c-Cbl-Dependent EphA2 Protein Degradation Is Induced by Ligand Binding

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Abstract
The EphA2 receptor protein tyrosine kinase is overexpressed and functionally altered in a large number of human carcinomas. Despite its elevated levels in cancer, the EphA2 on the surface of malignant cells demonstrates lower levels of ligand binding and tyrosine phosphorylation than the EphA2 on non-transformed epithelial cells. In our present study, we demonstrate that ligand-mediated stimulation causes EphA2 to be internalized and degraded. The mechanism of this response involves ligand-mediated autophosphorylation of EphA2, which promotes an association between EphA2 and the c-Cbl adaptor protein. We also show that c-Cbl promotes stimulation-dependent EphA2 degradation. These findings are important for understanding the causes of EphA2 overexpression in malignant cells and provide a foundation for investigating EphA2 as a potential target for therapeutic intervention.

Introduction
Malignant cells often demonstrate elevated levels of intracellular signaling by tyrosine kinases (1–3). Much recent interest has focused on receptor tyrosine kinases, which control many aspects of tumorigenesis (4–7) because receptor tyrosine kinases provide much-needed targets for therapeutic intervention (8–10). For example, antibody targeting of the HER2 receptor tyrosine kinase can treat certain breast cancers (11–13). In part, HER2 antibodies function by promoting the binding of adaptor molecules such as c-Cbl to HER2, and facilitating clearance of HER2 protein by degradation (14). However, HER2 is overexpressed on a narrow range of human cancers (15) and there remains a need to identify other molecules that might be similarly targeted.

EphA2 is a transmembrane receptor tyrosine kinase that is found at low levels on adult epithelial cells (16). EphA2 is one member of the Eph family of receptor tyrosine kinases, which are unique in that they recognize ligands, known as ephrins, which are anchored to the membrane of adjacent cells (17–19). However, the cellular functions of EphA2 in epithelial cells remain largely unknown (20–22).

EphA2 is frequently overexpressed in a large number of cancer cells (20, 23–27). Elevated EphA2 protein levels are observed in many different types of cancers, and the highest levels of EphA2 protein are consistently found on the most aggressive tumor cells (24, 27). For example, EphA2 protein levels are elevated in breast (25), prostate (24), and colon (20) carcinomas, as well as in aggressive melanomas (28). Furthermore, ectopic overexpression of EphA2 is sufficient to confer tumorigenic and metastatic potential upon non-transformed epithelial cells (27). Together, these findings implicate EphA2 as a powerful oncogene in metastatic cancer.

Despite its overexpression, the EphA2 on malignant cells exhibits lower levels of ligand binding than the EphA2 on non-transformed epithelial cells (25). This deficiency arises because malignant cells generally have unstable cell-cell contacts (29–31), which prevent EphA2 from productively interacting with its membrane-anchored ligands on neighboring cells (25, 27). In particular, EphA2-ligand binding is highly sensitive to the proper expression and function of the E-cadherin adhesion system (25). Due to decreased EphA2-ligand binding, the EphA2 on malignant cells has lower levels of tyrosine phosphorylation than the EphA2 in non-transformed epithelial cells that does bind ligand (25, 27). However, unlike other receptor tyrosine kinases, ligand binding is not necessary for EphA2 enzymatic activity (25). Instead, EphA2 ligands regulate EphA2 subcellular localization and its interactions with downstream adaptor and signaling proteins (25, 32, 33). Decreased levels of EphA2-ligand binding in cancer cells can be overcome in vitro by using either monoclonal antibodies or soluble forms of ligand, neither of which require stable cell-cell contacts to stimulate EphA2 (25, 32, 33). These strategies have been used to demonstrate that EphA2 ligands negatively regulate tumor cell growth and invasiveness (25, 32). Of particular note, ligand binding reverses the oncogenic effects of EphA2 overexpression (27).

Recent studies in our laboratory have sought to determine how EphA2 ligands reverse the oncogenic effects of EphA2 overexpression. In the present study, we demonstrate that ligand binding causes EphA2 to be internalized and degraded. We also study the mechanisms responsible for ligand-mediated EphA2 degradation and find that EphA2 interacts with the c-Cbl adaptor protein, which promotes EphA2 degradation.

Results
Stimulation Induces EphA2 Protein Degradation
EphrinA1-Fc is a soluble fusion protein in which the extracellular domain of the EphA2 ligand, Ephrin-A1, has been linked to the human immunoglobulin Fc region (33). To study the biochemical consequences of ligand stimulation of EphA2,
we treated MDA-MB-231 cultured breast carcinoma cells with 0–2 μg/ml EphrinA1-Fc. The samples were extracted and cell lysates were resolved by SDS-PAGE before Western blot analyses with EphA2-specific antibodies (D7). EphA2 immunoreactivity decreased by at least 80% in response to EphrinA1-Fc stimulation, and in a dose-dependent manner (Fig. 1A). When MDA-MB-231 cells were subsequently treated with 1 μg/ml EphrinA1-Fc for 0–24 h, EphA2 immunoreactivity began to decrease within 1 h. This decrease in EphA2 was durable, as low levels of EphA2 persisted for at least 24 h (data not shown).

Ligand binding similarly decreased EphA2 immunoreactivity in other cell models. Multiple cell systems in which EphA2 has low levels of endogenous ligand binding, as indicated by low levels of EphA2 tyrosine phosphorylation, were utilized. These models included PC-3, MDA-MB-435, MCFEphA2 (27), and EphA2-transfected LNCaP and NIH-3T3 cells. In each case, EphrinA1-Fc decreased EphA2 immunoreactivity in a dose- and time-dependent manner that was analogous to our results with MDA-MB-231 cells (Fig. 1B and data not shown).

The decreased EphA2 immunoreactivity did not reflect a generalized decrease in cellular proteins, because the levels of other proteins, including β-catenin, vinculin, or paxillin, were not changed in response to EphrinA1-Fc (Fig. 1A and B and data not shown). Thus, these proteins were used throughout this study as loading controls. These findings suggested that EphA2-ligand binding decreased EphA2 immunoreactivity in multiple cell model systems. In our subsequent studies that focused on the molecular mechanism of ligand-induced EphA2 protein degradation, we therefore primarily used MDA-MB-231 cells, which are a model of invasive human breast carcinomas, and PC-3 cells, which are a model for invasive human prostate carcinomas.

We next asked whether the decreased EphA2 immunoreactivity detected in cell lysates of ligand-stimulated MDA-MB-231 cells reflected a decrease in cell-surface associated EphA2. Cell-surface proteins were biotinylated and the cells were left untreated or stimulated with EphrinA1-Fc. Biotinylated proteins were immunoprecipitated with immobilized NeutrAvidin and were analyzed by Western blot analyses with EphA2-specific antibodies. Stimulation with EphrinA1-Fc led to a marked decrease in cell-surface associated EphA2 protein levels (Fig. 1C).

The EphA ligand used in these studies, EphrinA1, can stimulate EphA receptor tyrosine kinases other than EphA2 (19, 34). Therefore, we next asked whether decreased EphA2 protein levels following EphrinA1-Fc stimulation of cells was caused by EphA2 stimulation exclusively or indirectly through the stimulation of other EphA kinases. MDA-MB-231 cells were treated with B2D6, a monoclonal antibody that specifically recognizes an epitope on the extracellular domain of EphA2, and a secondary antibody (rabbit anti-mouse IgG). Western blot analyses of these cell lysates demonstrated that EphA2 cross-linking by B2D6 reduced EphA2 immunoreactivity (Fig. 2A). In contrast, neither the primary nor secondary antibodies alone changed EphA2 immunoreactivity. The membranes were stripped and reprobed with antibodies that recognize vinculin or β-catenin to confirm equal sample loading. Identical results were obtained in response to antibody-mediated aggregation of EphA2 on other cells systems, including PC-3 and MCFEphA2 cells (Fig. 2B and data not shown). Because EphrinA1-Fc and B2D6 antibody aggregation provided consistent results, these two stimuli were used interchangeably for the experiments described below.

We then sought to determine the mechanism for decreased EphA2 immunoreactivity in response to stimulation. One possibility was that loss of EphA2 immunoreactivity could have resulted from loss of a particular antibody epitope. However, lower levels of EphA2 were detected using multiple EphA2 antibodies, each of which recognizes a different epitope (data not shown). An alternative was that EphA2 stimulation induces EphA2 protein degradation. To test this idea, EphA2 protein stability was measured by pulse-chase analyses with or without stimulation by B2D6 cross-linking. EphA2 was metabolically labeled by incubating MDA-MB-231 cells in

![FIGURE 1.](image)

*Ligand activation decreases EphA2 immunoreactivity in a dose- and time-dependent manner. MDA-MB-231 breast cancer cells were incubated with 0–2 μg/ml EphrinA1-Fc at 37°C for 1 h. A. Equal amounts of cell lysates were resolved by SDS-PAGE and subjected to anti-EphA2 Western blot analysis (D7). The membrane was stripped and reprobed with β-catenin antibodies as a loading control. Note that EphA2 protein levels decrease in a dose-dependent manner following ligand activation, but β-catenin levels remain unchanged. The most substantial decrease in EphA2 levels was with 2 μg/ml EphrinA1-Fc treatment. B, PC-3 prostate cancer cells were left untreated (−) or were stimulated (+) with 1 μg/ml EphrinA1-Fc for 2 h. Cell lysates probed for EphA2 levels showed that ligand stimulation decreases EphA2 protein in PC-3 cells but does not alter the levels of the loading control β-catenin. C, MDA-MB-231 cells were incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin and were left untreated (−) or were stimulated (+) with 1 μg/ml EphrinA1-Fc for 3 h. Biotinylated proteins were immunoprecipitated (IP) with NeutrAvidin, and the presence of EphA2 protein was detected by Western blot analyses with EphA2-specific antibodies. Note that levels of cell-surface associated EphA2 decrease following EphrinA1-Fc treatment. Molecular mass standards are indicated to the left.*

1M. S. Kinch, unpublished observations.
Loading control decreases following antibody aggregation of EphA2, but the levels of the nonspecific staining at all time points (data not shown). Treated with secondary antibodies only had low levels of material appeared to have been internalized (Fig. 4A). Cells min of aggregation with secondary antibodies, EphA2 had EphA2 subcellular localization in metastatic cells (25). After 5 was observed, which is consistent with our previous studies of diffusion. Some aggregation of EphA2 within membrane ruffles surface of control samples (the 0 min time point) was mostly Fluorescence microscopy revealed that EphA2 at the cell untreated cells, little EphA2 degradation was detected over the 8-h time period (Fig. 3A). Upon EphA2 stimulation, however, EphA2 was rapidly degraded and had an estimated half-life of 3.5 h (Fig. 3B). These results indicated that the decreased EphA2 immunoreactivity detected following EphA2 stimulation represented EphA2 protein degradation.

EphA2 is Internalized in Response to Stimulation

Ligand-mediated aggregation of transmembrane receptors often precedes their internalization and degradation (35). Thus, we asked whether cell surface aggregation of EphA2 would similarly induce its internalization. To test this, the EphA2 on the surface of MDA-MB-231 cells was labeled with B2D6 antibodies and this EphA2 was aggregated using fluorescein-conjugated secondary antibodies for 0–60 min at 37°C. Fluorescence microscopy revealed that EphA2 at the cell surface of control samples (the 0 min time point) was mostly diffuse. Some aggregation of EphA2 within membrane ruffles was observed, which is consistent with our previous studies of EphA2 subcellular localization in metastatic cells (25). After 5 min of aggregation with secondary antibodies, EphA2 had clustered into distinct patches and after 20 min, much of this material appeared to have been internalized (Fig. 4A). Cells treated with secondary antibodies only had low levels of nonspecific staining at all time points (data not shown).

Multiple analyses confirmed that EphA2 was internalized in response to stimulation. First, we aggregated EphA2 with specific antibodies as described above and stripped the labeled antibodies from the cell surface using a low-pH medium. The EphA2 localization in acid-stripped samples was identical to that of samples in Fig. 4A (data not shown), which suggested that this EphA2 had been internalized. Immunoelectron microscopy confirmed that stimulated EphA2 had been internalized (Fig. 4B). For these studies, EphA2 was labeled at the surface of MDA-MB-231 cells with B2D6 antibodies and gold-conjugated secondary antibodies. The antibody incubation was performed on ice to permit binding but to prevent antibody-dependent receptor internalization before sample analysis. The samples were incubated at 37°C for 0–20 min to permit EphA2 internalization, and were then immediately processed for observation by electron microscopy using standard techniques. Before incubation at 37°C, gold-labeled EphA2 was diffusely distributed over the cell Fig. 4B, 0 min). Upon incubation at 37°C, EphA2 became clustered at the cell surface and was internalized within endosomal vesicles (Fig. 4B, 20 min). Thus, these studies demonstrate that antibody aggregation of EphA2 causes EphA2 internalization.

Stimulated EphA2 Interacts with c-Cbl

We next sought to determine the biochemical mechanism by which EphA2 is internalized and degraded in response to stimulation. In response to ligand binding, many receptor tyrosine

![Image](https://example.com/image1.png)

FIGURE 2. EphA2-specific antibody aggregation decreases EphA2 immunoreactivity. A, MDA-MB-231 breast cancer cells were incubated with EphA2-specific antibodies (B2D6, j alone, secondary antibodies alone (i), or aggregated at the cell surface with both (j + 2) for 1 h. Cell lysates were resolved by SDS-PAGE and EphA2 levels were assessed by Western blot analyses with anti-EphA2 antibodies. Note decreased EphA2 levels following aggregation of EphA2 with both primary and secondary antibodies relative to control samples. The blot was stripped and reprobed for the loading control vinculin. B, PC-3 cells were left untreated or EphA2 was aggregated with both EphA2-specific primary antibodies and secondary antibodies (j + 2) for 1 h. Again, EphA2 protein levels decrease following antibody aggregation of EphA2, but the levels of the loading control j-catenin remain constant.

![Image](https://example.com/image2.png)

FIGURE 3. Stimulation induces EphA2 protein degradation. A, MDA-MB-231 cells were metabolically labeled with Tran 35S-Label and EphA2 was left untreated (not treated (NT)) or was stimulated by antibody aggregation (j + 2). The radiolabel was chased for the indicated times and EphA2 was immunoprecipitated from cell lysates and resolved by SDS-PAGE. Note that whereas EphA2 in control cells (top) is stable over 8 h, stimulated EphA2 (bottom) is degraded. B, Quantitation of band intensities in A estimates that the half-life of antibody-aggregated EphA2 (■) is 3.5 h, whereas non-treated EphA2 in control cells (○) has a half-life greater than 8 h.
kinases undergo autophosphorylation, which creates docking sites for signaling or adaptor proteins (36, 37). Indeed, EphA2 was tyrosine phosphorylated within 10 min following either ligand binding or antibody aggregation (Fig. 5A). The phosphotyrosine (p-Tyr) content of EphA2 decreased over time, which coincided with decreased levels of EphA2 protein (data not shown).

The c-Cbl adaptor protein has been shown to preferentially associate with, and induce the degradation of, tyrosine-phosphorylated receptor tyrosine kinases (38–40). Thus, we postulated that an interaction with c-Cbl might facilitate EphA2 protein degradation. To test this hypothesis, we first examined the subcellular localization of EphA2 and c-Cbl before and after EphA2 stimulation. EphA2 was aggregated using B2D6 and fluorescein-conjugated secondary antibodies to track its localization. The samples were then immunostained for c-Cbl and examined by epifluorescence microscopy. In mock-stimulated cells, EphA2 exhibited a characteristic membrane-staining pattern that overlapped with the distribution of a population of c-Cbl (Fig. 5B). EphA2 and c-Cbl coclustered proximal to the cell membrane following EphA2 aggregation, and subsequently colocalized into intracellular vesicles (Fig. 5B). Vertical sectioning using confocal microscopy confirmed the colocalization of EphA2 and c-Cbl following ligand binding (Fig. 6A).

Based on the colocalization of EphA2 and c-Cbl in response to EphA2 activation, we asked if the two molecules could interact in vivo. To test this, we stimulated MDA-MB-231 cells with EphrinA1-Fc and asked if EphA2 could coinmunoprecipitate with c-Cbl. In designing this study, we considered that the EphrinA1-Fc itself might unintentionally precipitate EphA2 and thereby lead to an erroneous interpretation of the results. To overcome this, Sepharose beads were directly conjugated to c-Cbl antibodies [Cbl-protein A-Sepharose (PAS)] to prevent unwanted binding of EphrinA1-Fc and associated EphA2. MDA-MB-231 cells were then left untreated or stimulated with EphrinA1-Fc for 10 min at 37°C. This short duration of EphA2 stimulation was chosen to evaluate the protein interactions of EphA2 before its degradation. Cell lysates were precipitated with Cbl-PAS or with an irrelevant RPS. Relative to unstimulated controls, EphrinA1-Fc-stimulated cells had more EphA2 in association with c-Cbl. RPS-PAS precipitated very little EphA2, regardless of ligand stimulation (Fig. 6B). These experiments provide additional support for the hypothesis that ligand stimulation promotes the association of EphA2 with c-Cbl.

c-Cbl Regulates EphA2 Degradation

The activation-dependent association of EphA2 with c-Cbl led us to ask whether binding to c-Cbl contributed to activation-dependent EphA2 protein degradation. c-Cbl functions in part by targeting proteins for degradation via the proteasomal pathway (39, 40). Thus, our first experiments utilized the chemical proteasome inhibitor MG-132, which potently but reversibly decreases the degradation of ubiquitin-conjugated proteins by the 26S proteasome complex. PC-3 cell monolayers were treated with vehicle alone or 10 μM MG-132 and then left untreated or stimulated by EphrinA1-Fc for 2 h. MG-132 prevented 67% of activation-dependent EphA2 degradation (Fig. 6A). We were able to rule out that MG-132 had prevented EphA2 from productively responding to ligand because the EphA2 in cells that had been treated with both MG-132 and EphrinA1-Fc was highly tyrosine phosphorylated (Fig 7A, bottom panel). Similar results were obtained using MDA-MB-231 cells (data not shown).

To determine whether c-Cbl played a role in activation-induced degradation of EphA2, we blocked c-Cbl function.

**FIGURE 4.** EphA2 aggregation causes its internalization. **A.** The EphA2 on MDA-MB-231 breast cancer cells was stimulated using the anti-EphA2 antibody B2D6 and rhodamine-conjugated rabbit anti-mouse IgG secondary antibodies at 37°C for the times shown. Over time, EphA2 clustered at the cell surface (5 min) and was internalized (20 min). **B.** Immunoelectron microscopy was performed by labeling MDA-MB-231 cells with the anti-EphA2 antibody B2D6. EphA2 was then aggregated using gold-conjugated rabbit anti-mouse IgG secondary antibodies and 37°C for 20 min. Note that EphA2 clusters and is internalized into early endosomal compartments (big arrow) within 20 min of aggregation.

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using dominant-negative Cbl mutants. MDA-MB-231 cells were infected with a recombinant retrovirus that encodes v-cbl (41), which inhibits c-Cbl function by competing with it for binding to phosphorylated tyrosine residues (42). In the absence of EphA2 stimulation, vector-infected and v-cbl-infected cells had similar levels of EphA2 protein (Fig. 7B). Stimulation of EphA2 by antibody aggregation caused efficient degradation of EphA2 in control samples (68% degradation) but higher levels of EphA2 persisted in stimulated v-cbl-infected cells (25% degradation, Fig. 7B). Thus, v-Cbl prevented 63% of stimulation-induced EphA2 protein degradation (P < 0.03). Similar results were obtained using 70Z-Cbl (43) (P < 0.0004, data not shown). The inverse experimentation was also performed. Overexpression of c-Cbl in MDA-MB-231 cells was sufficient to decrease the levels of EphA2 protein (Fig. 7C). Thus, consistent data confirmed the link between c-Cbl and the regulation of EphA2 protein levels.

Discussion
The major finding of this study is that the EphA2 receptor tyrosine kinase is rapidly and efficiently degraded in response to ligand- or antibody-mediated stimulation. EphA2 levels in carcinoma cells decreased following treatment with stimuli that mimic the actions of endogenous ligand. We also demonstrate that EphA2 interacts with the c-Cbl adaptor protein in a stimulation-dependent manner and that proteasome and c-Cbl functions promote stimulation-dependent EphA2 protein degradation.

Recent studies by our laboratory and others have demonstrated that ligand stimulation of EphA2 on tumor cells has several biological consequences, including decreased tumor cell growth, migration, and invasiveness (25, 27, 32). At first glance, these biological outcomes were presumed to have arisen from direct induction of downstream signaling. Tyrosine-phosphorylated EphA2 interacts with a variety of adaptor and signaling proteins including PI 3-kinase (44), SHC, SHP2 (32), and SLAP (44). EphA2 also contains a consensus binding motif for c-Cbl (YxxxP) at tyrosine 813, although it is presently unclear whether this site is necessary or sufficient for the interactions described herein. Our present findings suggest that the biological consequences of ligand-mediated EphA2 stimulation may arise in part from the removal of EphA2 from the cell surface and its subsequent degradation. High levels of EphA2 are sufficient to confer oncogenic and metastatic potential upon non-transformed cells (27). It logically follows that stimulation-dependent degradation of EphA2 could negatively regulate malignant behavior by decreasing the expression of a powerful oncoprotein. In fact, recent studies
reveal that EphA2 can serve as a target for monoclonal antibodies and that growth-inhibitory EphA2 antibodies induce efficient degradation of EphA2 (45). However, we cannot exclude the possibility that active induction of downstream signals following EphA2 stimulation also contributes to the negative regulation of tumor cell growth, survival, and invasiveness. Thus, future studies should assess the relative contributions of direct EphA2 signaling versus receptor degradation as mechanisms of ligand-mediated inhibition of malignant cell behavior.

The interaction of EphA2 with c-Cbl has interesting implications for understanding the biological actions of both proteins. c-Cbl is an adaptor protein that interacts with certain tyrosine-phosphorylated proteins via an internal SH2 domain (38). c-Cbl has been shown to negatively regulate several receptor tyrosine kinases [e.g., epidermal growth factor receptor (46, 47), platelet-derived growth factor receptor (48), HER2 (14)] by promoting their ubiquitination and degradation following ligand stimulation (48, 49). This function involves a zinc finger domain in c-Cbl that functions as an ubiquitin ligase (49–51). Based on this knowledge, we sought to determine whether EphA2 becomes ubiquitinated in response to ligand binding, but technical limitations have precluded this line of investigation.

c-Cbl itself can be tyrosine phosphorylated (52) and serve as an adaptor for downstream signaling (53, 54). c-Cbl-mediated signal transduction is not well understood, but c-Cbl has been implicated in both the positive (55, 56) and negative (57) transmission of intracellular signals. Moreover, c-Cbl can become ubiquitinated (58) and such changes could also alter intracellular signaling. Although we cannot exclude that some of the biological consequences of EphA2 stimulation result from c-Cbl-mediated signaling, EphA2 stimulation did not alter the p-Tyr content or protein levels of c-Cbl (data not shown). Thus, our results suggest that c-Cbl primarily serves to facilitate EphA2 degradation.

Stable cell-cell contacts, particularly those mediated by E-cadherin, favor ligand-mediated EphA2 autophosphorylation (25), which could promote interactions between EphA2 and c-Cbl, and thereby favor EphA2 degradation. Malignant cells often have unstable cell-cell contacts (31, 59) and one prediction of our model is that the resulting decrease in EphA2-ligand binding (25, 26) allows EphA2 to accumulate at the cell surface. Our present findings suggest that decreased ligand binding might favor EphA2 accumulation, triggering a cycle that would increase the amount of EphA2 in tumor cells. Consistent with this, the highest levels of EphA2 protein are often have unstable cell-cell contacts (31, 59) and one prediction of our model is that the resulting decrease in EphA2-ligand binding (25, 26) allows EphA2 to accumulate at the cell surface. Our present findings suggest that decreased ligand binding might favor EphA2 accumulation, triggering a cycle that would increase the amount of EphA2 in tumor cells. Consistent with this, the highest levels of EphA2 protein are consistently found in aggressive tumor cells where EphA2 has a low p-Tyr content (27). Indeed, the levels of EphA2 protein can be disproportionately higher than EphA2 mRNA levels in malignant epithelial cells (26). Such a model may explain why high levels of EphA2 are found in a large number of human cancers.

The aforementioned model of EphA2 degradation was based upon artificial stimulation of EphA2 on tumor cells. It is presently unclear whether soluble ligands or antibodies faithfully reproduce the consequences of endogenous ligands, which are anchored to the surface of adjacent cells.
(60). For example, differences in the timing or duration of EphA2 stimulation, or changes in EphA2 subcellular localization in response to a soluble versus a membrane anchored, could have profound biochemical or biological consequences.

In summary, our present findings indicate that the EphA2 receptor tyrosine kinase is rapidly and efficiently degraded in response to antibody or ligand stimulation. Stimulated EphA2 becomes tyrosine phosphorylated and interacts with the c-Cbl adaptor protein, which promotes EphA2 degradation. Our findings have implications for understanding the prevalence of EphA2 overexpression in cancer cells and for the development of targeted therapeutics designed to selectively remove a powerful oncoprotein.

Materials and Methods

Antibodies and Reagents

The proteasome inhibitor, MG-132, was purchased from Calbiochem (San Diego, CA). Monoclonal antibodies specific for EphA2 (D7 and B2D6) were generated in the laboratory (61) or purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal antibodies specific for EphA2 and c-Cbl were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for vinculin and paxillin were a generous gift of Dr. K. Burridge (University of North Carolina). Monoclonal antibodies specific for β-catenin and c-Cbl were purchased from Transduction Laboratories (Lexington, KY) and 4G10 p-Tyr antibodies were purchased from Upstate Biotechnology. EphrinA1-Fc was the generous gift of Dr. B. Wang (Case-Western Reserve University).

Cells and Cell Culture

MDA-MB-231 cultured human breast tumor cells, and LNCaP and PC-3 cultured human prostate tumor cells were propagated in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotic solution as described (24, 25). MDA-MB-435 and MCF10a cells were cultured as described previously (27, 62).

Transfection

The HA-tagged human c-cbl in pGEMZ was a kind gift of W. Langdon (The University of Western Australia). HA-c-cbl was cut from this vector and cloned into the mammalian expression vector pCDNA3. It was then transfected into MDA-MB-231 cells using the FuGENE system according to the manufacturer’s recommendations (Roche, Indianapolis, IN) and cells were selected with 400 μg/ml G418. Transfectants were serially diluted and subcloned. Data reported are representative of at least three clones.

Immunoprecipitations and Western Blot Analyses

Cells were lysed and protein expression was analyzed by immunoprecipitation and Western blotting as described (24). For the Cbl immunoprecipitations, PAS beads were incubated overnight with c-Cbl polyclonal antibodies (Santa Cruz Biotechnology) and washed three times before using this material to precipitate cell lysates as described above. Immunoprecipitations with NeutrAvidin (Pierce, Rockford, IL) were performed by incubating 40 μl cross-linked immobilized NeutrAvidin in a 50% slurry with lysates of cell-surface biotinylated cells for 1.5 h at 4°C. To confirm equal sample loading, membranes were stripped and reprobed with antibodies.
specific for vinculin, β-catenin, or paxillin. To measure and compare protein levels, the autoradiograms were analyzed with KODAK 1D image analysis software. Band intensity was determined after correcting for nonspecific background.

**EphA2 Stimulation Studies**

The EphA2 on tumor cells was stimulated with a soluble EphrinA1 fusion protein (EphrinA1-Fc) or EphA2-specific antibodies (B2D6) as described (21, 25). Briefly, cells were incubated with ligand at 37°C for the times indicated, or incubated with antibodies on ice and then returned to 37°C for the given times. Mock-stimulated cells were incubated with ligand or antibodies on ice, but never returned to 37°C.

**Biotinylation of Cell Surface Proteins**

Cell-surface associated proteins were biotinylated by incubating adherent cells with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 30 min at room temperature on an orbital shaker. Excess biotin was removed, and the cells were then left unstimulated or stimulated with 1 μg/ml EphrinA1-Fc for 3 h. Biotinylated proteins were immunoprecipitated with immobilized NeutrAvidin (Pierce) and subjected to Western blot analyses with EphA2-specific antibodies.

**Metabolic Labeling**

MDA-MB-231 cells were cultured in 6-cm tissue culture dishes (Costar, Acton, MA) overnight at 37°C. The samples were then incubated in media lacking cysteine and methionine for 1 h before labeling with 200 μCi Tran35S-Label (ICN Radiochemicals, Irvine, CA) for 1 h. After removing the labeling medium, the samples were incubated for 0–8 h in standard cell culture medium supplemented with 1 mg/ml of L-cysteine and L-methionine (Sigma Chemical Co., St. Louis, MO). EphA2 antibody aggregation was performed coincident with the restoration of normal cell culture medium. The samples were resolved by SDS-PAGE, the gel was dried, and the resulting autoradiograms were analyzed using a Cyclone Storage Phosphor System (Packard, Meriden, CT). OptiQuant image analysis software (Packard) was used to measure band intensities.

**Immunofluorescence**

Cells were grown on glass coverslips overnight before fixation and extraction as described (63). The samples were stained with specific primary antibodies and rhodamine-conjugated donkey anti-mouse or FITC-conjugated donkey anti-rabbit antibodies (Chemicon International, Inc., Temecula, CA) and antibody binding was visualized using epifluorescence microscopy (BX60 Olympus, Lake Success, NY). Images were recorded by photomicroscopy using a 35-mm camera and Tmax 400 film (Eastman Kodak, Rochester, NY). To observe internalized antibodies, antibodies at the cell surface were stripped off of cell monolayers by incubation in a low-pH medium (pH 2) for 5 min on ice. Confocal images were taken with a Bio-Rad MRC1024-UV on a Nikon Diaphot 300 microscope.

**Electron Microscopy**

Cell monolayers were cultured in six-well tissue culture dishes at 37°C overnight. The samples were then incubated on ice with B2D6 anti-EphA2 antibodies for 20 min, rinsed, and incubated on ice with 10 nM colloidal gold-labeled goat anti-mouse IgG antibodies (Sigma). Cells were kept on ice or returned to 37°C for various times and then fixed in glutaraldehyde and prepared for electron microscopic evaluation according to standard methods.

**Retroviral Infections**

The v-chi cDNA constructs were a generous gift from Dr. W. Langdon (University of Western Australia) and were subcloned into the recombinant retroviral vector pR8-HygR (64). The resulting constructs were packaged into amphotropic retrovirus particles that were used to infect MDA-MB-231 cells as described (64). Infected cells were selected using hygromycin B (Alexis Corp., San Diego, CA) and drug-resistant colonies were pooled to generate bulk cultures that were utilized for the studies described herein.

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