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c-Cbl Regulates Glioma Invasion Through Matrix Metalloproteinase 2

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ABSTRACT

c-Cbl, a multifunctional adaptor and an E3 ubiquitin ligase, plays a role in such cytoskeleton-mediated events as cell adhesion and migration. Invasiveness of human glioma is dependent on cell adhesion, migration, and degradation of extracellular matrix (ECM). However, the function of c-Cbl in glioma invasion has never been investigated. We report here, for the first time, that c-Cbl plays a positive role in the invasion of ECM by SNB19 glioma cells. RNAi-mediated depletion of c-Cbl decreases SNB19 cell invasion and expression of matrix metalloproteinase 2 (MMP2). Consistent with these findings, SNB19 cells expressing wild-type, but not mutant c-Cbl show increased invasion and MMP2 expression. We demonstrate that the observed role of c-Cbl in invasion of SNB19 cells is not mediated by the previously shown effects of c-Cbl on cell adhesion and migration or on EGFR signaling. Together, our results suggest that c-Cbl promotes glioma invasion through up-regulation of MMP2. J. Cell. Biochem. 111: 1169–1178, 2010.

KEY WORDS: C-CBL; GLIOMA; INVASION; MMP2

The proto-oncogenic c-Cbl is a ubiquitously expressed mostly cytoplasmic protein, which functions as a multivalent adaptor and an E3 ubiquitin-protein ligase [reviewed in Rubin et al., 2005; Schmidt and Dikic, 2005; Thien and Langdon, 2005; Swaminathan and Tsygankov, 2006; Nau and Lipkowitz, 2008; Reddi et al., 2008; Tsygankov, 2008]. Its functional domains include a tyrosine kinase-binding (TKB) domain, a RING finger, a proline-rich region, a cluster of tyrosine phosphorylation sites and leucine zipper/ubiquitin-associated domain [reviewed in Rubin et al., 2005; Nau and Lipkowitz, 2008; Reddi et al., 2008; Tsygankov, 2008]. TKB binds to multiple protein tyrosine kinases (PTKs), a RING finger binds to an ubiquitin-conjugating enzyme E2 and is therefore required for E3 ubiquitin ligase activity of c-Cbl, a proline-rich region provides docking sites for SH3-containing proteins, the C-terminal tyrosine-phosphorylation sites bind to various SH2-containing proteins [Schmidt and Dikic, 2005; Swaminathan and Tsygankov, 2006; Nau and Lipkowitz, 2008; Tsygankov, 2008]. Several studies have shown that c-Cbl is involved in cytoskeletal events, such as cell spreading, adhesion, and migration. A mutant form of c-Cbl lacking the SH3-binding region alters morphology of fibroblasts by inhibiting the formation of actin lamellae, lamellipodia, and membrane ruffles [Scaife and Langdon, 2000]. Treatment of wild-type macrophages with c-Cbl-specific anti-sense oligonucleotides blocks their spreading on fibronectin (FN) [Meng and Lowell, 1998]. Furthermore, the knockout of c-Cbl gene leads to a decrease in migration for osteoclasts [Chiusaroli et al., 2003] and macrophages [Caveggion et al., 2003]. Previously, we also have shown that c-Cbl facilitates spreading, adhesion, and migration of Abl-transformed NIH 3T3 fibroblasts through the coordination of small GTPases including RhoA, Rac1, and Rap1 [Feshchenko et al., 1999; Teckchandani et al., 2001, 2005; Swaminathan et al., 2007; Lee et al., 2008].

Gliomas are the most common primary tumors of the central nervous system, accounting for 80% of adult primary brain tumors [Kleihues et al., 2002; Louis et al., 2007]. Gliomas are tumors of neuroepithelial tissue and are currently classified on the basis of morphological appearance: astrocytic, oligodendroglial, ependymal, and choroid plexus tumors. Astrocytomas, which are tumors composed predominantly of neoplastic astrocytes, amount to 80–85% of all gliomas [Kleihues et al., 2002; Louis et al., 2007]. World Health Organization grading is performed using a scale, from low

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(grade I) to high (grade IV), according to hallmarks of the tumor histological aberrations: nuclear atypia, mitotic activity, endothelial hyperplasia, and necrosis [Kleihues et al., 2002; Louis et al., 2007]. Grade IV astrocytomas [glioblastoma multiforme (GBM)] are very aggressive, invasive, destructive malignancies.

Despite aggressive treatment for GBM, which includes surgical resection followed by chemo- and radiation therapies, the median survival time remains <15 months. This is due to the active invasion of the adjacent healthy tissue by glioma cells, which precludes their complete removal by resection. The molecular mechanisms of glioma invasion are complex. First, invasive cells should detach from the original site and then adhere to extracellular matrix (ECM) proteins on the new site. This is followed by matrix degradation by glioma-produced proteases. Finally, glioma cells move through ECM. This process involves cell polarization, formation of membrane protrusions, including pseudopodia, lamellipodia, filopodia, and invadopodia, as well as cytoskeletal contraction [reviewed in Nakada et al., 2007; Stylli et al., 2008].

In spite of the involvement of c-Cbl in motility of various types of cells, including migration of transformed fibroblasts (see above) and in spite of the established role of c-Cbl as a regulator of epidermal growth factor receptor (EGFR) and other PTKs [Rubin et al., 2005; Thien and Langdon, 2005; Swaminathan and Tsygankov, 2006; Reddi et al., 2008], which affect proliferation, migration, invasion, and apoptosis of glioma cells [Lund-Johansen et al., 1990; Van Meter et al., 2004; Yamanaka et al., 2006; Rappl et al., 2008; Young et al., 2009], the role of c-Cbl in glioma invasion has never been investigated. The novel results reported here indicate that c-Cbl promotes glioma invasion through up-regulation of matrix metalloproteinase 2 (MMP2) essential for invasion of various tumors [Deryugina et al., 2001; Yeh et al., 2006], including glioma [Sawaya et al., 1996; Koul et al., 2001].

MATERIALS AND METHODS

CELLS

Human glioma cell line SNB19 was kindly provided by Dr. Marc Symons (Institute for Medical Research at North Shore-LIJ, Manhasset, NY). Human renal embryocarcinoma 293T cell was purchased from ATCC. Cells were grown in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (J R Scientific, Woodland, CA), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES buffer (Mediatech).

SIRNA TRANSFECTION

The siRNAs specific for mouse c-Cbl (ID: 60682, targeting to both human and mouse c-Cbl), human c-Cbl (ID: 121527 and ID: 121528), and the scrambled siRNA (*Silencer*® Negative Control #2 siRNA) were purchased from Ambion (Austin, TX). The siRNA transfections were performed using the X-tremeGENE siRNA transfection reagent (Roche Diagnostics, Mannheim, Germany). One day prior to transfection, cells were plated in 12-well plates in growth medium without antibiotics at a density 1×10^5/well and then grown overnight to achieve 30–50% confluency on the day of transfection. siRNA at a concentration of 100 nM in 50 μl of OPTI-MEM (Invitrogen, Carlsbad, CA) and transfection reagent (5 μl) in 50 μl of OPTI-MEM were mixed, incubated for 20 min at 25°C and then added to each well containing 500 μl of OPTI-MEM and 500 μl of growth medium without antibiotics. Transfection medium was replaced with growth medium 18 h after transfection.

PLASMIDS

A 1,463-bp fragment containing the human 3′-chromosomal flanking sequence of HIV-1 proviral vector was removed from the lentiviral vector pHR′-CMV-MCS [Hasham and Tsygankov, 2004] by cutting this vector with *Pst*I and then re-ligating it to reduce the vector size. To generate pCIG (pHR′-CMV-IRES-eGFP) transfer vector, IRES-EGFP was excised from pIRES2-EGFP (BD Bioscience, San Jose, CA) using *Hpa*I and *Sal*I and the excised fragment was inserted into shortened pHR'-CMV-MCS digested with *Xho*I and *Sal*I. Mutant forms of c-Cbl generated as described previously [Feshchenko et al., 1998] were excised from pAlterMAX (Promega, Madison, WI) and inserted into pCIG.

LENTIVIRAL TRANSDUCTION

293T cells were grown at 37°C in an incubator in the atmosphere containing 10% CO_2 for at least 1 week before transfection. 293T cells were plated in 100-mm Petri dishes in growth medium without antibiotics, cultured to achieve a density of ~90% confluency 24 h prior to transfection and split twofold. On a day of transfection, 13.5 μg of transfer vector (empty pCIG or a pCIG-based c-Cbl plasmid), 9 μg of packaging plasmid (pCMVΔ8.5), and 4.5 μg of envelope plasmid (pMD.G) were mixed in OPTI-MEM (Invitrogen) to yield a final volume of 1.5 ml. Both accessory plasmids have been kindly provided by Drs. Inder Verma and Didier Trono (Salk Institute) and are described in detail in Naldini et al. [1996]. Lipofectamine (Invitrogen) was diluted in OPTI-MEM (60 μl in 1.5 ml). Diluted DNA and Lipofectamine were vigorously mixed and incubated for 20 min at 25°C. The cells were washed with OPTI-MEM by swirling a plate, and growth medium was replaced with 5 ml of OPTI-MEM and DNA–Lipofectamine mixture was added to the cells. After 8-h incubation, transfection medium was replaced with 5 ml of fresh OPTI-MEM. At 24 h post-transfection, the culture supernatant was transferred to a 15-ml tube, and 5 ml of fresh OPTI-MEM was added to each plate. At 48 and 72 h post-transfection, supernatants were harvested as described above. All supernatants were centrifuged at 400*g* at 25°C for 5 min, filtered through a 0.45-μm filter, aliquotted and frozen at −70°C.

SNB19 cells were plated in 12-well or 6-well plates to achieve 40–60% confluency on the day of transduction. Polybrene (Sigma, stock solution: 10 mg/ml) was added in viral suspension to a final concentration of 10 μg/ml and the mixture of polybrene-virus was added to the plate. Viral suspension from the first harvest was used to transduce cells (100 or 300 μl/well in 12-well or 6-well plates, respectively). After overnight incubation, medium was replaced with regular growth medium. The EGFP expression was monitored and EGFP-positive cells were selected using fluorescence-activated cell sorter about 10 days after transduction.

MIGRATION ASSAY

Cell migration was studied in a 48-well microchemotaxis chamber (Neuroprobe, Gaithersburg, MD) as described previously [Teckchandani

et al., 2005]. PVP-free polycarbonate filters (VWR) with 8-μm pores were coated with human FN (10 μg/ml) in a shaker at 25°C overnight. Cells were washed three times with DMEM containing 0.2% fatty acid-free BSA and then added to the top wells (1×10^5 cells in 50 μl of the same medium). DMEM containing 0.2% calf serum (D0.2F), D10F (growth medium), epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ), or stromal cell-derived factor 1 (SDF1; Peprotech) was added to the bottom wells. After incubation for 16–18 h at 37°C, non-migrated cells on the top side of filter were scraped off and migrated cells on the bottom side of filter were fixed with methanol and stained with eosin/thiazine. Migrated cells were counted at a 20× magnification.

INVASION ASSAY

Invasion assay was performed using BD Biocoat™ Matrigel™ Invasion Chamber (BD Biosciences, Bedford, MA) following the manufacturer's protocol. Briefly, cells (1×10^5 cells in 500 μl of medium) were placed in the upper chamber and appropriate chemoattractants were placed in the lower chamber. After 22–24 h incubation at 37°C, the filter was fixed with 99% methanol (Fisher Scientific) for 10 min, rinsed with PBS, stained with 0.2% crystal violet (Fisher Scientific) for 10 min, and washed twice with water. Non-invading cells on the upper surface of the filter were then wiped off with a cotton-swab and washed with water twice. Invading cells on the bottom of the filter were counted using Olympus IX70 fluorescence microscope (Center Valley, PA).

WESTERN BLOTTING

Western blotting was performed using an Odyssey infrared imaging system (LI-COR, Lincoln, NE) following the manufacturer's protocol. In brief, cells were lysed in Western blotting lysis buffer [50 mM Tris–HCl (pH 8), 150 mM NaCl, 1% NP-40, 2 mM EDTA (pH 8), 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, aprotinin and leupeptin each at 10 μg/ml, 0.5% deoxycholic acid, and 0.1% SDS] for 30 min on ice. The lysates were clarified by centrifugation and protein concentrations were measured. Proteins were separated in SDS–polyacrylamide gel (8 or 10%) and transferred to nitrocellulose. Nitrocellulose was blocked in Odyssey blocking buffer (diluted twofold with PBS) for 1 h, and then incubated with a primary antibody followed by a secondary antibody. Both antibodies were diluted in Odyssey blocking buffer containing 0.1% Tween-20, and each incubation lasted 1 h. Proteins were visualized using an Odyssey scanner instrument, and the integrated intensity was normalized to the loading control.

GELATIN ZYMOGRAPHY

Gelatin (Sigma) was dissolved in distilled water at a concentration of 1%, autoclaved, cooled, and stored at 4°C. 10% SDS–polyacrylamide gel containing 0.1% of gelatin was polymerized and cell lysates were separated in this gel without prior boiling. The gel was washed in 2.5% Triton ×-100 in water for 1 h to remove residual SDS and then incubated in developing buffer [50 mM Tris–HCl (pH 7.5), 5 mM $CaCl_2$, 150 mM NaCl, 0.02% sodium azide] for 24 h at 37°C to promote activity of proteinases. The gel was stained with 0.5% Coomassie Brilliant Blue prepared in 30% ethanol, 10% acetic acid, and 1% formaldehyde overnight at 4°C. The gel was then destained with 40% methanol + 10% acetic acid twice for 10 min and then with 10% methanol + 10% acetic acid. The gel was visualized using an Odyssey scanner.

ANTIBODIES AND REAGENTS

The affinity-purified polyclonal antibodies (Ab) to c-Cbl, EGFR and the mouse monoclonal antibody (mAb) to c-Cbl were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAbs to EF1α, phosphotyrosine (4G10), neutralizing EGFR (LA1), and rabbit polyclonal Ab to EGFR were from Upstate Biotechnology (Charlottesville, VA). Mouse mAb to GAPDH was from Research Diagnosis (Flanders, NJ). Mouse mAbs to MMP2 and MMP9 were from Oncogene Research Products (Cambridge, MA). MMP15 (MT2-MMP) was from Lab Vision (Thermo Fisher Scientific, Fremont, CA). Mouse mAb to HA was from Roche Diagnostics.

The inhibitors of EGFR and MMPs: PD168393, GM-6001, and a specific MMP2 inhibitor I (*cis*-9-octadecenoyl-*N*-hydroxylamide) were from Calbiochem–Novabiochem, Corp. (La Jolla, CA). Another EGFR-specific inhibitor, AG 1478, was purchased from Tocris Cookson (Ellisville, MI). Human EGF and SDF1 were from Peprotech (Rocky Hill, NJ). AG 1478 and PD168393 were used at a concentration of 50 μM and 1–5 μM, respectively. Both GM6001 and MMP2 inhibitor I, were used at concentration of 25 μM.

STATISTICAL ANALYSES

The statistical analyses were performed using Prism® software (GraphPad Software, La Jolla, CA). Statistical analyses in three or more groups were performed using one-way analysis of variance (ANOVA) followed by the Bonferroni's *t*-test for multiple comparisons. Statistical differences between two groups were analyzed using unpaired two-side *t*-test.

RESULTS

ROLE OF C-CBL IN THE INVASION OF SNB19 GLIOMA CELLS

To evaluate the role of c-Cbl in glioma invasion, we depleted c-Cbl in the most aggressive invasive glioma cell line, SNB19, using c-Cbl-targeting siRNAs and then examined invasion of these cells using a transwell matrigel invasion assay. Transfection of two independent c-Cbl-targeting siRNAs, unlike that of scrambled siRNA used as negative control, dramatically reduced the level of endogenous c-Cbl (Fig. 1A). Invasion of SNB19 cells treated with c-Cbl-targeting siRNAs was significantly reduced as compared to that of scrambled siRNA-transfected cells (Fig. 1B,C).

We next characterized the role of functional domains of c-Cbl in its effect on glioma cell invasion. SNB19 stably expressing wild-type c-Cbl or its mutants, PTK binding-defective (G306E) and ubiquitylation-defective (C381A), were generated using lentiviral transduction. SNB19 cells transduced with an empty vector were generated as negative control. The HA-tagged wild-type and mutant c-Cbl were at least 20-fold overexpressed in comparison to endogenous c-Cbl in empty-vector control cells (Fig. 2A). Matrigel invasion of these cells was examined, and these results indicated that wild-type c-Cbl significantly increased the invasion of SNB19 cells, while mutants did not exert any effect (Fig. 2B,C). Thus, both independent approaches used to modulate the level of c-Cbl in

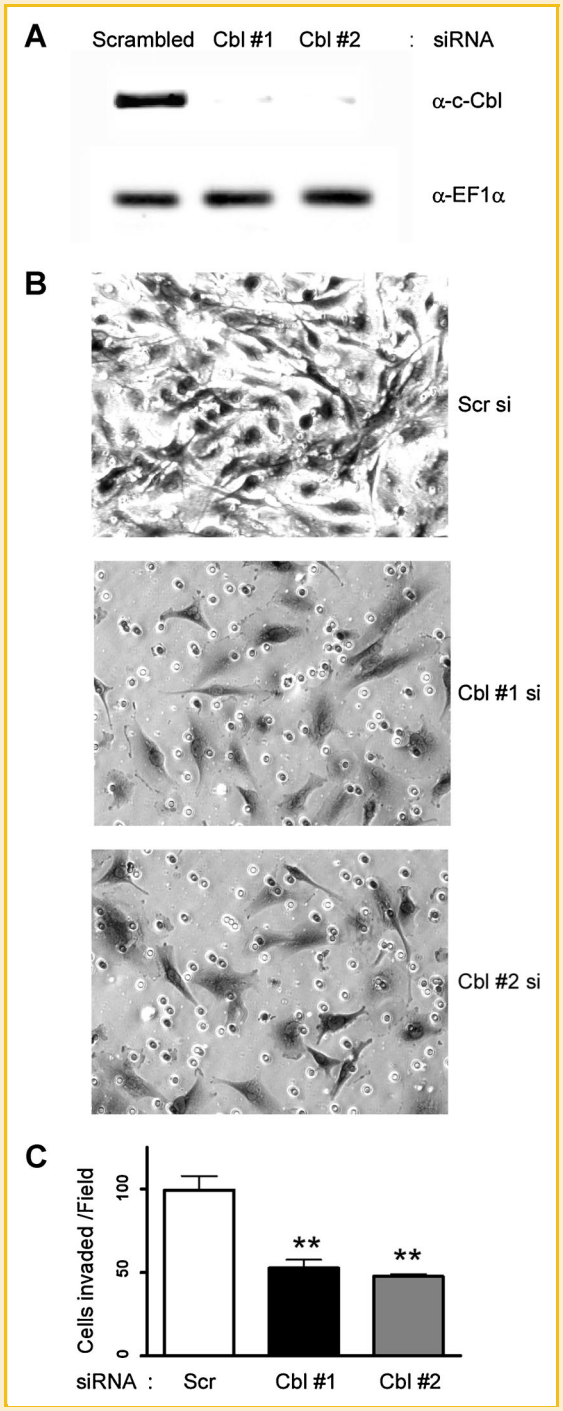


Fig. 1. Depletion of c-Cbl decreases invasion of SNB19 cells. SNB19 cells were transfected with siRNAs as indicated and harvested 72 h later. A: Cell lysates were immunoblotted with anti-EF1α (loading control) or anti-c-Cbl. SNB19 cells were examined for invasion as described in the Materials and Methods Section. B: Each phase-contrast image represents a randomly photographed field of invasion assays. C: The number of cells invaded per 20× microscopic field is plotted (mean ± SEM of six individual fields). Two asterisks (**) denote a significant difference ($P < 0.01$) between cells transfected with c-Cbl-targeting and scrambled siRNA. An independent representative experiment from a total of three is shown.

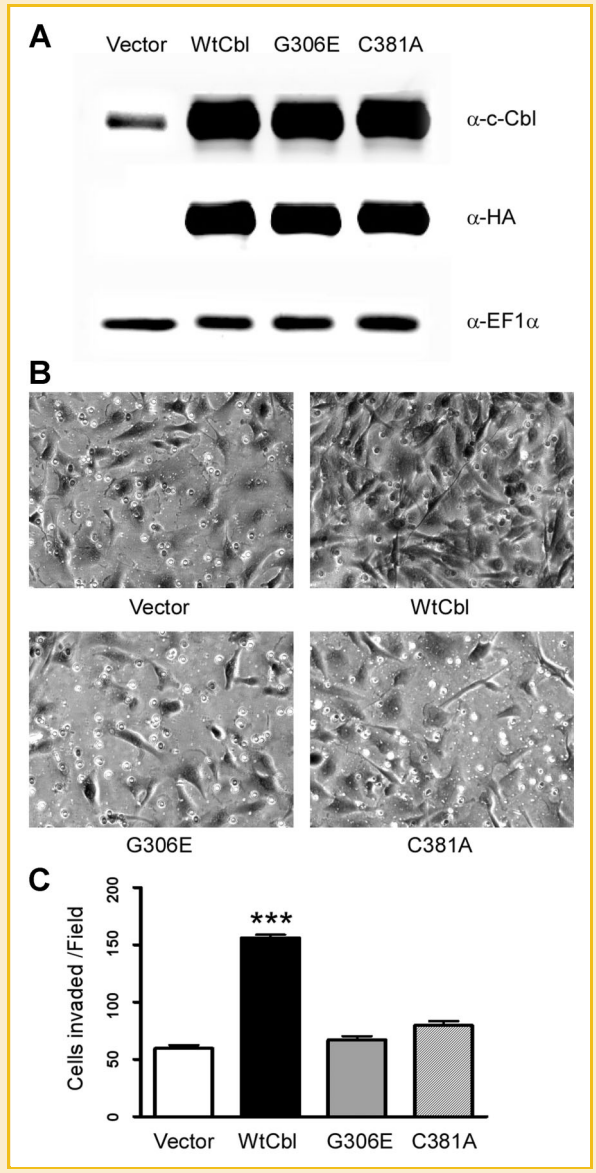


Fig. 2. Overexpression of wild-type c-Cbl increases invasion of SNB19 cells. SNB19 cells stably expressing wild-type c-Cbl, PTK binding-defective mutant (G306E), E2 binding-defective mutant (C381A), or transduced with an empty vector were examined as described in Figure 1. A: Cell lysates were immunoblotted with anti-EF1α (loading control), anti-HA, or anti c-Cbl. B: Randomly photographed fields of cell invasion assays. C: The number of cells invaded per 20× microscopic field is plotted (mean ± SEM of six individual fields). Three asterisks (***) denote a significant difference ($P < 0.001$) between cells overexpressing c-Cbl and vector control cells. No significant difference ($P > 0.05$) is seen between cells expressing mutant c-Cbl and vector control cells. An independent representative experiment from a total of five is shown.

SNB19 cells demonstrated that c-Cbl plays a positive role in regulation of SNB19 invasion. Furthermore, overexpression of c-Cbl mutants indicated that the E3 ubiquitin-protein ligase activity of c-Cbl is essential for its effect on glioma cell invasion.

Considering that invasion assays were carried out for 20–24 h, there was a possibility that an increase in invasion detected is, in fact, a result of differential proliferation of SNB19 cells expressing

different forms of c-Cbl. To address this issue, we examined proliferation of these cells and showed that it was not affected by overexpression of any form of c-Cbl studied (Fig. 3A), thus ruling out a possibility of a proliferation-related artifact.

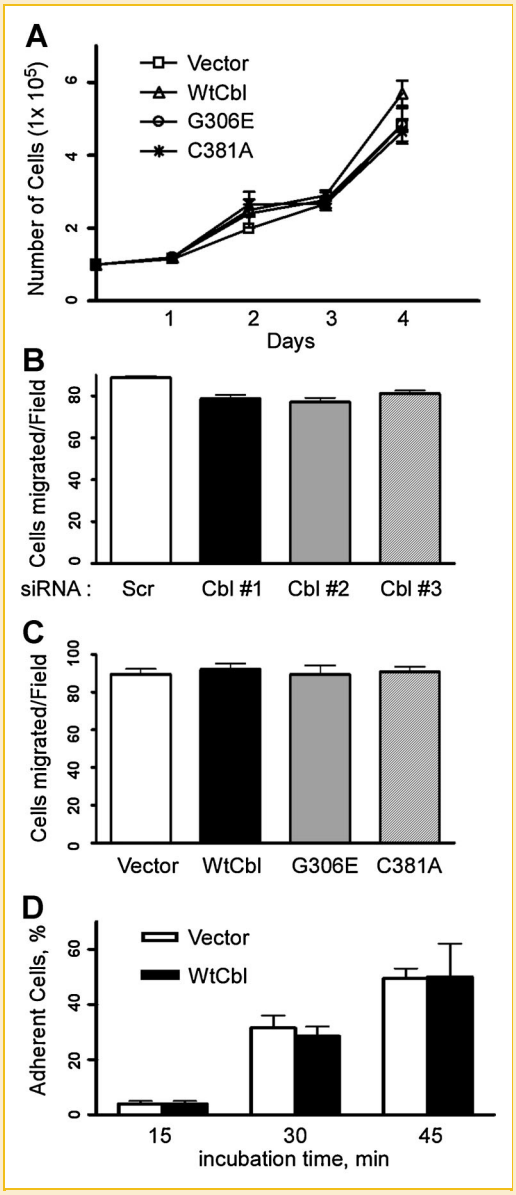


Fig. 3. Effects of c-Cbl on growth, migration, and adhesion of SNB19 cells. A: SNB19 cells stably expressing various forms of c-Cbl and transduced with an empty vector were examined for growth. Cells were plated in a six-well plate at a dose of 1×10^5/well in duplicates and counted as indicated. B: SNB19 cells were transfected with siRNAs as indicated, harvested 72 h later, and examined for migration as described in Materials and Methods Section. C: SNB19 cells stably expressing various forms of c-Cbl or empty vector were examined for migration. The number of cells migrated per 20× microscopic field is plotted (mean ± SEM of nine individual fields) in (B) and (C). D: SNB19 cells stably expressing wild-type c-Cbl or transduced with an empty vector were examined for adhesion. Cells were plated in a six-well plate at 1×10^6/well and incubated for the time indicated. Non-adherent cells were removed by washing cultures with PBS twice, and adherent cells were collected and counted. An independent representative experiment from a total of three is shown.

CONTRIBUTION OF CELL MIGRATION AND ADHESION TO THE C-CBL-DEPENDENT FACILITATION OF SNB19 INVASION

Glioma invasion is dependent on multiple biological processes including migration, adhesion, and degradation of ECM. Data published previously by us and other groups have shown that c-Cbl is critically involved in the regulation of cell migration (see Introduction). To determine whether c-Cbl plays a role in migration of SNB19 under conditions that did not require invasion, we modulated the level of c-Cbl in these cells using RNAi-mediated depletion (Fig. 3B) or overexpression (Fig. 3C) as described above. These experiments showed that neither depletion nor overexpression of c-Cbl had an effect on migration of SNB19 cells. To validate this finding, we examined SNB19 migration in response to other chemoattractants of these cells: EGF [Lund-Johansen et al., 1990] and the SDF1 chemokine [Bajetto et al., 2006]. While both EGF and SDF1 promoted cell migration in our experiments, neither form of c-Cbl modified migration induced by these stimuli (data not shown).

Since cell invasion involves transient adhesive interaction with ECM and since our previous studies showed that c-Cbl facilitates adhesion of Abl-transformed fibroblasts to ECM [Feshchenko et al., 1999; Teckchandani et al., 2001], we next analyzed the effect of c-Cbl on SNB19 cell adhesion. Cells overexpressing wild-type c-Cbl showed no significant change in the adhesive behavior as compared to vector control cells (Fig. 3D). These findings indicated that the invasion of SNB19 cells enhanced by c-Cbl does not result from an increase in their adhesion or migration.

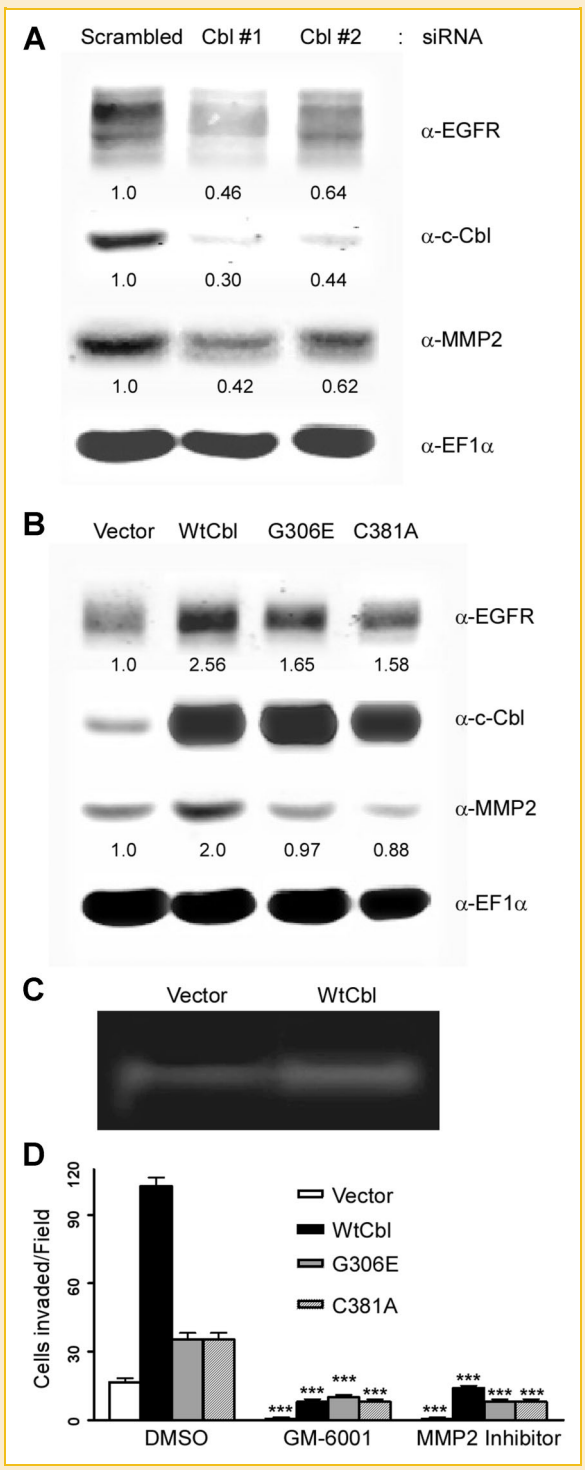
ROLE OF MMP2 IN THE C-CBL-DEPENDENT FACILITATION OF SNB19 INVASION

Considering that c-Cbl increases invasion of SNB19 cells, but not their migration or adhesion, we next considered a possibility that c-Cbl plays its role by promoting degradation of ECM. Many studies have shown a strong correlation between invasiveness of human gliomas and degradation of ECM by MMPs [reviewed in Rao, 2003; Martin and Matrisian, 2007]. Therefore, we examined the effect of c-Cbl on MMP2 production by SNB19 cells. Depletion of c-Cbl greatly decreased the level of MMP2 as compared to that in scrambled siRNA-transfected cells (Fig. 4A). Consistent with this result, overexpression of wild-type c-Cbl increased the level of MMP2 (Fig. 4B). Therefore, the effects of c-Cbl on invasion and MMP2 production were consistent with the notion that c-Cbl facilitates SNB19 invasion by upregulating MMP2. Furthermore, mutant forms of c-Cbl incapable of facilitating invasion did not increase the level of MMP2 (Fig. 4B). These results indicated that the effects of c-Cbl on invasion and MMP2 production were dependent on its E3 activity, thus further arguing that c-Cbl plays a role in invasion by regulating MMP2.

To further evaluate the role of c-Cbl in MMP2 regulation, we performed gelatin zymography with wild-type c-Cbl-overexpressing and vector control SNB19 cells (Fig. 4C). Consistent with the experiments based on MMP2 immunodetection, c-Cbl overexpression increased the enzymatic activity of MMP2 in SNB19 lysate. Thus, these results indicated that c-Cbl upregulates both protein level and overall activity of MMP2. We also tested other MMPs including MMP9, one of most characterized MMPs involved in ECM degradation, and MT2-MMP (MMP15), which increases in glioma

invasion in response to SDF1 [Zhang et al., 2005], but their expression was not changed by c-Cbl (data not shown).

To determine the role of MMPs in c-Cbl-facilitated invasion of SNB19 cells, we analyzed the effect of MMP inhibitors using matrigel invasion assays with SNB19 cells stably overexpressing various forms of c-Cbl and vector control cells. Both the broad-spectrum and the MMP2-specific inhibitors, dramatically reduced invasion of SNB19 cells, blocking or at least diminishing the facilitating effect of c-Cbl overexpression. Thus, no significant difference in invasion was seen between cells expressing wild-type c-Cbl and those expressing mutant c-Cbl in the presence of GM-6001. Although this difference remained significant upon treatment with an MMP2-specific inhibitor, the ratio between invasion abilities of cells expressing wild-type and mutant c-Cbl decreased from ~2.8 in control to ~1.7 in inhibitor-treated cultures (Fig. 4D). Therefore, MMP2 activity is essential for a c-Cbl-dependent increase in the invasion of SNB19 cells.



ROLE OF EGFR SIGNALING IN THE C-CBL-DEPENDENT FACILITATION OF SNB19 INVASION

Since EGFR signaling facilitates glioma invasion and upregulates MT1-MMP in glioma cell lines [Van Meter et al., 2004] and MMP2 in other cancers [Yeh et al., 2006] and since c-Cbl is a known regulator of EGFR, we were prompted to determine whether or not the function of c-Cbl in SNB19 invasion involves EGFR. First of all, we characterized the effect of EGF on migration and invasion of SNB19 cells overexpressing various forms of c-Cbl. These experiments indicated that EGF is a chemoattractant comparable to serum, a well-established chemoattractant of SNB19 cells [Lund-Johansen et al., 1990]. Responses of SNB19 to serum and EGF depend on c-Cbl expression in a similar fashion; overexpression of neither wild-type nor mutant c-Cbl affected their migration under conditions not requiring invasion for motility (data not shown), whereas only wild-type, but not mutated c-Cbl dramatically upregulated invasion of SNB19 cells towards both serum and EGF (Fig. 5A).

We then analyzed the effect of c-Cbl on a steady-state level of EGFR in SNB19 cells. Depletion of endogenous c-Cbl decreased, while overexpression of wild-type c-Cbl increased it (Fig. 4A,B). This effect was unexpected, since c-Cbl is widely thought to downregulate EGFR, and therefore, we examined the effect of c-Cbl on EGFR in SNB19 cells using a short-term time course of EGFR degradation in response to EGF as done in most studies of the c-Cbl-dependent downregulation of EGFR. As expected, c-Cbl

Fig. 4. Effects of c-Cbl on MMP2 level in SNB19 cells. SNB19 cells (A) transfected with siRNAs as indicated or (B) stably expressing various forms of c-Cbl were analyzed as described in Figures 1A and 2A. Bands were quantified and the relative levels of proteins are shown at the bottom of each band. C: SNB19 cells stably expressing wild type c-Cbl or transduced with an empty vector were starved in DMEM for 24 h and lysed. Their lysates were used to perform gelatin zymography. D: SNB19 cells stably expressing various forms of c-Cbl as indicated were examined for invasion in the presence of GM6001, a pan-MMP inhibitor, or the MMP2-specific inhibitor I, or DMSO (vehicle control). Invasion was analyzed as described in Figure 1. Three asterisks (***) denote a significant difference ($P < 0.001$) for each indicated cell type between control and MMP inhibitor-treated culture. Furthermore, Bonferroni's multiple comparison tests show the following a significant difference among cells expressing various forms of c-Cbl within the inhibitors-treated culture. In GM6001, WtCbl, G306E, C381A versus vector: $^{**}P < 0.01$; G306E, C381A versus WtCbl; G306E versus C381A: $P > 0.05$, no significant difference (NS). In the MMP2-specific inhibitor I, WtCbl versus vector: $^{***}P < 0.001$; G306E, C381A versus vector: $^{**}P < 0.01$; G306E, C381A versus WtCbl: $^{*}P < 0.05$; G306E versus C381A: $P > 0.05$ (NS). An independent representative experiment from a total of three is shown.

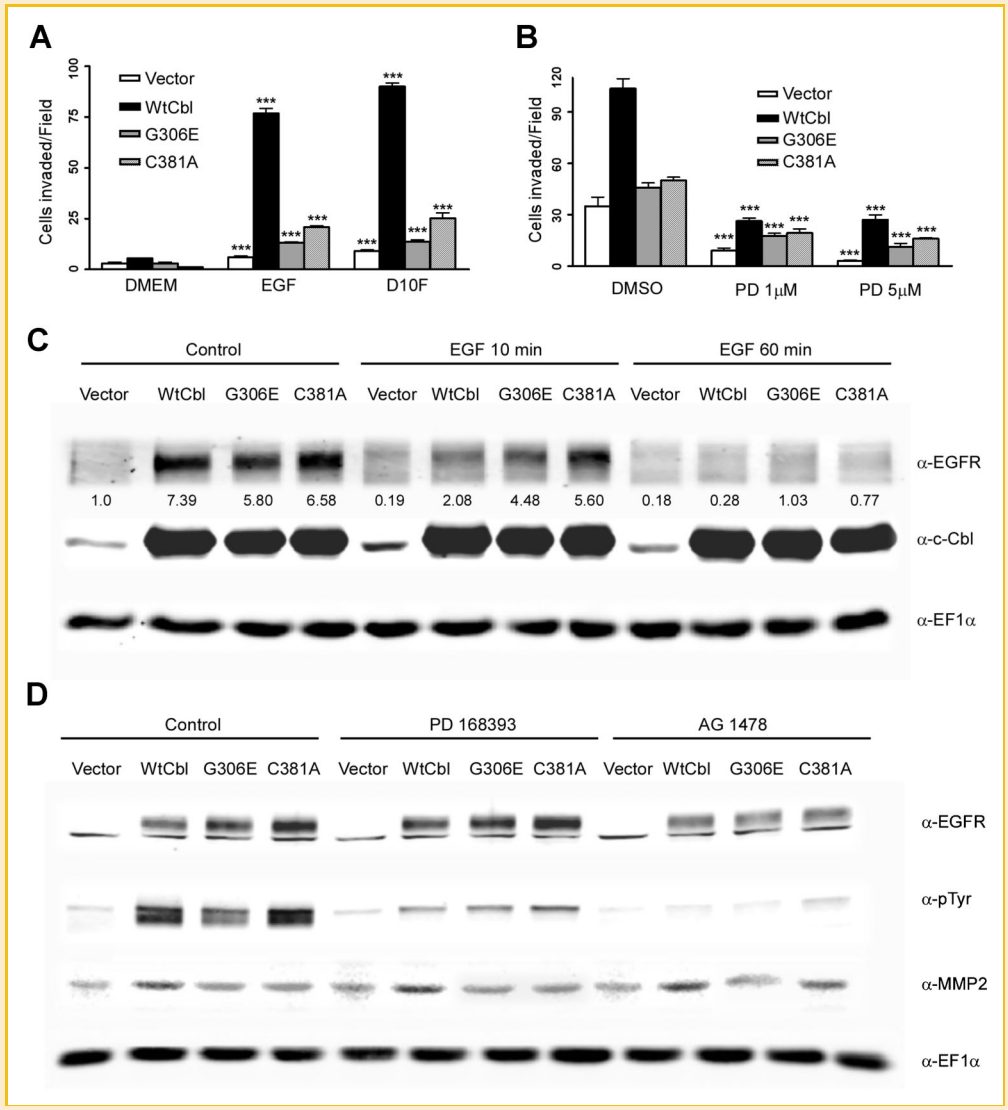


Fig. 5. Role of EGFR signaling in the effects of c-Cbl on SNB19 cells. A: SNB19 cells stably expressing various forms of c-Cbl were examined for invasion in response to the chemoattractants indicated as described in Figure 1. Invasion was analyzed as described in Figure 1. Three asterisks (***) denote a significant difference ($P < 0.001$) for each indicated cell type between control and EGF- or D10F-treated culture. B: SNB19 cells stably expressing various forms of c-Cbl were examined for invasion in response to serum in the presence of inhibitors, as indicated. Invasion was analyzed as described in Figure 4D. Three asterisks (***) denote a significant difference ($P < 0.001$) for each indicated cell type between control and EGFR inhibitor-treated culture. As described in Figure 4D, the significant difference is shown among cells expressing various forms of c-Cbl within the inhibitors-treated culture. In PD168393 1 μM, vector, G306E, C381A versus WtCbl: $^{***}P < 0.001$; C381A versus vector: $^{**}P < 0.01$; G306E versus vector: $^{*}P < 0.05$; G306E versus C381A: $P > 0.05$ (NS). In PD 168393 5 μM, WtCbl, G306E versus vector and C381A versus WtCbl: $^{***}P < 0.001$; vector versus C381A and WtCbl versus G306E: $^{**}P < 0.01$; G306E versus C381A: $P > 0.05$ (NS). C: SNB19 cells stably expressing various forms of c-Cbl were starved in DMEM for 24 h and stimulated with 100 ng/ml of EGF as indicated. Cell lysates were immunoblotted with the antibodies indicated. Bands were quantified and the relative levels of EGFR are shown at the bottom of each band. D: SNB19 cells stably expressing various forms of c-Cbl were incubated with EGFR inhibitors or DMSO (as vehicle control) as indicated and stimulated with 100 ng/ml of EGF for 3 min. Cell lysates were immunoblotted with the indicated antibodies. An independent representative experiment from a total of three is shown.

downregulated EGFR in SNB19 cells under these conditions (Fig. 5C). Therefore, the positive effect of wild-type c-Cbl on the steady-state level of EGFR is not caused directly by c-Cbl-dependent EGFR degradation, but is due, most likely, to a complex feedback loop mediated by the effect of c-Cbl on EGFR under steady-state quiescent conditions in cell culture. The notion that these effects of c-Cbl on EGFR are not mediated by the same mechanisms is further supported by the finding that the well-characterized c-Cbl-mediated downregulation of EGFR is dependent on the E3 activity of c-Cbl (see Introduction) as also seen in Figure 5C (wild-type c-Cbl is clearly more potent in down regulating EGFR than c-Cbl mutants at 10 min), whereas an increase in the steady-state level of EGFR is seen in cells expressing not only wild-type, but also mutant c-Cbl (Figs. 4B and 5C,D).

To examine the role of EGFR in SNB19 invasion, we applied specific EGFR inhibitors in matrigel assays and demonstrated that these inhibitors substantially decreased invasion of SNB19 cells overexpressing any form of c-Cbl studied (Fig. 5B and data not

shown). Notably, even in the cells, in which EGFR was inhibited (Fig. 5D), causing a dramatic decrease in invasion ability, the facilitating effect of c-Cbl was to a significant extent preserved (Fig. 5B); in spite of the robust effect of EGFR inhibitors on cell invasion in our system, comparable to that of MMP inhibitors (see above), the difference between invasion of cells expressing wild-type c-Cbl and those expressing mutant c-Cbl was consistently maintained in EGFR-inhibited cultures at the level seen in control cultures (~2-fold) and remained statistically significant (Fig. 5B). Taken together, these results argue that although EGFR signaling is key in SNB19 invasion, changes in the steady-state level of EGFR in SNB19 cells expressing c-Cbl do not seem to mediate the facilitating effect of wild-type c-Cbl on SNB19 invasion.

Considering that our results indicated that c-Cbl is likely to facilitate SNB19 invasion by upregulating MMP2, we further analyzed a link between EGFR and MMP2 in the context of the role played by c-Cbl in this invasion by determining the effect of EGFR inhibitors on MMP2 production. These experiments demonstrated that EGFR inhibitors, while reducing invasion of SNB19 cells, exert no significant effect on the level of MMP2 (Fig. 5D). These findings together with the lack of correlation between the effects of c-Cbl on steady-state level of EGFR, on the one hand (increased by wild-type and mutant c-Cbl), MMP2 levels and invasion, on the other (increased by wild-type, but not mutant c-Cbl), argue that c-Cbl facilitates SNB19 invasion primarily through its effect on MMP2.

DISCUSSION

The results reported in this study indicate, for the first time, that c-Cbl plays a positive role in the invasion of ECM by SNB19 glioma cells (Figs. 1 and 2). To evaluate the potential biological significance of the contribution of c-Cbl to glioma cell invasion, the effect of c-Cbl depletion can be compared to those for proteins that are thought to play an important role in glioma invasion, focusing on studies conducted in SNB19 cells. The magnitude of the effect of c-Cbl depletion (~2-fold) is comparable to those shown for the depletion of DDR1 kinase [Yamanaka et al., 2006], pharmacological inhibition of p38/JNK signaling [Lin et al., 2008], ADP-ribosylation factor 6 depletion [Hu et al., 2009], Trio, Ect2, or Vav3 GTP/GDP-exchange factor depletion [Salhia et al., 2008], antibody-mediated neutralization of the matricellular protein CCN1/Cyr61 and urokinase plasminogen activator [Young et al., 2009]. Based on this comparison, the role of c-Cbl in glioma cell invasion should be considered significant.

Because, we have previously shown that c-Cbl facilitates adhesion, spreading, and migration of transformed fibroblasts [Feshchenko et al., 1999; Teckchandani et al., 2001, 2005; Swaminathan et al., 2007; Lee et al., 2008] and because adhesion and motility are critical elements of the invasion process [Nakada et al., 2007; Stylli et al., 2008], it appeared reasonable to evaluate the role of c-Cbl in adhesion and migration of SNB19 cells. However, this analysis indicated that c-Cbl does not play a role in these processes when SNB19 cells are not invading ECM, thus suggesting that facilitation of glioma invasion by c-Cbl is mediated by an event specifically regulating invasion, such as ECM degradation.

Considering that MMPs play a key role in the ECM degradation associated with cancer cell invasion, metastasis, and angiogenesis [reviewed in Rao, 2003; Martin and Matrisian, 2007], thus representing a group of possible mediators of the observed effect of c-Cbl, we analyzed how depletion and overexpression of c-Cbl affect production of MMPs in SNB19 cells. Among human MMPs, MMP2, and MMP9 are most characterized. Hence, we focused on analyzing their role in the c-Cbl-dependent facilitation of SNB19 invasion. Expression and activity of MMP2 is upregulated by c-Cbl, while expression of MMP9 remains unchanged (Fig. 4 and data not shown). Furthermore, experiments utilizing specific MMP inhibitors have indicated that MMP2 is crucial for c-Cbl-facilitated invasion in SNB19 cells (Fig. 4). Therefore, although not only MMP2 is involved in cell invasion and although identifying other proteases that degrade ECM for c-Cbl-enhanced SNB19 invasion is necessary for further elucidating the mechanism of this invasion, it is clear that upregulation of MMP2 by c-Cbl is key for facilitation of this process. Taken together, our results strongly argue that c-Cbl plays a positive role in regulating SNB19 glioma cell invasion acting through upregulation of MMP2.

It has previously been shown that both adaptor functions and E3 ubiquitin-protein ligase activity of c-Cbl are involved in the regulatory role of c-Cbl in cytoskeleton-mediated events, such as cell adhesion and motility (see Introduction Section). Therefore, both possibilities have initially been considered in our system. However, the lack of detectable tyrosine phosphorylation of either endogenous or overexpressed c-Cbl under conditions inducing robust invasion of SNB19 cells as well as the failure of pharmacological inhibitors of PI3K, a key mediator of cytoskeletal effects of c-Cbl mediated by its tyrosine phosphorylation [Feshchenko et al., 1999], to reduce SNB19 invasion ruled out a crucial role of adaptor-type interactions in the observed effect of c-Cbl in SNB19 cells (data not shown). In contrast, mutations disrupting the ability of c-Cbl to ubiquitylate TKB-binding substrates severely impaired the c-Cbl-dependent facilitation of SNB19 invasion (Fig. 2). It is important that the positive effect of c-Cbl on both invasion and MMP2 production was blocked by these mutations (Figs. 2 and 4), further arguing that the observed increased in invasion is due to the c-Cbl-dependent upregulation of MMP2.

The essential role of E3 activity and TKB binding in the effect that c-Cbl exerts on invasion and MMP2 production in SNB19 indicates that c-Cbl acts in this process by ubiquitylating a TKB-interacting substrate. However, the identity of this substrate remains uncertain. EGFR is one of the best-characterized substrates of c-Cbl, which is downregulated by c-Cbl in many biological systems [reviewed in Rubin et al., 2005]. In agreement with these data, c-Cbl promotes degradation of EGFR immediately following treatment with EGF (Fig. 5C). Interestingly, the effect of c-Cbl on the steady-state level of EGFR in SNB19 cells (Fig. 4B) appears to be unrelated to the ability of c-Cbl to ubiquitylate and downregulate EGFR (Fig. 5C). Although the reason for this discrepancy is unclear, it appears that the observed effect of c-Cbl on the steady-state level of EGFR is not caused directly by c-Cbl-dependent ubiquitylation of EGFR, but is

mediated through a mechanism involving regulation of gene expression. This may be a feedback loop upregulated to compensate for interference of c-Cbl in intracellular homeostasis.

Although EGFR contributes significantly to SNB19 invasion (Fig. 5A,B), as expected, our results do not support the notion of EGFR being a key mediator of the facilitating role of c-Cbl in SNB19 invasion, because pharmacologic inhibition of EGFR, while suppressing SNB19 invasion (Fig. 5B), does not exert a significant effect on MMP2 (Fig. 5D). Furthermore, the effects of c-Cbl mutants on EGFR, on the one hand, and on MMP2 and invasion, on the other, differ; both wild-type and mutant increase steady-state level of EGFR, although to a different extent, while only wild-type c-Cbl, but not mutant c-Cbl, increases MMP2 levels and invasion (Figs. 2C and 4B).

It appears that c-Cbl exerts its effect on invasion through ubiquitylating, possibly causing degradation of a protein inhibiting a signaling pathway that is critical for MMP2 production yet independent of EGFR. Alternatively, c-Cbl may activate a positive regulator of MMP2 production by inducing its ubiquitylation without degradation. Indeed, degradation-independent regulation of activity and interactions by ubiqutylation has been shown for several proteins [Shao et al., 2003; Zhang et al., 2003; Suetsugu et al., 2004; Cestra et al., 2005]. A definitive answer to this question will require the identification of ubiquitylation substrates of c-Cbl in SNB19 cells.

Considering the key role of EGFR-mediated signaling in glioma invasion [reviewed in Huang et al., 2009], attempts have been made to use anti-EGFR therapy for the treatment of glioma [Mellinghoff et al., 2005; Scott et al., 2007; van den Bent et al., 2009]. However, glioma is frequently resistant to anti-EGFR therapy [Li et al., 2003; Bianco et al., 2005; Mellinghoff et al., 2007]. Our results indicate that c-Cbl may play an important role in promoting glioma invasion. Therefore, therapies targeting c-Cbl, such as RNAi and, possibly, pharmaceuticals that disrupt functional interactions between c-Cbl and the relevant ubiquitylation substrate(s) may improve therapeutic modalities in treatment of glioma.

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