Tumor-inhibitory Antibodies to HER-2/ErbB-2 May Act by Recruiting c-Cbl and Enhancing Ubiquitination of HER-2\(^1\)

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Abstract

Overexpression of HER-2/ErbB-2, a homologue of the epidermal growth factor receptor, is associated with poor prognosis, and an ErbB-2-specific antibody is therapeutic when administered to patients with metastatic breast cancer. To understand the mechanism underlying immunotherapy, we concentrated on antibody- and epidermal growth factor-induced degradation of ErbB-2. We show that enhanced degradation is preceded by poly-ubiquitination of ErbB-2. This process necessitates recruitment of the c-Cbl ubiquitin ligase to tyrosine 1112 of ErbB-2. Consequently, mutagenesis of this site retards antibody-induced degradation. Thus, the therapeutic potential of certain antibodies may be due to their ability to direct ErbB-2 to a c-Cbl-regulated proteolytic pathway.

Introduction

HER-2/ErbB-2 is a receptor tyrosine kinase that is a member of the EGF\(^3\)-receptor family. By serving as a shared coreceptor for multiple stratal growth factors, ErbB-2 cooperates with ErbB-1 (EGF receptor) and the neuregulin receptors (ErbB-3 and ErbB-4; Ref. 1). Unlike low expression in normal adult cells, ErbB-2 is overexpressed in ~20–35% of human breast, gastric, lung, and prostate cancer. ErbB-2 overexpression in breast carcinomas is correlated with poor prognosis, especially of node-positive cases (reviewed in Ref. 2). Extensive work in animal models has indicated that blocking ErbB-2 with mAbs can reduce the rate of tumor growth. These studies led to successful clinical tests of a humanized anti-ErbB-2 mAb (3). Furthermore, patients with metastatic breast cancer showed improved responses when treated with a combination of chemotherapy and the mAb (4). Two potential mechanisms may underlie immunotherapy: (a) mAbs can inhibit the interaction of ErbB-2 with other family members (5); and (b) they can accelerate ErbB-2 degradation (6, 7).

Accelerated degradation of ErbB-1 following EGF binding (“down-regulation”) involves receptor ubiquitination and sorting of internalized molecules in the early endosome (8). The specific ubiquitin ligase involved in this process has been recently identified as c-Cbl (9, 10), a major substrate of many tyrosine kinases (11). Because mAb-induced down-regulation of ErbB-2 is related to the process promoted by EGF binding to ErbB-1, we addressed the possibility that immunotherapy targets ErbB-2 to a c-Cbl-regulated ubiquitination/degradation pathway. Here, we show that a tumor-inhibitory mAb can enhance a Cbl-mediated process of ubiquitination and degradation of ErbB-2.

Materials and Methods

Materials. mAbs directed against ErbB-2 have been described previously (5). A polyclonal rabbit antiserum was raised against a synthetic peptide comprising the 15 COOH-terminal amino acids of the human ErbB-2. EGF (human, recombinant) and an anti-HA mAb were purchased from Boehringer Mannheim. Antibodies were radiolabeled with Na\(^{125}\)I (Amersham) by using the chloramine T method. The isolation of a recombinant v-Cbl-GST protein and its subsequent binding to gluthatione-agarose has been described previously (10). The lysis buffer contained 25 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Na-deoxycholate, 1% NP40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na\(_4\)VO\(_4\), 5 \(\mu\)g/ml leupeptin, and 5 \(\mu\)g/ml aprotinin. Washing solution contained 25 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol.

Construction and Transfection of Expression Vectors. To generate mutated ErbB-2 proteins, we used an oligonucleotide-directed mutagenesis method with a site-directed mutagenesis kit (QuickChange; Stratagene, La Jolla, CA). Tyrosine 1112 was mutated to a phenylalanine using the oligonucleotide 5’-CTCTACAGCGTGTCATGAGGACCC. The transmembranal valine 659 was replaced by a glutamic acid with the oligonucleotide 5’-GTCTCTGGCTGGAGGGCATCTGCTG. The double mutant EF was generated by introducing the V659E mutation into a receptor construct containing the Y1112F mutation. Expression vectors were introduced into CHO cells by using the Lipofectamine transfection method (Life Technologies, Inc., Bethesda, MD) and into HEK-293T cells by using the calcium phosphate precipitation method.

Determination of mAb Effect on Tumor Growth in Vivo. N87 cells (5 \(\times\) 10\(^6\)) were injected s.c. into CD1/nude mice, followed by three i.p. injections of the mAbs on days 3, 7, and 10. Tumor parameters were measured once a week with calipers, and tumor volume was calculated according to the formula: tumor volume = length \(\times\) width \(\times\) height.

Receptor Down-Regulation. Cells grown in 24-well plates were incubated at 37°C for various time intervals with or without mAb L26 (20 \(\mu\)g/ml) in binding buffer (0.01% albumin in DMEM). Cells were then rinsed three times in ice-cold binding buffer. Surface-bound antibody molecules were removed by use of low \(p\)H wash (8). The level of receptor residing on the cell surface was then determined by incubating the cells at 4°C with a radiolabeled mAb L26 for 1.5 h.

Detection of Ubiquitinated Proteins. The ubiquitinated form of ErbB-2 was detected in immunoprecipitates prepared from cells that were cotransfected with a plasmid encoding a HA-tagged ubiquitin [a gift from Dirk Bohmann (European Molecular Biology Laboratory, Heidelberg, Germany)] and the appropriate ErbB-2 construct. The receptor was immunoprecipitated from whole cell lysates with a rabbit serum. Ubiquitin levels were determined by immunoblotting with anti-HA antibodies.

Receptor Turnover. Confluent cell monolayers grown in 90-mm plates were biosynthetically labeled with \(^{35}\)S-methionine for 16 h. The cells were then chased at 37°C for different time intervals, in the presence or absence of mAb L26. ErbB-2 was immunoprecipitated and separated by gel electrophoresis. Labeled proteins were detected by directly exposing the dried gel to an X-ray film.

In Vitro Binding of v-Cbl to ErbB-2. HEK-293T cells grown in 90-mm plates were transfected with pCDNA3 plasmids encoding either ErbB-1 (1 \(\mu\)g) or ErbB-2 (2 \(\mu\)g). Twenty-four h later, cultures were split into two plates, and forty-eight h after transfection cells were treated with EGF (10 min at 37°C). Cell lysates were then prepared, cleared from debris, and incubated with glutathion-agarose beads that were precoupled to a v-Cbl-GST fusion protein.
After 2 h of incubation at 4°C, the beads were washed twice and bound proteins were dissociated in boiling sample buffer prior to gel electrophoresis.

### Results and Discussion

The L26 mAb Down-Regulates ErbB-2 from the Surface of Human Cancer Cells and Inhibits Their Tumorigenic Growth in Mice. We have previously shown that antibodies directed at several distinct epitopes of ErbB-2 inhibit tumor growth in vivo (5). Although this suggests a variety of mechanisms underlying the inhibitory effect, the ability of mAbs to increase receptor turnover seems common to many tumor-inhibitory mAbs (6, 7). To elucidate the underlying mechanism, we selected antibody L26 as an example for mAbs capable of strong tumor inhibition. This previously described mAb (5) decreased the growth in nude mice, of human gastric carcinoma cells (N87) overexpressing ErbB-2, in a dose-dependent manner (Fig. 1A). Mice receiving a total dose of 2 mg of purified L26 demonstrated an almost undetectable tumor growth along a period of 35 days. Because it has previously been shown that tumor-inhibitory mAbs can induce the internalization of antibody-receptor complexes whereas a non-inhibitory mAb cannot (7), we tested the ability of L26 to down-regulate ErbB-2 from the surface of N87 cells. As shown in Fig. 1B, the amount of surface ErbB-2 declined significantly when cells were exposed to mAb L26, reaching 50% of the initial level within 2 h. This suggests that the enhanced receptor internalization caused by L26, in the presence of mAbs L26 or N28 (each at 20 μg/ml). ErbB-2 was immunoprecipitated (IP) by using a mixture of mAbs specific to its extracellular domain and immunoblotted (IB) with a rabbit polyclonal serum. Untreated cells served as controls (lanes labeled —). B, CHO cells were cotransfected with an ErbB-2 expression vector, along with plasmids encoding a HA-tagged ubiquitin. Forty-eight h later, cell monolayers were treated for 30 min at 37°C with or without mAb L26 (20 μg/ml). ErbB-2 ubiquitination was detected by IB receptor precipitates (top) or whole cell lysates (bottom) with an anti-AH antibody. CHO cells were transfected, as described above, in the presence of a plasmid encoding ErbB-1. Following stimulation by EGF (100 ng/ml, 10 min), cells were lysed and ErbB-2 was specifically precipitated under conditions that break ErbB-1-ErbB-2 heterodimers. The precipitated receptor was examined for ubiquitination, as described above. Whole cell lysates were subjected to anti-ErbB-2 IB to compare receptor expression (bottom).

Fig. 1. Tumor inhibition and down-regulation of cell surface expressed ErbB-2 by the L26 mAb. A, athymic mice received a s.c. injection of 5 × 10⁶ N87 human gastric cancer cells that overexpress ErbB-2. Three, 7, and 10 days later, mAb L26 was injected i.p. at a total dose of 0.1 mg (●), 0.25 mg (▲), or 2 mg (▲▲). Tumor volumes were measured at the end of the indicated time periods. Saline-injected mice were used for control (○). Bars represent SDs for groups of six mice. B, N87 cells were grown to confluence in 24-well plates, washed with binding buffer, and incubated at 37°C for the indicated time intervals in the presence (●) or absence (▲) of mAb L26 (20 μg/ml). Monolayers were then rinsed twice and stripped of surface-bound antibody by using a 7-min long incubation in a low pH buffer. The level of ErbB-2 molecules that remained at the cell surface was determined by binding of a radiolabeled mAb L26. Each point represents the average ± SD of duplicates.

Fig. 2. Antibody- and ligand-induced degradation of ErbB-2 involves increased receptor ubiquitination. A, N87 cells were incubated at 37°C for the indicated time intervals in the presence of mAbs L26 or N28 (each at 20 μg/ml). ErbB-2 was immunoprecipitated (IP) by using a mixture of mAbs specific to its extracellular domain and immunoblotted (IB) with a rabbit polyclonal serum. Untreated cells served as controls (lanes labeled —). B, CHO cells were cotransfected with an ErbB-2 expression vector, along with plasmids encoding a HA-tagged ubiquitin. Forty-eight h later, cell monolayers were treated for 30 min at 37°C with or without mAb L26 (20 μg/ml). ErbB-2 ubiquitination was detected by IB receptor precipitates (top) or whole cell lysates (bottom) with an anti-AH antibody. C, CHO cells were transfected, as described above, in the presence of a plasmid encoding ErbB-1. Following stimulation by EGF (100 ng/ml, 10 min), cells were lysed and ErbB-2 was specifically precipitated under conditions that break ErbB-1-ErbB-2 heterodimers.
CHO cells were cotransfected with plasmids encoding the WT form of ErbB-2 or the transforming mutant (V659E), along with a c-Cbl vector (+) or the respective empty vector (−). Total cell proteins were examined for receptor expression 48 h later by immunoblotting (IB) with an anti-ErbB-2 antibody. C, amino acid sequence alignment of the predicted Cbl-binding sites of ErbB-1 (10), ErbB-2, and ZAP-70 (18) is shown. The consensus sequence for c-Cbl (18), and we have shown that a similar motif surrounding tyrosine 1045 of ErbB-1 mediates the effect of Cbl on the EGF receptor (10), aligning the amino acid sequence of ErbB-2 with the predicted Cbl-binding sites of the corresponding human proteins.

By using mutagenesis, we replaced this tyrosine with a phenylalanine (mutant denoted Y1112F). To test the effect of the mutation on c-Cbl docking, we coexpressed the Y1112F mutant, or the WT form, together with the expression vector encoding ErbB-1 and ErbB-2 (either WT or Y1112F), and 24 h later receptor molecules were immunoprecipitated (IP) and their ubiquitination was detected by using an anti-HA antibody (top). Alternatively, extracts were first incubated with an immobilized v-Cbl-GST protein and association with ErbB-2 was analyzed by IB (bottom). Note the up-smearing of ErbB-2, which is attributed to poly-ubiquitination of the WT form. D, HEK-293T cells were transfected with plasmids encoding ErbB-1 and ErbB-2 (either WT or Y1112F), and 24 h later cell lysates were exposed for 10 min to EGF (100 ng/ml). Control cultures (Cont.) were mock-treated prior to cell lysis. Cell extracts were directly analyzed by IB with an anti-ErbB-2 antibody (bottom). Alternatively, extracts were first incubated with a immobilized v-Cbl-GST protein and association with ErbB-2 was assayed by IB (top). Note the up-smearing of ErbB-2, which is attributed to poly-ubiquitination of the WT form. E, ubiquitination effects of EGF (left) and a bivalent Fab fragment of the L26 mAb (right) were examined in CHO cells. For stimulation by EGF, the WT form of ErbB-2 and a Y1112F mutant were introduced along with a plasmid encoding ErbB-1. Antibody stimulation was tested in cells expressing the V659E mutant of ErbB-2, and a double mutant (V659E and Y1112F) denoted EF. Ubiquitination was detected in receptor immunoprecipitates (top), and the ErbB-2 level was determined in whole cell lysates (bottom).
To examine the putative c-Cbl docking site in cells and its relevance to receptor poly-ubiquitination, we transiently expressed Y1112F, or the WT form, together with ErbB-1. Although basal ubiquitination has been observed under these conditions, EGF stimulation was able to enhance ubiquitination of the WT form, but not of the single residue mutant (Fig. 3E). Thus, tyrosine 1112 is essential for ubiquitination of ErbB-2 in heterodimers. To test this requirement in homodimers, we introduced the Y1112F mutation into the constitutively dimerized transforming mutant V659E (producing a double mutant, denoted EF). An increase in ubiquitination of the EF receptor could not be induced by a bivalent fragment of L26, but ubiquitination of the original V659E receptor, bearing a tyrosine at position 1112, was readily induced (Fig. 3E), probably because L26 stabilized dimers. A high level of basal ubiquitination detected for the double mutant, could be the result of differing expression levels (see total cellular protein in the bottom panel).

Alternatively, additional mechanisms, independent of tyrosine 1112, may be involved in ErbB-2 ubiquitination. One example is ubiquitination by the benzoquinone geldanamycin that involves an interaction of ErbB-2 with a member of the HSP90 family of stress proteins (19). Taken together, the results presented in Fig. 3 attribute a role to c-Cbl in ligand-, antibody-, and also in mutation-induced ubiquitination of ErbB-2.

**Enhanced Turnover of ErbB-2 Is Dependent On the Putative c-Cbl Docking Site.** To study the involvement of the putative c-Cbl docking site in the induction of ErbB-2 degradation, we used HEK-293T cells. These cells enable very high expression of transfected plasmids, which was particularly useful for testing effects on the rate of ErbB-2 turnover. Similar to observations made with N87 tumor cells (Fig. 1B and 2A), mAb L26 induced a decrease in the level of the WT ErbB-2 expressed in HEK-293T cells (Fig. 4A, right). However, mAb treatment of cells expressing the mutant Y1112F could not reproduce this effect (Fig. 4A, left), indicating that the putative c-Cbl docking site is essential for antibody-induced degradation of ErbB-2. Similarly, stimulation by EGF decreased the level of the WT (Fig. 4B), but not of the Y1112F, form of ErbB-2 (Fig. 4B). Monitoring ErbB-2 turnover by using metabolic labeling confirmed the short half-life of the V659E transforming mutant relative to the WT form (Fig. 4C). We then used this mutant, the ubiquitination in CHO cells of which was stimulated by mAb L26, to examine the involvement of tyrosine 1112 in antibody-induced ErbB-2 turnover. Treatment with mAb L26 rapidly decreased the amount of the V659E mutant (Fig. 4D, right), but double mutant EF molecules harboring a phenylalanine at the putative c-Cbl-docking site were not degraded after mAb treatment. This confirms the importance of tyrosine 1112 for antibody-induced degradation of ErbB-2 and suggests that c-Cbl directs mAb-stimulated ErbB-2 molecules to proteasomal/lysosomal degradation, as it does for other receptors on stimulation with their natural ligands.

Cbl proteins emerge as pivotal players in the process, leading to inactivation of tyrosine kinase signaling pathways (9, 10). Relative to other growth factor receptors, the coupling of c-Cbl to ErbB-2 seems very weak, which may explain why this interaction escaped detection by previous studies (17, 20). Nevertheless, the realization that breast cancer immunotherapy directs HER-2/ErbB-2 to this major pathway of negative regulation is consistent with several observations. For example, targeted inactivation of the c-cbl gene in mice resulted in mammary hyperplasia, perhaps because c-Cbl negatively regulates the action of many mammary gland-derived growth factors (21). Consistent with negative regulation by c-Cbl, infection of an ErbB-2/Neu-driven neuroblastoma with a c-cbl-
encoding retrovirus caused tumor retardation in mice. The exact mechanism allowing c-Cbl to enhance ubiquitination of target proteins is unknown, but similar to other ubiquitin ligase complexes, it may involve association with still unknown ancillary proteins. Predictably, detailed understanding of ErbB-2 ubiquitination and degradation by a c-Cbl-mediated process will yield improvements in immunotherapy. For example, drugs known to enhance ubiquitination and degradation of ErbB-2, such as geldanamycin and herbimycin A (19), may enhance c-Cbl-mediated immunotherapy. Likewise, mAbs that better recruit ErbB-2 to the c-Cbl-regulated endocytic pathway are expected to act as superior therapeutic agents. Future studies will have to resolve the intricate machinery leading to ErbB-2 degradation.

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References


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