Introduction

The epidermal growth factor (EGF) receptor (EGFR) is involved in various aspects of cell growth, survival, differentiation, migration, and invasion (1, 2). EGFRs are often present in excessive amounts in human breast cancers. In most cases, the mechanisms underlying the increased levels of receptor in human breast cancers are not known, although they often cannot be attributed to gene amplification (3, 4). An important mechanism for the termination of EGFR signaling is via c-Cbl-catalyzed EGFR ubiquitination and degradation (5–7). Aberrant accumulation of EGFR with enhanced EGF-coupled signaling occurs in cells when the E3 ligase activity of c-Cbl is compromised (7, 8). This information suggests that the elevated levels of EGFR in human cancer cells may be associated with impaired receptor degradation.

Abstract

Overexpression of epidermal growth factor receptor (EGFR) contributes to increased cell proliferation and migration in breast cancer. However, mechanisms of EGFR overexpression remain elusive and often cannot be attributed to gene amplification. In NIH3T3 fibroblasts, active Cdc42 inhibits c-Cbl-regulated EGFR degradation to induce cellular transformation. Here, we use two EGFR-overexpressing breast cancer cell lines, MDA-MB-231 and BT20, as models to test the hypothesis that up-regulated Cdc42 activity impairs c-Cbl-mediated EGFR degradation and contributes to EGFR overexpression. We show that silencing Cdc42 significantly reduces EGFR signaling on the expression of c-Cbl-N480, a c-Cbl mutant that is not regulated by Cdc42 and blocks Cdc42-induced transformation but still binds and ubiquitinitates EGFR. Expression of c-Cbl-N480 in NIH3T3 cells inhibits the binding of c-Cbl to EGFR and thus prevents c-Cbl from catalyzing receptor ubiquitination (29). This leads to the aberrant accumulation of EGFR and sustained EGF-stimulated extracellular signal-regulated kinase (ERK) activation in NIH3T3 cells resulting in malignant transformation (29).

Evidence suggests that a positive feedback loop may exist between Cdc42 and EGFR. Treatment of cells with EGF stimulates the activation of Cdc42 (28). Activated Cdc42, through an interaction with its target/effecter, cloned-out-of-library (p85Cool-1)/p21-activated kinase (PAK)–interactive exchange factor (β-Pix), inhibits the binding of c-Cbl to EGFR and thus prevents c-Cbl from catalyzing receptor ubiquitination (29). Cdc42, Rac, and Rho not only sufficiently transform fibroblasts, but also are required for Ras transformation (18, 23–26). Unlike Ras, which is activated primarily by point mutations that impair its GTPase activity in human cancers, Rho family members are activated by changes in upstream regulators (27).

Rho family GTPases are well known for their effects on the actin cytoskeleton (9). Activation of RhoA induces the formation of stress fibers (10). Activation of Rac is required for the formation of lamellipodia and membrane ruffles and is thought to be the driving force for cell movement (11). Activation of Cdc42 triggers the formation of filopodia or microspikes (12–14). Cdc42 also controls cell polarity in response to external directional cues (15). Improper regulation of cell migration can contribute to pathologic processes, including tumor cell invasion and metastasis (16).

Cdc42 has also been implicated in the regulation of cell growth (17, 18). Recently, it has been reported that levels of Cdc42, Rac1, and RhoA are elevated in many different human cancers, including human breast cancers (19–21), and that they contribute to enhanced mitogenic signaling (22). Cdc42, Rac, and Rho not only sufficiently transform fibroblasts, but also are required for Ras transformation (18, 23–26). Unlike Ras, which is activated primarily by point mutations that impair its GTPase activity in human cancers, Rho family members are activated by changes in upstream regulators (27).
Materials and Methods

Materials. Antibodies against EGFR, Cdc42, and Rac1 were purchased from BD Transduction Laboratories (Franklin Lakes, NJ). Antibodies against RhoA and Cbl (C-15) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rhodamine-conjugated phalloidin and Alexa 488–conjugated goat anti-mouse antibody were purchased from Molecular Probes (Eugene, OR). Antibody against phosphorylated p44/42 mitogen-activated protein kinase was purchased from Cell Signaling (Danvers, MA). Antibody against β-actin was from Sigma (St. Louis, MO). Paxillin antibody (BD Transduction Laboratories) was a kind gift of Paul A. Randazzo (Laboratory of Cellular Oncology, Center for Cancer Research, National Cancer Institute, Bethesda, MD). Recombinant EGFR and LipofectAMINE were from Invitrogen (Carlsbad, CA). SMARTpool short interfering RNA (siRNA) targeting human Cdc42 and human c-Cbl and control nontargeting siRNA were purchased from Dharmacon (Chicago, IL).

Cell culture, plasmids, and stable cell lines. MDA-MB-231 breast cancer cells [American Type Culture Collection (ATCC), Manassas, VA] were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic. BT20 breast cancer cells (ATCC) were cultured in MEM supplemented with 10% FBS and antibiotic/antimycotic. Stable cell line selection was achieved using G418 (18). Cells stably expressing c-Cbl-N480 were generated as outlined in Wu et al. (29). Transient transfections of siRNAs and plasmids were done using LipofectAMINE according to the manufacturer’s protocol.

EGFR down-regulation and EGFR-dependent ERK activation assays. Cells were cultured in six-well plates for 24 hours and then serum starved for 12 hours. Thereafter, cells were treated with 100 ng/mL EGF. At the indicated times, cells were harvested, and whole-cell lysates were subjected to SDS-PAGE. The levels of EGFR and phosphorylated p44/42 ERK were assessed by Western blot analysis with anti-EGFR and anti-phosphorylated p44/42 ERK antibodies.

Growth rates and soft agar growth assays. Growth rates and soft agar growth assays with various stable cell lines were done as reported previously (18, 29). To study the effect of Cdc42-specific siRNA on the growth rates of MDA-MB-231 and BT20 cells, cells were transfected with 100 nmol/L Cdc42-specific siRNA or control nontargeting siRNA. Cells were then cultured in medium supplemented with 10% FBS and at the indicated times, trypsinized, and counted. Trypan blue staining was used to assess cell death.

GTPase activity pull-down assay. A construct expressing glutathione S-transferase (GST)-rhokin Rho-binding domain (RBD; GST-RBD) was a kind gift of Drs. M.A. Wozniak and P.J. Keely (University of Wisconsin-Madison, Madison, WI). GTPase activity pull-down assays were done using GST-p21-binding domain of PAK (PBD) or GST-RBD as described previously (31).

Migration assay. Boyden chamber migration assays, fixation, and staining were done as described previously (32).

Results

Cdc42 siRNA suppresses EGFR signaling to ERK and reduces MDA-MB-231 cell proliferation. MDA-MB-231 cells, which overexpress EGFR but lack EGFR gene amplification (4), are a good model cell line for studying the causes of EGFR overexpression. In an attempt to understand better the relationship between EGFR overexpression and the activity of Cdc42 in human breast cancer cells, we compared total protein levels of endogenous Cdc42 and EGFR among MDA-MB-231, BT20, and MCF-7 breast cancer cells. As shown in Fig. 1A, the levels of endogenous Cdc42 were similar between the three cell lines. In contrast, the expression of endogenous EGFR in MDA-MB-231 and BT20 cells was significantly higher than in MCF-7 cells (Fig. 1A, top). The levels of EGFR in BT20 cells were slightly higher than in MDA-MB-231 cells. A comparison between levels of activated Cdc42 under serum-free conditions showed that MDA-MB-231 cells had ~3-fold and BT20 cells had at least 10-fold higher levels of activated Cdc42 compared with MCF-7 (Fig. 1A, PBD pull down, bottom), implicating that the activity of Cdc42 is correlated with the expression of EGFR among these three different breast cancer cell lines. Figure 1B showed that silencing Cdc42 significantly reduced levels of endogenous EGFR and the activity of ERK in MDA-MB-231 cells. These data further indicated that Cdc42 was necessary for the stability of EGFR in MDA-MB-231 cells.

We next analyzed the effect of Cdc42 knockdown on cell proliferation. Expression of Cdc42-specific siRNA inhibited MDA-MB-231 cell growth (Fig. 1C). Minimal reduction in cell numbers was observed 6 to 8 days after Cdc42-specific siRNA transfection. By staining with trypan blue, it was determined that ~40% of cells transfected with Cdc42-specific siRNA died by day 6 compared with 10% of cells transfected with control nontargeting siRNA (data not shown). We next determined if Cdc42-specific siRNA transfection altered the DNA content of cells, which might cause the effect on cell proliferation. As depicted in Fig. 1D, no changes in DNA content were detected at either early or late time points following siRNA transfection. Figure 1E contains representative micrographs of differences in cell numbers and morphology observed at 6 days after siRNA transfection.

In NIH3T3 fibroblast cells, stable expression of active Cdc42 is transforming due to the formation of a tripartite complex containing active Cdc42, p85Cool-1/βPix, and c-Cbl, which blocks c-Cbl-regulated down-regulation of EGFR (29). If the reduction of EGFR induced by Cdc42-specific siRNA is through c-Cbl-regulated EGFR degradation, then silencing endogenous c-Cbl should increase the levels of EGFR. Transfection of c-Cbl-specific siRNA for 48 hours in MDA-MB-231 cells increased EGFR protein levels ~3-fold compared with cells transfected with control nontargeting siRNA (Fig. 1F). The same is true when c-Cbl siRNA was transfected in BT20 cells (data not shown). These data indicated that endogenous c-Cbl functions in down-regulating EGFR and that reduced c-Cbl-mediated down-regulation of EGFR is able to contribute to EGFR overexpression.

Expression of c-Cbl-N480 increases the rate of EGFR degradation and inhibits cell proliferation. We have found previously that c-Cbl-N480, which is a c-Cbl mutant that does not associate with active Cdc42 but still binds and ubiquitinates EGFR, sufficiently blocks Cdc42-induced cellular transformation (29). We then hypothesized that expression of c-Cbl-N480 would reduce tumorigenic activity of MDA-MB-231 cells by enhancing the rate of EGFR degradation. We first examined whether c-Cbl-N480 affected the down-regulation of EGFR. Following serum starvation, different cell lines were treated with 100 ng/mL EGF for the indicated times. Figure 2A (top) shows that, in vector control cells, EGFR levels were sustained for 90 minutes. In contrast, reductions in EGFR protein levels began at 30-minute post-treatment, and receptor levels were sustained for 90 minutes. In NIH3T3 fibroblast cells, stably expressing c-Cbl-N480 (Fig. 2A, clones 11, 25). However, in cells with low expression of c-Cbl-N480 (14), EGFR levels were sustained following EGF treatment similar to vector control cells. Figure 2B (top) shows the relative amounts of c-Cbl-N480 expressed in the different MDA-MB-231 stable cell lines. Endogenous levels of c-Cbl did not differ between vector control cells and cells with different levels of c-Cbl-N480 overexpression (data not shown).

The enhanced rate of receptor degradation in MDA-MB-231 cells stably expressing high levels of c-Cbl-N480 may explain why the basal protein levels of EGFR in clones 11 and 25 were ~2- to 3-fold lower than in vector control cells (compare EGFR protein levels at time 0 minute). We obtained similar results from independent
experiments done at steady state, which showed that levels of EGFR were reduced in cells expressing c-Cbl-N480 (clones 11 and 25) compared with vector control MDA-MB-231 cells (data not shown).

We next examined the effect of c-Cbl-N480 expression on EGF-coupled ERK activation. Basal levels of activated ERK were significantly higher in vector control cells than in cells expressing high levels of c-Cbl-N480 (Fig. 2C; time 0 minute). In vector control cells, there was no significant increase in ERK activation at 15-minute post-EGF treatment, and levels of activated ERK were sustained over the 90-minute time course. These data indicate that ERK was constitutively active in MDA-MB-231 cells, consistent with data presented in Hoshino et al. (33). In contrast, c-Cbl-N480 clones 11 and 25 cells had a significant increase in ERK activation that peaked within 15-minute EGF treatment, began to reduce at 30 minutes, and was dramatically decreased after 90-minute EGF treatment (Fig. 2C). Sustained activation of ERK on EGF stimulation, as seen in vector control cells, was restored when low levels of c-Cbl-N480 (clone 14) were expressed in cells, although a slight increase in ERK activation was observed at 15-minute post-EGF treatment. Overall, the kinetics for EGF-coupled ERK activation in cells with high levels of c-Cbl-N480 is significantly changed, such that ERK is no longer constitutively activated.

MDA-MB-231 cells stably expressing c-Cbl-N480 were then tested for their ability to grow in soft agar and low serum. Figure 2D showed that MDA-MB-231 cells grew in soft agar, whereas c-Cbl-N480 clones 11 and 25 cells were unable to grow in soft agar. The ability of MDA-MB-231 cells to grow in soft agar was partially restored in cells that express c-Cbl-N480 at relatively low levels (clone 14). Fig. 2D (top) is representative micrograph of the growth in soft agar observed. MDA-MB-231 cells grew effectively under low-serum conditions, whereas c-Cbl-N480 clones 11 and 25 cells were incapable of growing in low-serum medium (Fig. 2E). Again, the ability of MDA-MB-231 cells to grow in low serum was partially recovered in cells that expressed low levels of c-Cbl-N480 (clone 14).
Taken together, these data indicated that the inhibitory effects of c-Cbl-N480 on MDA-MB-231 cells were dependent on the relative amount of c-Cbl-N480 expressed in cells. These data also suggested that overexpression of EGFR in MDA-MB-231 cells was due, at least in part, to reductions in c-Cbl-regulated EGFR degradation.

Figure 2. Stable expression of c-Cbl-N480 restores EGFR degradation and blocks EGFR-regulated cell proliferation. A, MDA-MB-231 cells stably expressing vector or hemagglutinin (HA)–tagged c-Cbl-N480 were serum starved for 12 hours and then stimulated with 100 ng/mL EGF for the indicated times. Whole-cell extracts were analyzed by Western blot for EGFR protein levels. Actin Western blot analysis was done to confirm equal protein loading. Clones 11, 14, and 25 are three unique clones with different expression levels of HA-tagged c-Cbl-N480. B, top, protein levels of HA-tagged c-Cbl-N480 in MDA-MB-231 grown in 10% FBS medium were determined by Western blot using anti-HA antibody; bottom, actin Western blot analysis was done to confirm equal protein loading. C, experimental procedures were described as in Fig. 2A. Following serum starvation, cells were treated with 100 ng/mL EGF for the indicated times. Levels of phosphorylated p44/42 ERK and actin were determined by Western blot. D, anchorage-independent growth of MDA-MB-231 vector control cells and cells stably expressing c-Cbl-N480 (clones 11, 14, and 25). Cell colonies were determined 14 days following seeding in soft agar. Columns, mean of three independent experiments; bars, SD. Top, representative phase-contrast micrographs of growth observed. Magnification, ×200. E, growth profiles (1% FBS) of MDA-MB-231 vector control cells and cells stably expressing c-Cbl-N480. Points, mean of three independent experiments; bars, SD.

EGFR to Cdc42, Rac1, and RhoA signaling pathways regulate the morphology of MDA-MB-231 cells. MDA-MB-231 is a highly invasive and poorly differentiated carcinoma cell line. Under phase-contrast microscopy, MDA-MB-231 cells (vector control) displayed a spindled and mainly bipolar shape with extended protrusions at

Figure 3. Stable expression of c-Cbl-N480 alters MDA-MB-231 cell morphology and relative levels of GTP-bound Cdc42, GTP-bound Rac1, and GTP-bound RhoA. A, cells were seeded on fibronectin-coated coverslips overnight and then analyzed. Phase-contrast micrographs were taken at a ×200 magnification. Actin staining was done using rhodamine-conjugated phallolidin. Paxillin staining was done using mouse anti-paxillin antibody followed by Alexa 488–conjugated goat anti-mouse antibody. Magnification, ×600. Open arrows, lamellipodia/membrane ruffles; arrowheads, stress fibers; closed arrows, focal adhesions. B, Cdc42, Rac1, and RhoA activities were assessed in GTPase activity pull-down assays. PBD of PAK was used for GST pull-down of activated Cdc42 and Rac1. Recombinant GST-RBD was used for GST pull-down of activated RhoA. GTP-bound Cdc42, Rac1, and RhoA were determined by Western blot analysis. C, c-Cbl-N480 11 cells were transiently transfected with a pcDNA3 vector encoding EGFR, HA-Cdc42-Q61L, or HA-Rac1-Q61L. Cells were then fixed, permeabilized, and stained for F-actin (red) and EGFR (green) or HA (green) to detect transfected cells. Yellow, overlap between red (Actin) and green (EGFR, Cdc42-Q61L, or Rac1-Q61L). Microscopy was done using a Bio-Rad 2100 confocal microscope (Hercules, CA) attached to a Nikon E800 microscope (Melville, NY). Images were taken using a ×60 Plan Apo/numerical aperture (NA) 1.4 lens with a 2× zoom. Magnification, ×1,200.
both ends of the cell body (Fig. 3A); their morphology did not differ from nontransfected parental MDA-MB-231 cells (data not shown). In contrast to vector control cells, c-Cbl-N480 11 cells were large and flat and lacked protrusions extending from the cell body (Fig. 3A, phase). MDA-MB-231 cells had lamellipodia, whereas cells expressing c-Cbl-N480 (clone 11) lacked lamellipodia and had robust stress fibers (Fig. 3A, actin staining). In vector control cells, paxillin mainly localized at membrane ruffles, whereas, in c-Cbl-N480-expressing cells (clone 11), paxillin was found at prominent focal adhesions (Fig. 3A, paxillin staining). MDA-MB-231 cells expressing low levels of c-Cbl-N480 (clone 14) exhibited a more flattened morphology than vector control cells but lacked robust stress fibers observed in clone 11 cells and instead had prominent lamellipodia similar to vector control cells (Fig. 3A, right).

Rho family GTPases play key roles in regulating the actin cytoskeleton. This led us to hypothesize that differences in the activity of Rho family GTPases between vector control cells and cells expressing c-Cbl-N480 (clone 11) may be responsible for the morphologic changes shown in Fig. 3A. GTP-bound Cdc42 and GTP-bound Rac1 were detected using PBD pull-down assays in cells cultured in growth medium supplemented with 10% FBS (Fig. 3B). Levels of active Cdc42 and Rac1 in c-Cbl-N480 clone 11 cells were significantly decreased compared with vector control cells (Fig. 3B). The activity of RhoA exhibited an inverse relationship with the activity of Cdc42 and Rac1, such that c-Cbl-N480 clone 11 cells contained a higher level of active RhoA than vector control cells (Fig. 3B, bottom). The elevated level of RhoA on the expression of c-Cbl-N480 is likely the cause of the increased stress fibers and focal adhesions, consistent with another report (34). We next asked whether c-Cbl-N480-induced changes in MDA-MB-231 morphology could be rescued by overexpressing EGFR. On overexpression of EGFR, cells became elongated, contained protrusions and membrane ruffles, and lacked strong stress fibers (Fig. 3C, top). Overexpression of constitutively active Cdc42-Q61L or Rac1-Q61L in c-Cbl-N480 clone 11 cells also restored cells to a morphology similar to vector control MDA-MB-231 cells (Fig. 3C, middle and bottom). Taken together, these data suggested that enhanced EGFR signaling leads to increased levels of active Cdc42 and Rac1 and decreased levels of activated RhoA, resulting in significant lamellipodium formation in MDA-MB-231 cells. These data also supported the idea that a positive feedback loop may exist between Cdc42 and EGFR in MDA-MB-231 cells.

Increasing the rate of EGFR degradation suppresses cell migration. Given that expression of c-Cbl-N480 altered cell morphology and the activity of Rho family GTPases, we then asked whether these changes affected cell motility. Cells were analyzed using a wound-healing assay (Fig. 4A). By 24-hour postwounding, vector control cells had migrated into the wound, such

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**Figure 4.** Stable expression of c-Cbl-N480 in MDA-MB-231 cells inhibits cell migration. A, wound-healing assays were done on cells seeded in RPMI 1640 supplemented with 10% FBS in 12-well plates. MDA-MB-231 vector control cells and cells expressing c-Cbl-N480 clone 11 were seeded overnight and then scratch wounded across the cell monolayer using a 1,000 μl micropipette tip. Phase-contrast images were taken at the indicated times. Magnification, ×200. B, cells seeded overnight in 12-well plates containing fibronectin (12 μg/mL)-coated coverslips were wounded as described in (A). At the indicated times, cells were then fixed in PBS containing 3.7% formaldehyde, permeabilized using PBS containing 0.1% Triton X-100, and stained for actin (left) and paxillin (middle). F-actin was stained using rhodamine-conjugated phalloidin. Paxillin was stained using mouse anti-paxillin antibody followed by Alexa 488–conjugated goat anti-mouse antibody. Right, merged images. Microscopy was done using a Bio-Rad 2100 confocal attached to a Nikon E800 microscope. Images were taken using a 60× Plan Apo/NA 1.4 lens. Open arrows, membrane ruffles (a-c and g-i); arrowheads, stress fibers (d, l, j, and l); closed arrows, focal adhesions (e, f, k, and l). Magnification, ×600. C, Boyden chamber assays were used to analyze migration at 4-hour postseeding. Fibronectin was the chemoattractant. Columns, mean number of cells per field migrated to the lower membrane from three independent experiments; bars, SD. Six wells per cell line and 10 fields per well were counted.
that it was 70% closed, and by 48 hours, the wound was completely closed. Wound closure was significantly slower in cells expressing c-Cbl-N480 (clone 11) compared with vector control cells. By 48 hours, cell migration in these cells had only resulted in 75% closure of the wound. We next examined cytoskeletal remodeling at the wound edge 90-minute and 24-hour post wounding. At the wound edge, vector control cells exhibited lamellipodia and membrane ruffles with the ruffling activity predominantly restricted to the front edge where paxillin mainly located (Fig. 4B, a-c). In contrast, cells expressing c-Cbl-N480 (clone 11) displayed robust stress fibers and focal adhesions and lacked membrane ruffles (Fig. 4B, d-f). By 24-hour post wounding, at the edge of the wound, vector control MDA-MB-231 cells became individualized and still had lamellipodia oriented toward the wound, whereas MDA-MB-231 cells expressing c-Cbl-N480 remained in close contact to each other and exhibited no ruffling activity (Fig. 4B, g-l). Significant differences in lamellipodium formation at the wound edge, which is thought to be the driving force for the cell movement, argue against the enhanced rate of wound closure for vector control cells being due to the difference in the rates of cell growth between vector control cells and cells expressing c-Cbl-N480. In Boyden chamber migration assays, the rate of cell migration of c-Cbl-N480 clone 11 cells was significantly slower than vector control cells (Fig. 4C). Cells with low levels of c-Cbl-N480 protein (clone 14) migrated to a level close to that of vector control cells.

The results shown in Fig. 5A showed a significant reduction in migration was observed following Cdc42-specific siRNA transfection, indicating that the activity of Cdc42 is involved in cell migration of MDA-MB-231 cells. By 72-hour post wounding, cell migration in cells transfected with Cdc42-specific siRNA had only resulted in 35% to 45% closure of the wound. In contrast, by 72-hour post wounding, the wound was ~95% closed in cells transfected with control nontargeting siRNA. Similar results were obtained when examining the effects of Cdc42-specific siRNA on cell migration using a Boyden chamber assay (Fig. 5B).

**Reducing Cdc42 protein levels or stably expressing c-Cbl-N480 in BT20 breast cancer cells decreases EGFR protein levels, cell proliferation, and motility.** We next determined if the above observations would generalize to another EGFR-overexpressing cell line. In BT20 cells, transfection with Cdc42-specific siRNA resulted in ~80% reduction in Cdc42 protein levels and 50% reduction in EGFR protein levels (Fig. 6A, inset, lane 2) compared with cells transfected with control nontargeting siRNA (Fig. 6A, inset, lane 1). There was no detectable activity of ERK in BT20 cells transfected with either control nontargeting siRNA or Cdc42-specific siRNA (data not shown). The rate of cell growth was significantly inhibited in BT20 cells transfected with Cdc42-specific siRNA compared with those transfected with control nontargeting siRNA (Fig. 6A).

c-Cbl-N480 overexpression in BT20 cells had similar effects on EGFR as found in MDA-MB-231 cells overexpressing c-Cbl-N480. Following EGF treatment, the protein levels of EGFR were rapidly decreased in cells expressing c-Cbl-N480 (Fig. 6B, third) compared with vector control cells where significant amounts of EGFR were detected over the 6-hour incubation with EGF (Fig. 6B, third). Figure 6C contains the protein expression of c-Cbl-N480 in BT20 cells. Similar to MDA-MB-231 cells, expression of c-Cbl-N480 in BT20 cells did not affect endogenous c-Cbl protein levels (data not shown). In BT20 cells, very little ERK activity was detected before the EGF treatment, which differed from MDA-MB-231 cells where ERK was constitutively activated (compare Fig. 6D, top, 0 minute, with Fig. 2C, top, 0 minute). Following EGF treatment, levels of activated ERK in vector control cells increased significantly and remained activated for 6 hours consistent with the slowed rate of EGFR degradation in BT20 cells. In cells overexpressing c-Cbl-N480, ERK was activated following EGF treatment. However, the extent of ERK activation was significantly lower than in vector control cells, and the levels of activated ERK were decreased at 6-hour post-treatment (Fig. 6D). Consistent with the effect of overexpressing c-Cbl-N480 on cell growth in MDA-MB-231 cells, the rate of BT20 cell growth in low serum (1% FBS) was inhibited in cells expressing c-Cbl-N480 (Fig. 6E).

Next, we tested whether c-Cbl-N480 overexpression in BT20 cells had similar effects on Rho GTPase activation as in MDA-MB-231 cells. BT20 cells expressing c-Cbl-N480 had increased levels of activated Cdc42 and Rac1 compared with vector control cells (Fig. 6F). Interestingly, levels of activated RhoA were below the level of detection for both vector control and c-Cbl-N480-overexpressing cells (data not shown). This was different from c-Cbl-N480-expressing MDA-MB-231 cells, which had increased RhoA activity compared with vector control cells (Fig. 3B). These data may explain why c-Cbl-N480-expressing BT20 cells lacked prominent stress fibers (data not shown). Cell migration as measured using Boyden chamber assays was also significantly inhibited in BT20 cells expressing c-Cbl-N480 compared with vector control cells (Fig. 6G). In wound-healing assays, BT20 vector control cells required 4 days for complete wound closure, whereas wounds in BT20 cells overexpressing c-Cbl-N480 remained incompletely closed at 4 days (data not shown).

![Figure 5](cancerresearch.org)
Discussion

Loss of c-Cbl-regulated EGFR degradation is an important mechanism contributing to EGFR overexpression in breast cancer. Deregulation of growth factor receptor tyrosine kinases, including EGFR, is linked to a large number of malignancies (35). In human breast cancer, EGFR is often overexpressed but rarely amplified at the gene level (3, 4). The ubiquitin ligase activity of c-Cbl is critical for the normal balance between EGFR signaling and degradation (5–8). Escape from Cbl-regulated down-regulation of EGFR is an important mechanism contributing to the over-expression and enhanced activity of receptor (35).

Here, we present data that suggest a novel mechanism contributing to the overexpression and enhanced activity of receptor (35). The effects of c-Cbl-N480 on MDA-MB-231 and BT20 cells share significant similarity with those found for Cdc42-specific siRNA, in that stable expression of c-Cbl-N480 enhances EGFR degradation, reduces EGFR-stimulated ERK activation, and inhibits cell proliferation. c-Cbl-N480 expression also decreases Cdc42 activity in both MDA-MB-231 and BT20 cells. Because Cdc42 is activated downstream of EGFR, reduced Cdc42 activity may be a consequence of increased EGFR degradation caused by expression of c-Cbl-N480, and in turn, decreased Cdc42 activity may allow for increased rates of EGFR degradation. These data strongly suggest that evasion of c-Cbl-regulated EGFR degradation due to elevated levels of active Cdc42 is a mechanism contributing to EGFR overexpression in MDA-MB-231 and BT20 cells.

EGFR signaling regulates cell migration through the activity of Rho family GTPases. EGFR signaling has been shown to activate Cdc42 as well as Rac1 (9, 28). Here, we show that, in MDA-MB-231 cells, Cdc42 and Rac1 activity is low (Fig. 3A and B). Previous work has shown that efficient motility of colon carcinoma cells requires low Rho and high Rac activities, which are both achieved through the activation of ERK signaling (34). Consistent with these observations, our results indicate the activity of Cdc42, Rac1, and RhoA in MDA-MB-231 cells is correlated with EGFR signaling. On the expression of c-Cbl-N480, increased EGFR down-regulation results in decreased ERK activation, decreased GTP-bound Cdc42 and GTP-bound Rac1, and increased GTP-bound RhoA (Fig. 3B). These changes in Rho...
family GTPase activities likely explain the significant morphologic changes of MDA-MB-231 cells from an invasive morphology (vector control) to a relatively normal epithelial cell shape (c-Cbl-N480 clone 11; Fig. 3A) and the altered cell migration (Fig. 4A-C). These findings are supported by evidence that the morphologic changes of MDA-MB-231 cells induced by c-Cbl-N480 (clone 11) can be rescued to that of vector control cells by overexpressing EGFR, constitutively active Cdc42 or Rac1 (Fig. 3C). Thus, in MDA-MB-231 cells, constitutively activated Cdc42 and Rac1 are likely the consequence of EGFR overexpression and constitutively activated ERK signaling.

MDA-MB-231 cells rarely display filopodia, although both Cdc42 and Rac1 are constitutively activated. This may be due to the amount of endogenous Rac1 protein being significantly higher than endogenous Cdc42 in MDA-MB-231 cells (data not shown), resulting in a dominant effect of active Rac1 on cells. Like in Swiss 3T3 fibroblasts, in which Cdc42 has been placed upstream of Rac1 (12), activation of Cdc42 may further activate Rac1 in breast cancer cells so that strong lamellipodium formation occurs. We also observed that silencing Cdc42 did not block the formation of lamellipodia in MDA-MB-231 cells (data not shown) but was required for cell migration as assessed by wound healing and Boyden chamber assays (Fig. 5A and B). This supports the idea that directional migration is dependent on Cdc42 due to its role in the regulation of cell polarity (15, 36).

The low levels of active RhoA are likely due to negative regulation of RhoA downstream of EGFR. One possible mechanism contributing to low RhoA activity may be through the ERK pathway. Up-regulated ERK signaling in MDA-MB-231 cells may stimulate the expression of Fra-1 leading to an inhibition of RhoA activity (34). Interestingly, RhoA activity is increased in c-Cbl-N480 clone 11 MDA-MB-231 cells. On the expression of c-Cbl-N480, ERK signaling is down-regulated, and this may in turn result in the activation of RhoA in MDA-MB-231 cells.

Overexpression of c-Cbl-N480 in BT20 cells similarly reduced levels of activated Cdc42 and Rac1 compared with vector control BT20 cells (Fig. 6F). However, unlike MDA-MB-231 cells, there is no detectable active RhoA in either c-Cbl-N480-expressing BT20 cells or control BT20 cells. This may explain why in BT20 cells c-Cbl-N480 does not alter the level of stress fibers observed (data not shown). This difference could be due to ERK not being constitutively active in BT20 cells despite EGFR overexpression.

Taken together, these data suggest a positive feedback loop between EGFR and Cdc42 contributes to the increased EGFR protein levels, signaling, cell proliferation, and migration observed in MDA-MB-231 and BT20 cells. The mechanism we propose here may also apply to other human cancer cells that overexpress EGFR. Disrupting this feedback loop, by preventing Cdc42 regulation of c-Cbl and restoring EGFR degradation, warrants further investigation as a potential therapeutic target in breast cancer treatment.

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Growth and Motility Inhibition of Breast Cancer Cells by Epidermal Growth Factor Receptor Degradation Is Correlated with Inactivation of Cdc42

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