligase, Cbl, and thereby blocks receptor ubiquitination and degradation. The resulting accumulation of EGFRs gives rise to excessive mitogenic signaling and cellular transformation.

Given these findings, we might predict that the aberrant expression or regulation of Cdc42-GEFs would also induce cellular transformation by altering the normal down-regulation of EGFRs. We were particularly interested in examining such a possibility using the Cdc42-specific GEF, intersectin-L, because a shorter version of this protein, intersectin-S, is implicated in endocytic events and has some capability for transforming fibroblasts (23, 29). We suspected that the deregulation of the Cdc42-GEF activity of intersectin-L would result in the hyperactivation of Cdc42, as well as the potential disruption of EGFR endocytosis, and could thus be especially potent in stimulating cellular transformation. Although indeed it turned out that a truncated version of intersectin-L lacking the EH domains (SH3A-C2) was very effective at transforming fibroblasts, as indicated by its ability to stimulate colony for-
Information in soft agar and to promote growth in low serum, the mechanism responsible for its transforming activity was not what we had originally expected and, in fact, turned out to be rather interesting and novel.

Particularly surprising was our finding that the ability of different intersectin-L constructs to transform NIH 3T3 cells did not directly correlate with their ability to promote the activation of Cdc42. For example, although an intersectin-L construct comprising just the DH, PH, and C2 domains (DH-C2) was very effective in stimulating the GDP-GTP exchange activity of Cdc42 and promoting Cdc42 activation in cells, it was rather ineffective in stimulating the transformation of fibroblasts. On the other hand, SH3A-C2, an intersectin-L construct containing all five SH3 domains together with the DH, PH, and C2 domains, was only capable of modest Cdc42-GEF activity both in vitro and in cells, but it was extremely effective in inducing the transformation of fibroblasts as assayed by colony formation in soft agar. Moreover, although the underlying mechanism responsible for transformation by constitutively active, fast-cycling Cdc42 mutants was the accumulation of EGFRs and prolonged EGF-coupled signaling, this was not the case for SH3A-C2-induced transformation. Cells expressing this intersectin-L construct did not show increased levels of EGFRs or a sustained EGF-coupled signaling to ERK. Nonetheless, SH3A-C2-induced transformation was dependent on the Cdc42-GEF activity of SH3A-C2, as mutating the DH domain and blocking this activity completely inhibited its transforming activity. Thus, although it was necessary that SH3A-C2 increased the levels of GTP-bound Cdc42 to stimulate colony formation in soft agar, apparently the amounts of activated Cdc42 generated in SH3A-C2-expressing cells were not sufficient to adequately sequester Cbl and reduce EGFR degradation. This then suggested that the SH3A-C2-induced activation of Cdc42, together with another SH3A-C2-promoted activity, was necessary to give rise to the potent stimulation of growth in soft agar.

One interesting possibility regarding an additional SH3A-C2-promoted activity seemed to be Ras, as previous studies have shown that intersectin-S, which lacks the DH and PH domains and thereby is ineffective in promoting the activation of Cdc42, is capable of binding via its SH3 domains to Sos (27). Likewise, we found that SH3A-C2 was capable of forming a stable complex with Sos and promoting the activation of Ras in cells, whereas Ras activation was blocked by mutating conserved tryptophan residues in the first and third SH3 domains (i.e. SH3A and SH3C). It is worth noting that mutating the conserved tryptophan residues within these domains did not inhibit the binding of WASP, a well known interaction partner of intersectin-L that has been shown to help activate the Cdc42-GEF activity of the full-length intersectin protein, ap-

![Image of SH3A-C2 binds Sos and activates Ras](image-url)
parently by reversing an autoinhibitory intramolecular interaction between the SH3 and DH domains (22). The mutations of the conserved tryptophan residues in SH3A and SH3C also did not have any effect on the ability of SH3A-C2 to activate Cdc42. However, importantly, mutations that prevented SH3A-C2 from functionally coupling to Sos and Ras also completely blocked the transforming activity of this intersectin-L construct.

Thus, intersectin-L appears to be capable of coordinating the activation of both Ras and Cdc42, the former occurring through interactions with Sos and the latter being a result of directly functioning as a specific GEF for Cdc42. Presumably, the actions of intersectin-L are tightly regulated, such that its ability to activate Ras- and Cdc42-coupled pathways occurs when the proper growth-promoting signals are received. Apparently, the SH3A-C2 construct is only partially effective in its ability to...
activate Cdc42 (at least when compared with a construct that lacks the SH3 domains (i.e. DH-C2)); still, this construct is nonetheless able to generate sufficient amounts of activated Cdc42 as well as activated Ras via interactions with Sos, so that these GTP-binding proteins can work together to elicit a transforming signal (Fig. 9). As discussed above, excessive signaling to ERK is not responsible for SH3A-C2-induced cellular transformation. Although SH3A-C2 at best only weakly activates JNK and PI3K, both of these signals are essential for transforming activity. It is also worth noting that EGF-stimulated JNK and PI3K activities are reduced in cells expressing transforming activity. It is also worth noting that EGF-stimulated JNK and PI3K activities are reduced in cells expressing the SH3A-C2(WS) mutant that is defective for binding Sos and activating Ras. This suggests that some portion of the EGF-mediated stimulation of JNK and PI3K occurs via endogenous intersectin (24), such that the Ras activation-defective SH3A-C2(WS) construct has a dominant-negative inhibitory effect on these signals.

Overall, these findings suggest a novel and somewhat unexpected mechanism by which Ras and Cdc42 cooperate to derail the normal control of cell growth and raise some interesting implications regarding other situations where it has been observed that Ras transformation relies on the activation of Cdc42 (16). A puzzling question that emerges from these studies is why DH-C2, which effectively activates Cdc42 in cells, does not mimic the actions of the constitutively active Cdc42(F28L) mutant and give rise to cellular transformation. The answer may lie in the apparent differences in the cellular localization of DH-C2 versus Cdc42(F28L) or SH3A-C2. We have found that the predominant location of both Cdc42(F28L) and SH3A-C2 is perinuclear, whereas DH-C2 is diffusely distributed throughout the cell. Thus, it may be that at least some of the Cdc42-signaling activities necessary for transformation need to originate from the Golgi (30). Future studies will be directed toward addressing this and a number of other interesting questions that now arise from these findings, such as how full-length intersectin-L is regulated normally so that excessive signaling through Ras or improper cooperation between Ras and Cdc42 does not occur.

REFERENCES

1. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
2. Ridley, A. J., and Hall, A. (1992) Cell 70, 389–399
3. Nobes, C. D., and Hall, A. (1995) Cell 81, 53–62
4. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) Nature 390, 635–636
5. Schmitz, A. A., Govek, E.-E., Bottner, B., and van Aelst, L. (2000) Exp. Cell Res. 361, 1–12
6. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes, R. O. (2000) Nature 406, 532–535
7. Qiu, R. G., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995a) Nature 374, 457–459
8. Qiu, R. G., Chen, J., McCormick, F., and Symons, M. (1995b) Proc. Natl. Acad. Sci. U. S. A. 92, 11781–11785
9. Wu, W.-J., Tu, S., Yang, W., Nolbant, P., Hahn, K., and Cerione, R. A. (2000) J. Biol. Chem. 275, 27421–27427
10. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1029–1040
11. Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. C. (1999) Science 286, 309–312
12. Jambaou, L., van der Kammen, R. A., and Collard, J. C. (1995) Nature 375, 338–340
13. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes, R. O. (2000) Nature 406, 532–535
14. Qiu, R. G., Chen, J., McCormick, F., and Symons, M. (1995a) Nature 374, 457–459
15. Qiu, R. G., Chen, J., McCormick, F., and Symons, M. (1995b) Proc. Natl. Acad. Sci. U. S. A. 92, 11781–11785
16. Qiu, R. G., Chen, J., McCormick, F., and Symons, M. (1997) Mol. Cell. Biol. 17, 3449–3458
17. Wu, W. J., Tu, S., and Cerione, R. A. (2003) Cell 114, 715–725
18. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., O’Brian, J. P., Der, C. J., Kay, B. K., and McPherson, P. S. (2000) J. Biol. Chem. 275, 27421–27427
19. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1029–1040
20. Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. C. (1999) Science 286, 309–312
21. Hussain, N. K., Jenna, S., Glogauer, M., Quinn, C. C., Wasiak, S., Guipponi, M., Antonarakis, S. E., Kay, B. K., Stossel, T. P., Lamarche-Vane, N., and McPherson, P. S. (2001) Nat. Cell Biol. 3, 927–932
22. Hussain, N. K., Yamabai, M., Ramjaun, A. R., Guy, A. M., Baranes, D., O’Bryan, J. P., Der, C. J., Kay, B. K., and McPherson, P. S. (1999) J. Biol. Chem. 274, 15671–15677
23. Hussain, N. K., Yamabai, M., Ramjaun, A. R., Guy, A. M., Baranes, D., O’Bryan, J. P., Der, C. J., Kay, B. K., and McPherson, P. S. (2000) EMBO J. 19, 1263–1271
24. Taylor, S. N., and Shalloway, D. (1996) J. Biol. Chem. 271, 7259–7264
25. Zammanian, J. L., and Kelly, R. B. (2003) Mol. Biol. Cell 14, 1624–1637
26. Zhu, K., Debreceni, B., Li, R., and Zheng, Y. (2000) J. Biol. Chem. 275, 25993–26001
27. Tong, X. K., Hussain, N. K., de Heuvel, E., Kurakin, A., Ahi-Jaroude, E., Quinn, C. C., Olson, M. F., Marais, R., Baranes, D., Kay, B. K., and McPherson, P. S. (2000) J. Biol. Chem. 275, 1621–1627
28. spouses, A., Ahi-Jaroude, E., Quinn, C. C., Olson, M. F., Marais, R., Baranes, D., Kay, B. K., and McPherson, P. S. (2000) J. Biol. Chem. 275, 1621–1627
29. Adams, A., Thorn, J. M., Yamabai, M., Kay, B. K., and O’Bryan, J. P. (2000) J. Biol. Chem. 275, 27414–27420
30. Wu, W. J., Erickson, J. W., and Cerione, R. A. (2000) Nature 405, 808–814

a J.-B. Wang, W. J. Wu, and R. A. Cerione, unpublished results.