Epidermal Growth Factor-dependent Regulation of Cdc42 Is Mediated by the Src Tyrosine Kinase*

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Cdc42 is a member of the Rho subfamily of Ras-related GTP-binding proteins and has been implicated in a wide range of cellular processes and signaling activities. Among its best known actions is the regulation of actin cytoskeletal architecture, because microinjection of activated Cdc42 was found to give rise to filopodia formation, whereas the closely related Rac and Rhos proteins were implicated in membrane ruffling and in the generation of actin stress fibers, respectively (1–4). A number of lines of evidence have also implicated Cdc42 in the generation of actin stress fibers, respectively (1–4). A number of lines of evidence have also implicated Cdc42 in the generation of actin stress fibers, respectively (1–4). A number of lines of evidence have also implicated Cdc42 in the generation of actin stress fibers, respectively (1–4).

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A number of different extracellular stimuli including epidermal growth factor (EGF) have been shown to mediate the regulation of the activation-deactivation (GTP-binding/GTPase) cycle of Cdc42. The addition of EGF to NIH 3T3 cells is accompanied by a rapid increase in the levels of GTP-bound Cdc42 (11), as read-out by using an assay that detects the activation-dependent binding of Cdc42 to its limit-binding domain on PAK (often referred to as the PBD for p21-binding domain), a serine/threonine kinase that serves as a cellular target for Cdc42 and Rac. Additional indications that EGF stimulates the activation of Cdc42 in cells come from the findings that the interactions of Cdc42 with its target/effectors, IQGAP, which serves to interface Cdc42 with the actin cytoskeleton (12, 13), and ACK2, a nonreceptor tyrosine kinase (14), are stimulated following the addition of this growth factor (13, 14). Moreover, we have found that Cdc42 can influence EGF receptor sorting and degradation. The Cdc42-specific target, ACK2, together with its tyrosine phosphosubstrate, SH3PX1 (also known as sorting nexin 9), was shown to stimulate the sorting and degradation of EGF receptors (15). It also was found that ACK1 and ACK2 are able to bind to clathrin (16, 17), which presumably represents an early step in the ACK/SH3PX1-mediated sorting of EGF receptors. Activated (GTP-bound) Cdc42 negatively regulates the binding of ACK/SH3PX1 to clathrin (16) and thus may serve to set the timing for EGF receptor sorting. Likewise, recently we have found that activated forms of Cdc42 block the binding of the Cbl ubiquitin ligase to EGF receptors, thereby providing a mechanism for regulating the timing of receptor ubiquitination (18).

In this study, we have set out to determine how EGF receptors direct the activation of Cdc42 in cells. Because we had originally identified Cdc42 through its ability to serve as a phosphosubstrate for the EGF receptor in reconstituted phospholipid vesicle systems (19), we were especially interested in the possible link between phosphorylation and the EGF-dependent activation of Cdc42. Here we show that Cdc42 undergoes EGF-dependent tyrosine phosphorylation in cells; however, this phosphorylation event is not essential for the activation of Cdc42 but does appear to influence its ability to bind to RhodGDI, which plays an essential role in Cdc42-mediated cellular signaling and transformation (20). We further...
demonstrate that the EGF-dependent activation of Cdc42 is mediated through the Src tyrosine kinase and Vav2, a widely distributed member of the Dbl family of GEFs, which like the hematopoietic-specific Vav1 protein can bind directly to the EGF receptor (21–26). Thus, EGF receptor activation exerts multiple regulatory influences on the signaling capability of Cdc42.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—EGF was purchased from Calbiochem. Monoclonal antibodies to hemagglutinin (HA) and anti-Myc were obtained from Covance. Monoclonal anti-phosphotyrosine G410 antibody and the cDNAs encoding constitutively active mouse Src (Src(Y329F)) and kinase-defective mouse Src (Src(K297R)) in the pUSE vector were obtained from Upstate Biotechnology, Inc. The Src kinase inhibitor PP2 was from Calbiochem.

Cell Culture and Preparation of Cell Extracts—COS-7 and HEK293 cells were maintained in a humidified 7% CO2 environment in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and with 10% fetal bovine serum. The cells were washed with ice-cold phosphate-buffered saline and then resuspended in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM β-glycerol phosphate, 10 μg/ml leupeptin, and 10 μg/ml aprotonin) for 15 min at room temperature prior to use.

Src Kinase Assay—Recombinant human wild-type Src, expressed in SF9 insect cells as a histidine-tagged protein and purified by nickel-nitritotriacetic acid-agarose chromatography as an active kinase (>40,000 units/mg of protein where 1 unit = 1 pmol of Src substrate peptide phosphorylated per minute), was purchased from Upstate Biotechnology, Inc. Ten μg of Escherichia coli-expressed GST-Cdc42, prepared as previously described (27), were added to 10 μl of Src reaction buffer (100 mM Tris-HCl, pH 7.2, 125 mM MgCl2, 25 mM MnCl2, 2 mM EGTA, 0.25 mM sodium orthovanadate, and 2 mM dithiothreitol). Five μl of active Src protein (20 μg of kinase activity) were then added to the reaction mixture and incubated with 100 μl of α[γ-32P]ATP and 5 μM ATP for 10 min (final assay volume, 100 μl). Ten μl of SDS loading buffer were added to stop the reaction, and the reaction mixture was then subjected to SDS-polyacrylamide gel electrophoresis.

Molecular Constructs—Point mutants were generated using polymerase chain reaction from a cDNA encoding Cdc42 that had been subcloned into the BamHI-EcoRI site of pcDNA3. The cDNAs encoding different Cdc42 constructs were then transiently expressed in COS-7 cells as HA-tagged proteins.

Assaying the Activation of Cdc42 in Cells Using the Limit Cdc42/Rac-binding Domain from PAK—Activation of cellular Cdc42 was assayed as previously described (11, 28), based on a procedure originally developed for Ras (29). COS-7 cells were transiently transfected with the cDNA for wild-type Cdc42 in the pcDNA3 vector. The cells were allowed to grow in the presence of 10% fetal bovine serum for 24 h and thenstarved for 4 h, followed by stimulation with 50 ng/ml of EGF. Twenty minutes after stimulation, the cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 20 mM β-glycerol phosphate, 20 μM GTP, 1 mM sodium orthovanadate, and 10 μM each of leupeptin and aprotonin) and then incubated with 50 μg of GST-PBD (for p21-binding domain from PAK). The cells were rocked at 4 °C for 3 h. GST-PBD was then precipitated with glutathione-agarose beads, washed three times with lysis buffer, and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using the indicated antibodies.

Cellular Transformation—NIH 3T3 cells stably expressing Cdc42(F29L) and Cdc42(F29L,Y64A) were generated, and their abilities to form colonies in soft agar were assayed as outlined in Tu et al. (10) and Wu et al. (18).

RESULTS

Cdc42 Is Phosphorylated in Cells by the Src Kinase in an EGF-dependent Manner—The mammalian Cdc42 was originally identified using a reconstitution assay to search for possible participants in the EGF receptor-coupled signaling pathway (19). Specifically, Cdc42 was phosphorylated in an EGF-dependent manner in reconstituted phospholipid vesicles containing the purified EGF receptor and crude extracts from bovine brain membranes. Thus, we were interested in seeing whether Cdc42 was also phosphorylated in a similar fashion in living cells and whether this was important for the EGF-stimulated activation of the GTP-binding protein. Fig. 1 (A and B, top left panels) shows that when either COS-7 or HEK293 cells transiently expressing HA-tagged Cdc42 were treated with 50 ng/ml of EGF, the GTP-binding protein was phosphorylated in an EGF-dependent manner, as read-out by Western blotting with an anti-phosphotyrosine antibody (4G10). The phosphorylation of Cdc42 peaked within 10 min of treatment with the growth factor and was sustained for 30 min (data not shown). As will be demonstrated below, the phosphorylation can be significantly attenuated by specific mutations of Cdc42 and can be obtained with Myc-tagged Cdc42, thus ruling out the trivial possibility that the HA tag was the site of phosphorylation.

Because we had earlier found that purified Cdc42 showed little ability to be phosphorylated directly by the purified EGF receptor in vitro, we suspected that the EGF-stimulated phosphorylation of Cdc42 in cells was mediated through another tyrosine kinase. One interesting candidate was Src, because it has been shown that the Src tyrosine kinase acts downstream from the EGF receptor (30–33). Thus, we transiently expressed the kinase-defective mouse Src(K297R) mutant, together with HA-tagged Cdc42, in both COS-7 and HEK293 cells that were then treated with EGF. As shown in Fig. 1 (A and B, middle left panels), kinase-defective Src(K297R) blocked the EGF-dependent phosphorylation of Cdc42 in each of these cell types. Likewise, the treatment of cells with the Src kinase inhibitor PP2 eliminated the EGF-dependent phosphorylation of Cdc42 (Fig. 1, A and B, bottom left panels).

In the absence of EGF treatment, expression of wild-type Src did not catalyze the phosphorylation of HA-tagged Cdc42 in cells (Fig. 2A, top panel), whereas the constitutively active
mouse Src(Y529F) mutant was able to elicit the tyrosine phosphorylation of Cdc42. Activated (GTPase-defective) Cdc42(Q61L) appeared to show a stronger phosphorylation signal in this experiment; however, what we have found from several experiments is that after correcting for the levels of expression of the HA-tagged Cdc42 proteins, there are little or no significant differences between wild-type and activated (GTP-bound) Cdc42.

Insect cell-expressed, purified Src kinase was able to phosphorylate directly purified, E. coli-recombinant Cdc42 in vitro. An example is shown for GST-Cdc42(Q61L) in Fig. 2B (top panel); the same was also true for GST-Cdc42 wild type (data not shown). Thus, overall, the results presented in Figs. 1 and 2 (A and B) indicated that the EGF-stimulated phosphorylation of Cdc42 was mediated by Src.

Tyrosine 64 on Cdc42 Is a Major Site of Phosphorylation by the Src Kinase—We next set out to identify the tyrosine residue(s) that is phosphorylated in an EGF- and Src kinase-dependent manner. One particularly attractive residue was tyrosine 64, which is present within the Switch II domain (i.e. a conformationally sensitive region first identified in Ras (34)), because it lies within a sequence (EDYDR) that is favorable for phosphorylation by certain tyrosine kinases including Src (35). As shown in Fig. 2B (upper panel), the Cdc42(Y64F) mutant did not serve as a phosphosubstrate when assayed in vitro. The same was true when examining either the EGG-dependent or Src(Y529F)-catalyzed phosphorylation of Cdc42(Y64F) or Cdc42(Y64E) in cells (Fig. 2C, upper right panel), whereas changing the tyrosine residue at position 40, located in the Switch I region of Cdc42, to a cysteine did not diminish phosphorylation (upper left panel). Changing tyrosine 64 to either a phenylalanine or glutamic acid within an activated (Q61L) background also significantly inhibited phosphorylation. However, the fact that some hint of phosphorylation was sometimes detectable on both the Cdc42(Q61L,Y64F) and Cdc42(Q61L,Y64E) mutants (Fig. 2C, upper left panel) suggested that although tyrosine 64 was likely to be the primary phosphorylation acceptor site, secondary phosphorylation sites may become accessible when Cdc42 is in the GTP-bound state.

Is the Phosphorylation of Cdc42 Necessary for Its Interactions with Target/Effectors?—Thus far, we have not found any indication that the EGF- and/or Src-catalyzed phosphorylation of Cdc42 directly influences its interactions with known target/effectors. Fig. 3 shows the results of experiments where Myc-tagged Cdc42(Q61L) was transiently expressed with either wild-type Src or Src(Y529F) and together with different HA-tagged target/effectors in COS-7 cells. There were no detectable differences in the ability of activated Cdc42 to be co-immunoprecipitated with these targets (Fig. 3, left panels) when the GTP-binding protein was phosphorylated in a Src(Y529F)-dependent manner (Fig. 3, right panels). The same was true when examining the inter-
actions of activated Cdc42 with two endogenous targets, IQGAP and MRCK.

The Src-catalyzed Phosphorylation of Tyrosine 64 on Cdc42 Is Accompanied by an Enhanced Binding to RhoGDI—Structural studies of the Cdc42-RhoGDI complex have demonstrated that the Switch II domain of Cdc42 forms an important part of the binding interface with RhoGDI (36). Thus, we examined whether the Src-catalyzed phosphorylation of tyrosine 64 on Cdc42 influenced its interactions with this regulatory protein. This was done by co-expressing HA-tagged wild-type Cdc42 or the Cdc42(Q61L) mutant, which also binds RhoGDI, with Myc-tagged RhoGDI and either wild-type Src or the constitutively active Src(Y529F) mutant. As shown in Fig. 4, co-expression of the Cdc42 proteins with activated Src(Y529F), under conditions that resulted in the phosphorylation of Cdc42 (top panel), caused an enhancement in the amount of RhoGDI that was co-immunoprecipitated with the anti-HA-tagged GTP-binding protein (bottom panel), compared with that detected when Cdc42 was co-expressed with wild-typeSrc. We could not detect the association of RhoGDI with Cdc42 proteins that were mutated at position 64. The RhoGDI typically appeared as a smear on the anti-Myc Western blots after its immunoprecipitation from COS-7 cells, which may mean that this regulatory protein was susceptible to phosphorylation in cells. At the present time, we know very little regarding how Cdc42-RhoGDI interactions are regulated or why the tyrosine phosphorylation of Cdc42 might enhance its ability to bind RhoGDI. It is interesting that the x-ray crystal structure for the Cdc42-RhoGDI complex (36) shows that tyrosine 64 of Cdc42 comes into close proximity of lysine residues 43 and 52 from RhoGDI, and so one attractive possibility was that the negative charge accompanying the phosphorylation of tyrosine 64 was stabilized by the positive charges provided by these basic residues. However, if this were the case, it might have been expected that simply changing tyrosine 64 to glutamic acid would have enhanced the interactions between Cdc42 and RhoGDI, but this did not occur (Fig. 4, bottom panel). Thus, it may be that other mechanisms account for the enhanced binding of RhoGDI that accompanies the EGF/Src-dependent tyrosine phosphorylation of Cdc42.

The phosphorylation of Cdc42 and its effects on Cdc42-RhoGDI interactions may have important signaling consequences. We have previously shown that the constitutively active Cdc42(F28L) mutant induces the transformation of NIH 3T3 fibroblasts, as read-out either by the growth of cells in low serum or their ability to form colonies in soft agar (7). Recently, we have also demonstrated an essential role for RhoGDI in Cdc42-induced cellular transformation (20). This then raises the question of whether a phosphorylation-defective Cdc42 mutant that is impaired in its ability to bind RhoGDI is still able to transform fibroblasts. The data presented in Fig. 5 shows that this is not the case. A Cdc42(F28L,Y64A) double mutant, which can be stably expressed to levels that are within 50–70% of the expression levels for Cdc42(F28L), is severely compromised both in its ability to stimulate growth in low serum and to form colonies in soft agar. Although this does not definitively prove that the phosphorylation of Cdc42 at position 64 is essential for transformation, it does indicate that tyrosine 64 is a critical residue and is consistent with the notion that the phosphorylation of this tyrosine residue, and the resultant enhanced binding of RhoGDI, contribute to the transformation signal.

Is the Src-catalyzed Phosphorylation of Cdc42 Essential for the EGF-dependent Activation of the GTP-binding Protein?—We had previously shown that treatment of NIH 3T3 cells with EGF led to a relatively rapid increase in the levels of GTP-bound (activated) Cdc42 (11), as read-out by an assay that examines the binding of this GTP-binding protein to its limiting-binding domain on PAK (called the PBD). The same is true for COS-7 cells transiently transfected with HA-tagged Cdc42, as shown in Fig. 6 (top panel). In these cells, the EGF-dependent activation of Cdc42 persists through 30 min of treatment with the growth factor. When COS-7 cells were transiently transfected with constitutively active Src(Y529F), there was an obvious increase in the basal levels of GTP-bound Cdc42 (i.e. in the absence of any growth factor treatment; Fig. 6, middle panel). This was done by co-expressing HA-tagged wild-type Cdc42 or the Cdc42(Y64F) and Cdc42(Y64E) mutants, together with either wild-type mouse Src or the constitutively active Src(Y529F) and Myc-tagged GDI. The Cdc42 proteins were immunoprecipitated using an anti-HA antibody, and the precipitates were then subjected to Western blot analysis using either an anti-phosphotyrosine antibody to assess the phosphorylation state of Cdc42 (top panel), an anti-HA antibody to determine the relative levels of the Cdc42 proteins (middle panel), or an anti-Myc antibody to examine the association of Cdc42 with RhoGDI (bottom panel). For the EGF-dependent activation of the GTP-binding protein, when COS-7 cells were transiently transfected with cDNAs encoding HA-tagged wild-type Cdc42, Cdc42(Q61L), or the phosphorylation-defective Cdc42 mutants (Cdc42(Y64F) and Cdc42(Y64E)), together with either wild-type mouse Src or the constitutively active Src(Y529F) and Myc-tagged GDI. The Cdc42 proteins were immunoprecipitated using an anti-HA antibody, and the precipitates were then subjected to Western blot analysis using either an anti-phosphotyrosine antibody to assess the phosphorylation state of Cdc42 (top panel), an anti-HA antibody to determine the relative levels of the Cdc42 proteins (middle panel), or an anti-Myc antibody to examine the association of Cdc42 with RhoGDI (bottom panel).
panel), suggesting that the activated Src kinase can promote the activation of the GTP-binding protein. However, when the COS-7 cells were transiently transfected with a cDNA encoding the kinase-defective Src mutant (Src(K297R)), both the basal and EGF-stimulated activation of Cdc42 were completely abolished (Fig. 6, bottom panel). These results indicate that like the EGF-stimulated phosphorylation of Cdc42, the EGF-promoted activation of this GTP-binding protein is mediated by the Src kinase.

We therefore asked the question of whether the Src-catalyzed phosphorylation was directly responsible for the EGF- and Src-mediated activation of Cdc42. However, the data presented in Fig. 7 shows that this was not the case. The ability of Src(Y529F) to promote the activation of Cdc42, as monitored by the ability of GST-PBD to precipitate GTP-bound Cdc42 (Fig. 7, middle panel), occurred independently of whether Cdc42 could be phosphorylated at tyrosine 64 (Fig. 7, top panel). Thus, the HA-tagged Cdc42(Y64F) mutant was as effective as HA-tagged wild-type Cdc42 in its ability to undergo activation and thus to be precipitated by GST-PBD (Fig. 7, middle panel). Note that in these experiments, the GTPase-defective Cdc42(Q61L) mutant served as a positive control for the PBD assay and was able to bind to GST-PBD in the presence and absence of Src(Y529F).

**What Is the Underlying Mechanism for the EGF- and Src-dependent Activation of Cdc42?**—Because the Src-catalyzed phosphorylation of Cdc42 did not directly result in its activation, we suspected that the EGF receptor and Src were acting through a GEF. One potentially interesting candidate was intersectin-1L, because this is a highly specific GEF for Cdc42 (37), which we have shown binds to the Cdc42-target/effector ACK2, a nonreceptor tyrosine kinase.2 Although intersectin-1L appears to be exclusively found in neuronal cells, a second related form (intersectin-2L) appears to be more broadly distributed. Because we had earlier shown that Src associates with ACK2 (38), we wondered whether an ACK-Src complex might bind to and phosphorylate intersectin-2L, which in turn could stimulate its GEF activity toward Cdc42. However, thus far, we have not obtained any evidence for a link between Src kinase activity and the ability of the intersectin proteins to function as Cdc42-GEFs.

We then turned our attention to another member of the Dbl

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* W. Smith and R. Cerione, unpublished data.
family of GEFs, Vav. The originally identified Vav protein (Vav1) was shown to be hematopoietic-specific, whereas a second form, Vav2, was found to be much more widely distributed. It has been well established that the Vav proteins are activated following their phosphorylation by Src and related tyrosine kinases (24–26) and that Vav2 exhibits GEF activity toward Cdc42 as well as Rac (39, 40). Fig. 8 shows that HA-tagged Vav2 can be phosphorylated in cells upon their treatment with EGF (bottom panel) or in the absence of growth factor addition when the cells expressed activated Src(Y529F) (top panel). Moreover, under these conditions, Vav2 can promote the activation of Cdc42 in cells, as read-out by the PBD assay. When Vav2 was transiently co-expressed with HA-tagged wild-type Cdc42 in COS-7 cells, the EGF-dependent activation of Cdc42 was enhanced (Fig. 9, compare C and D, left panels). In some cases (e.g. panel F), the basal activation of Cdc42 was enhanced when overexpressing Vav2, suggesting that some background activation of this GEF can occur. However, when Src(Y529F) was co-expressed with Vav2, the basal activation of Cdc42 was significantly elevated such that it was comparable with the levels of EGF-stimulated activation (Fig. 9A, left panel), whereas expression of the kinase-defective Src(K297R) effectively suppressed both the basal and EGF-dependent activation of Cdc42 (Fig. 9E, left panel). Overall, these results argue for a signaling pathway leading from the EGF receptor, through the Src kinase to Vav2, that is responsible for the EGF-dependent activation of Cdc42. Apparently Vav2 is a limiting component in this pathway, at least in COS-7 cells, because its expression enhanced the levels of Cdc42 activation. The results presented in Fig. 9F (left panel) further verify that the phosphorylation of Cdc42 at tyrosine 64 is not required for the EGF-dependent, Vav2-mediated activation of the GTP-binding protein.

DISCUSSION

The mechanisms by which different types of extracellular stimuli (growth factors, hormones, and integrins) mediate the activation and regulation of signaling activities through Rho family GTP-binding proteins are still being delineated. Cdc42, in particular, serves as an important control switch for growth factors and other types of extracellular stimuli through its ability to direct changes in cell shape and motility and to influence cell cycle progression and apoptosis. We have found that Cdc42 is very responsive to the treatment of cells with EGF, resulting in an activation of this GTP-binding protein within 3–5 min of growth factor addition. The ability of EGF to promote the activation of Cdc42 may provide a means by which this growth factor can influence cell morphology and motility, given that activated forms of Cdc42 have been shown to trigger changes in the actin cytoskeletal architecture (3, 4). However, as will be discussed in more detail below, another and perhaps more important reason for linking the EGF receptor to Cdc42 activation comes from recent work suggesting that Cdc42 influences the timing of EGF receptor sorting and degradation.

The various participants in the EGF-dependent regulation of the sorting and processing of its receptor are still being identified. Recently, we showed that the Cdc42-specific target/effector ACK2 works together with its phosphosubstrate SH3PX1/sorting nexin 9 to promote EGF receptor sorting and degradation (15). These findings likely explain why overexpression of ACK2 in fibroblasts led to a marked inhibition of cell growth (41). The ability of ACK2 to phosphorylate SH3PX1 appears to be essential for proper control of EGF receptor sorting, because the timing of receptor degradation was disrupted when kinase-defective ACK2 was expressed in cells (15). The cellular activation of ACK2 is absolutely dependent on the ability of Cdc42 to become activated, as expression of the dominant-negative mutant Cdc42(T17N) blocks ACK2 activity, whereas EGF treatment, by promoting the activation of Cdc42, stimulates ACK2 activity (14). Cdc42 also regulates negatively the binding of ACK2 to the heavy chain of clathrin (16), which may have an important regulatory effect on EGF receptor sorting if the ACK2-clathrin interaction is a necessary step for ACK2 and SH3PX1 to help direct the sorting process. Activ...
Cdc42 also does not appear to directly influence the binding of activated Cdc42 to the Cool-1 (for cloned-out-of-library)/H9252 (for PAK-interactive exchange factor) protein (18). The EGF- and Src-dependent phosphorylation of Cdc42 primes ACK2 to bind and phosphorylate SH3PX1. However, only when Cdc42 disengages from the ACK2/SH3PX1 complex, presumably because of GTP hydrolysis, would ACK2/SH3PX1 associate with clathrin, which may be necessary to ensure that these sorting participants are properly conveyed to the endosomes. It is interesting that we have also found that Cdc42 regulates the Cbl-catalyzed ubiquitination of EGF receptors (18), which has been shown to be essential for receptor degradation (42, 43). Taken together, these results have led us to propose that the EGF-dependent activation of Cdc42 provides a timing mechanism for the sorting and degradation of EGF receptors.

Overall, these findings then lead to the important question of how Cdc42 activation comes under the regulation of EGF receptors. In the work presented here, we show that the Src tyrosine kinase and the Dbl family member, Vav2, represent two key participants in the pathway connecting EGF receptor activation to the stimulation of GDP-GTP exchange on the Cdc42 protein. Various lines of evidence have implicated Src as being downstream of the EGF receptor (30–33), although the exact mechanism by which the treatment of cells with EGF results in the activation of the Src tyrosine kinase has not yet been determined. However once activated, Src can phosphorylate Vav2, thereby stimulating its GEF activity, a mechanism that has been well established (39, 40, 44). The activated Vav2 then directly catalyzes GDP-GTP exchange on Cdc42. This can occur relatively quickly and presumably at the plasma membrane, most likely in a manner analogous to the coupling between EGF receptor activation and the stimulation of GDP-GTP exchange on Ras, although in the latter case, this involves the recruitment of a Grb2-SOS complex to the membrane rather than an activation of SOS through phosphorylation.

In light of the connection between EGF receptors, Src kinase activity, and Cdc42 activation and the fact that Cdc42 is phosphorylated on tyrosine 64 in an EGF- and Src-dependent manner in cells, it is interesting that this phosphorylation event does not directly influence the activation of this GTP-binding protein. The EGF- and Src-dependent phosphorylation of Cdc42 also does not appear to directly influence the binding of activated Cdc42 to any of its known target/effector proteins. However, this phosphorylation event is correlated with an enhanced binding of Cdc42 to RhoGDI. This is particularly interesting in light of recent findings in our laboratory that the binding of activated Cdc42 to RhoGDI is essential for the ability of the constitutively active Cdc42(F28L) mutant to transform cells (20). In fact, we find that the Cdc42(P28L,Y64A) double mutant, which is phosphorylation-defective and impaired in its ability to bind RhoGDI, is also incapable of transforming NIH 3T3 fibroblasts. The role of RhoGDI in Cdc42-mediated transformation is likely linked to the requirement for activated Cdc42 to participate in a trafficking activity (45). The ability of RhoGDI to bind to activated forms of Cdc42 and to release them from membranes may help to regulate the movement of Cdc42 between different cellular (membrane) locations from which it can engage target/effectors important for the transforming signal. We would suspect that RhoGDI plays a similar role in ensuring the proper participation of activated Cdc42 in normal growth factor–coupled signaling and cell growth regulation.

Thus, EGF receptor and Src activation may modulate Cdc42 function in two important ways (Fig. 10). First, it promotes the rapid activation of Cdc42 such that this GTP-binding protein can engage target/effectors at or near the plasma membrane that ultimately participate in the sorting and degradation of EGF receptors, as well as targets that may be necessary for helping to coordinate actin cytoskeletal changes with receptor down-regulation. Second, it promotes the interactions of Cdc42 with RhoGDI that may be important for allowing Cdc42 to bind to targets in different membrane locations with the proper timing necessary to coordinate receptor processing with other cell growth signals, such as those involving trafficking events at the Golgi (45). It will be interesting in the future to better understand exactly how Cdc42 activation is maintained for...
sustained periods in cells and if in fact, RhoGDI contributes to this through its ability to inhibit GTP hydrolysis (46), thus enabling Cdc42 to engage targets in the Golgi such as the γ-coatomer subunit, which also is essential for cell growth and transformation (45).

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. Noes, C. D., and Hall, A. (1995) Cell 81, 53–62
4. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995) Mol. Cell. Biol. 15, 1942–1952
5. Cerione, R. A., and Zheng, Y. (1996) Curr. Opin. Cell Biol. 8, 216–222
6. Whitehead, I. P., Campbell, S., Rossman, K. L., and Der, C. J. (1997) J. Biol. Chem. 272, 1199–1206
7. Lin, Q., Lo, C. G., Cerione, R. A., and Yang, W. (2002) J. Biol. Chem. 277, 14748–14751
8. Qiu, R. G., Chen, J., McCormick, F., and Symons, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11781–11785
9. Lin, R., Bagrodia, S., Taylor, S. J., Jordan, K. A., VanAelst, L., and Cerione, R. A. (2002) Biochemistry 41, 12350–12358
10. Tu, S. S., Wu, W. J., Yang, W., Nolbant, P., Hahn, K., and Cerione, R. A. (2002) J. Biol. Chem. 277, 2997–3005
11. Tu, S., and Cerione, R. A. (2001) EMBO J. 15, 2761–2768
12. Hart, M. J., Callow, M. G., Souza, B., and Polakis, P. (1996) J. Biol. Chem. 271, 26852–26859
13. Erickson, J. W., Zhang, C., Kahn, R. A., Evans, T., and Cerione, R. A. (1996) J. Biol. Chem. 271, 26850–26854
14. Yang, W., and Cerione, R. A. (1997) J. Biol. Chem. 272, 24819–24824
15. Lin, Q., Lo, C. G., Cerione, R. A., and Yang, W. (2002) J. Biol. Chem. 277, 10134–10138
16. Yang, W., Lo, C. G., Dispensa, T., and Cerione, R. A. (2001) J. Biol. Chem. 276, 17469–17473
17. Teo, M., Tan, L., Lim, L., and Manser, E. (2001) J. Biol. Chem. 276, 18392–18396
18. Wu, W. J., Tu, S. S., and Cerione, R. A. (2003) Cell 114, 715–725
19. Hart, M. J., Polakis, P. G., Evans, T., and Cerione, R. A. (1990) J. Biol. Chem. 265, 5990–6001
20. Lin, Q., Puij, R. N., Yang, W., and Cerione, R. A. (2003) Curr. Biol. 13, 1469–1472
21. Bustelo, X. R., Ledbetter, J. A., and Barbacid, M. (1992) Nature 356, 68–71
22. Bustelo, X. R., and Barbacid, M. (1992) Science 256, 1196–1199
23. Margolis, B., Hu, P., Katzav, S., Li, W., Oliver, J. M., Ulrich, A., Weiss, A., and Schlessinger, J. (1992) Nature 356, 71–74
24. Crespo, P., Schuherl, K. E., Ostrom, A. A., Gutkind, J. S., and Bustelo, X. R. (1997) Nature 385, 169–172
25. Bustelo, X. R. (2000) Mol. Cell. Biol. 20, 1461–1477
26. Chiarlello, M., Marinissen, M. J., and Gutkind, J. S. (2001) Nat. Cell Biol. 3, 580–586
27. Wu, W. J., Lin, R., Cerione, R. A., and Manser, D. (1998) J. Biol. Chem. 273, 16655–16658
28. Bagrodia, S., Taylor, S. J., Jordan, K. A., VanAelst, L., and Cerione, R. A. (1998) J. Biol. Chem. 273, 23633–23636
29. Taylor, S. J., and Shalloway, D. (1996) Curr. Biol. 6, 1621–1627
30. Bouton, A. H., Kanner, S. B., Vines, R. R., Wang, H. C., Gibbs, J. B., and Parsons, J. T. (1991) Mol. Cell. Biol. 11, 945–953
31. Maa, M. C., Chen, J., McCormick, F., and Symons, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6981–6985
32. Biscardi, T. S., Maa, M. C., Tice, D. A., Cox, M. E., Leu, T. H., and Parsons, S. J. (1999) J. Biol. Chem. 278, 3835–3841
33. Thomas, S. M., and Brugge, J. S. (1997) Annu. Rev. Cell Dev. Biol. 13, 513–609
34. Milburn, M. V., Tong, L., Devos, A. M., Bruger, A., Yamaizumi, Z., Nishimura, S., and Kim, S.-H. (1990) Science 247, 939–945
35. Brown, M. T., and Cooper, J. A. (1996) Biochim. Biophys. Acta 1287, 121–149
36. Hoffmann, G. R., Nassar, N., and Cerione, R. A. (2000) Cell 100, 345–356
37. Hussain, N. K., Jenna, S., Glogaver, M., Quinn, C. C., Wasiak, S., Gui, M., Antonarakis, S. E., Kay, B. K., Stossel, T. P., Lamarche-Vane, N., and McPherson, P. S. (2001) Nat. Cell Biol. 3, 927–932
38. Yang, W., Lin, Q., Guan, L. J., and Cerione, R. A. (1999) J. Biol. Chem. 274, 8524–8530
39. Liu, B. P., and Burridge, K. (2000) Mol. Cell. Biol. 20, 7160–7169
40. Ahe, K., Rosselet, R., Liu, B., Ritola, K. D., Chiang, D., Campbell, S. L., Burridge, K., and Der, C. J. (2001) J. Biol. Chem. 276, 39973–39978
41. Levkowitz, G., Waterman, H., Zamir, E., Zam, Z., Oved, S., Langdon, W., Beguinot, L., Geiger, B., and Yarden, Y. (1998) Genes Dev. 12, 3663–3674
42. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tseng, K., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1029–1040
43. Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1029–1040
44. Aghazadeh, B., Lowry, W. E., Huang, X. Y., and Rossmann, M. K. (2000) Cell 102, 625–633
45. Wu, W. J., Erickson, J. W., and Cerione, R. A. (2000) Nature 405, 800–804
46. Hart, M. J., Maru, Y., Leonard, D., Witte, O. N., Evans, T., and Cerione, R. A. (1992) Science 256, 812–815
