A rodent oncogenic mutant of the Neu receptor tyrosine kinase is a useful experimental model because overexpression of the respective receptor, namely HER2/ErbB-2, in human malignancies is associated with relatively aggressive diseases. Here we show that the oncogenic form of Neu is constitutively associated with the product of the c-neu proto-oncogene and is part of a large complex that includes the phosphoinositide 3-kinase and Shc. Ectopic expression of c-Cbl, a ubiquitin-protein isopeptide ligase specific to activated tyrosine kinases, causes rapid removal of Neu from the cell surface and severely reduces signaling downstream of oncogenic Neu. c-Cbl-induced down-regulation of Neu involves covalent attachment of ubiquitin molecules and requires the carboxyl-terminal domain of Neu. The negative effect of c-Cbl is antagonized by v-Cbl, a virus-encoded oncogenic truncated form of c-Cbl. In an in vivo model, infection of a Neu-transformed neuroblastoma with a c-Cbl-encoding retrovirus caused enhanced down-regulation of Neu and correlated with tumor retardation. Our results implicate c-Cbl in negative regulation of Neu and offer a potential target for treatment of HER2/ErbB-2-positive human malignancies.

Two ErbB family members, ErbB-1 (also called the epidermal growth factor (EGF)1 receptor) and ErbB-2 (also called Neu and HER2), are implicated in human cancer; genetic aberrations of the respective two genes cause their oncogenic activation (reviewed in Ref. 1). The neu/neu/erbB-2 oncogene was initially identified as an activated oncogene using a DNA transfection strategy (2). The human counterpart of the gene has been independently isolated using related human or viral erbB probes and termed HER2 (3) or erbB-2 (4–6). The neu oncogene differs from c-neu/erbB-2 by a point mutation replacing a valine for a glutamate at position 664, which lies within the transmembrane region (7). The resulting oncogenic protein is constitutively active as a tyrosine kinase (8) due to its ligand-independent dimeric state (9). Although a similar mutation has not been reported in human cancers, oncogenic activation of Neu has been frequently used as a genetic model for breast cancer development in rodents (10–14). Moreover, this protein is considered a major target for anti-tumor therapies that use kinase inhibitors or monoclonal antibodies (1). A humanized monoclonal antibody specific to ErbB-2 has been recently approved for treatment of metastatic breast cancers overexpressing ErbB-2 (15–17). Therapeutic anti-ErbB-2 antibodies are thought to enhance down-regulation and degradation of ErbB-2, but the underlying mechanism is still unknown (18, 19).

Activation of ErbB-2 through overexpression, mutagenesis, or stimulation of EGFR/ErbB-2 chimeras with EGF results in the recruitment of several substrates. The list of ErbB-2 substrates includes phospholipase Cγ (20, 21), phosphatidylinositol (PI) 3-kinase (22, 23), Shc (24), c-Src (25–27), the GTPase-activating protein (20, 28), protein tyrosine phosphatase 1D (29), and GRB-7 (30). The status of one potential substrate of Neu/ErbB-2, namely c-Cbl, is currently unclear. This issue is important because recent studies have identified the c-Cbl adaptor protein as a negative regulator of activated receptor tyrosine kinases, including the receptors for EGF (31–34), the platelet-derived growth factor (35), and colony-stimulating growth factor-1 (36). Consistent with a negative regulatory role, c-Cbl-deficient mice exhibit hyperplasia of mammary ducts (37), a process known to be regulated by EGF-like growth factors. Recruitment of c-Cbl to the EGF receptor plays a key role in sorting the receptor to late endosomes, and it involves receptor ubiquitination and degradation (32, 33). By using a cell-free ubiquitination assay, we and others (34, 38, 39) have shown that Chl is a component of the ubiquitin conjugation machinery, and it qualifies as a ubiquitin-protein isopeptide ligase or a ligase ancillary protein.

In view of the pathological significance of Neu/ErbB-2, identification of negative regulators of this oncogenic receptor is of clinical importance. We previously reported that the proto-oncogenic c-Cbl protein is specifically recruited by ErbB-1/EGF receptor but not by other ErbB proteins, including an EGFR/ ErbB-2 chimeric protein, which we stimulated with EGF (40). A similar conclusion was drawn by Muthuswamy et al. (41) who used ErbB receptor chimeras containing the synthetic ligand-binding domain of FK506-binding protein, although some residual recruitment of c-Cbl by ErbB-2 has been observed by these authors. Apparently, ErbB-2 can weakly interact with c-Cbl, either directly or indirectly through ErbB-1. Consistent with this model, recruitment of c-Cbl by ErbB-1 was reduced when newly synthesized ErbB-2 molecules were trapped in the endoplasmic reticulum (42). Because Neu/ErbB-2 was shown to undergo both clathrin-mediated receptor down-regulation (43)
and poly-ubiquitination (44, 45), we have addressed the existence and relative strength of Cbl interactions with ErbB-2 by using the most active natural form of the receptor, namely the transforming rodent Neu protein. Here we report that C-cbl is physically associated with and phosphorylated by the Neu oncoprotein. In agreement with this conclusion, overexpression of c-Cbl can attenuate oncogenic signaling by Neu. Our results attribute the negative effect of c-Cbl to rapid removal of Neu from the cell surface, which impairs activation of downstream effectors and early response genes.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Lines—** Streptavidin conjugated to horseradish peroxidase (HRP) was purchased from Amersham Pharmacia Biotech. Biotin-X-NHS was from Calbiochem. All other chemicals, including EGF, were purchased from Sigma. An antibody to the Shc protein was purchased from Transduction Laboratories. Anti-p85/P1 3-kinase was from Upstate Biotechnologies Inc. Anti-hemagglutinin (HA) mAb 12CA5 was purchased from Roche Molecular Biochemicals. The murine mAb B10 to Neu has been previously described (8). For immunoblot analysis of Neu and c-Cbl we used a polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). The active form of MAPK was detected with an antibody kindly provided by Rony Seger (Weizmann Institute of Science). Anti-phosphotyrosine expression plasmids for Neu, P1, P2, v-Cbl, and Cbl-C were previously described (32, 46). The retroviral c-cbl vector was constructed by digesting pGEM4z-c-Cbl (kindly provided by W. Langdon) with BamHI and SauI and sub-cloning into the corresponding sites of the pBabe-Puro plasmid. NIH-3T3, B104, B104-1-1 (neu-transformed 3T3-fibroblasts), 3T3-v-Src, 3T3-sp-FGF, Rat-1, and Rat-1-EJ (ras-transformed fibroblasts) cells were grown in Dulbecco’s modified Eagle’s medium. Transfected CHO cells were cultured in Dulbecco’s modified Eagle’s/F-12 medium (1:1 ratio). The 32D sub-line overexpressing either the human ErbB-2 or ErbB-2 together with ErbB-1 (denoted D2 or D12, respectively (47)) were grown in interleukin-3-containing RPMI medium. Media were supplemented with 10% fetal calf serum (Biological Industries, Beit-hemek, Israel). Transfection of CDNAs and Luciferase Reporter Assay—Expression vectors were introduced to CHO cells using the LipofectAMINE™ transfection method (Life Technologies, Inc.) according to the manufacturer’s instructions. In short, sub-confluent CHO monolayers, which were grown in 10-cm culture dishes, were co-transfected with 2 μg of new constructs, either alone or in combination with 3 μg of wild type or mutant c-cbl vectors. The total amount of DNA in each transfection was normalized with the pcDNA3 plasmid. The SRE-luciferase reporter assay was performed as described previously (48) after transient transfection of the indicated vectors along with a reporter pSRE-Fluc (4 μg) containing one copy of SRE cloned upstream to the fos minimal promotor and a luciferase gene (49).

**Immunoprecipitation and Immunoblotting Analyses—** Cells were grown in 10-cm dishes, washed briefly with ice-cold phosphate-buffered saline (PBS), and solubilized for 30 min at 4 °C with lysis buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 100 μM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 mM sodium fluoride (NaF), and 2 mM sodium orthovanadate (Na3VO4). Cell lysates were cleared at 4 °C by centrifugation (12,000 × g; 10 min). Antibodies were coupled either to Sepharose-protein A or to goat anti-mouse-IgG-conjugated agarose beads (Sigma). The beads were incubated while shaking for 1 h at 4 °C. Immunoprecipitates were washed three times with HNTG solution (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol), mixed with gel sample buffer, heated for 5 min at 95 °C, and subjected to electrophoresis on 7.5% polyacrylamide gels. After electrophoresis, proteins were electro-pherirectionally transferred onto nitrocellulose filters and subjected for 2 h at 22 °C in a blocking solution (1% low fat milk, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.025% Tween 20). Antibodies were added in the same solution and incubated at 4 °C for 12–16 h. Filters were washed extensively with TBST solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.025% Tween 20) and reacted for 40 min at 4 °C with HRP-conjugated protein A or with HRP-conjugated goat anti-mouse immunoglobulins. Signals were detected by using the enhanced chemiluminescence method (ECL, Amersham Pharmacia Biotech).

**Cell Surface Biotinylation and Ubiquitination Assay—** Monolayers of cells grown in 10-cm dishes were washed three times with ice-cold PBS and then incubated for 45 min at 4 °C with 0.5 mg/ml of a water-soluble Biotin-X-NHS (Calbiochem) dissolved in borate buffer (10 mM boric acid and 150 mM NaCl, pH 8.0). Coupling of biotin was blocked by extensive washes with a solution of 15 mM glycine in PBS. Cells were then incubated for different time intervals at 37 °C. To evaluate the amounts of cell surface receptors, cleared cell extracts were subjected to immuno precipitation and gel electrophoresis. Visualization of the biotinylation sites of the receptors was performed with the HRP-conjugated streptavidin and developed with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). The ubiquitinated form of Neu was detected in immunoprecipitates prepared from cells that were cotransfected with a plasmid encoding a hemagglutinin (HA)-tagged ubiquitin (a gift from Dirk Bohmann, EMBL, Heidelberg, Germany) and a nev expression vector. The receptor was immunoprecipitated from whole cell lysates with a monoclonal antibody (B10), and its ubiquitination levels were determined by immunoblotting with anti-HA antibodies.

**Recombinant Retroviral Infection and in Vivo Tumor Growth in Nude Mice—** The pBabe-Puro-Cbl retroviral vector was transiently transfected to HEK-293T cells along with the ψ-MLV helper-virus vector using the calcium phosphate precipitation method. Two days later, conditioned media containing viral particles were sequentially collected every 6 h and used to infect the rat neuroblastoma B104 cell line. Puromycin-resistant clones that overexpressed c-Cbl were examined by Western blot analysis. The in vivo tumor growth assay was performed with two groups of eight CD1/nude male mice at the age of 6 weeks (obtained from the Experimental Animals Center of the Weizmann Institute of Science). Animals were subcutaneously injected with either B104-Cbl or a control B104-Puro sub-line (2 × 106 cells per animal, suspended in 0.5 ml PBS), and tumor dimension parameters were measured after 24 days. Tumor volume was calculated according to the following formula: length × width × height. To validate volume measurements, a correlation between tumor volume and tumor weight was verified after mice were sacrificed.

**RESULTS**

**Neu and Several Other Oncogenic Pathways Increase Tyrosine Phosphorylation of c-cbl—** c-cbl has previously been shown to associate with phosphotyrosine-containing protein complexes induced by different mitogenic signals (50). Analysis of a series of oncogene-transformed cells revealed that c-cbl is constitutively associated with tyrosine-phosphorylated protein complexes in fibroblasts transformed by the oncogenic form of Neu, a transforming secreted fibroblast growth factor (sp-FGF) (51) and viral Src (v-Src), but not in Ras-transformed cells (Fig. 1A). The respective untransformed control cells exhibited no such association of c-Cbl, but activation with EGF led to tyrosine phosphorylation of the protein in untransformed Rat-1 cells (Fig. 1A). A reciprocal experiment in which c-Cbl was first immunoprecipitated and then the resolved complexes were probed with an anti-phosphotyrosine antibody confirmed prominent phosphorylation of the 120-kDa c-cbl protein (Fig. 1B).

Intriguingly, the relative amount of the c-cbl protein in v-Src-transformed cells was 10-fold lower than in other cell lines (Fig. 1, lower panels), suggesting the existence of additional regulatory mechanisms affecting the status of the c-cbl protein.

Because Neu/ErbB-2 is the preferred heterodimeric partner of the ErbB family (42), we expected that overactivation of ErbB-1, which is endogenously expressed in NIH-3T3 cells, recruits c-Cbl to Neu. To test possible interactions between ErbB-2 homodimers and c-cbl, we used ErbB-2-transformed cells derived from the 32D cell line, a murine myeloid cell line that expresses no endogenous ErbB family member (47). ErbB-2 homodimers were induced by using mAb N28 directed to the extracellular part of ErbB-2. Upon activation with the mAb, c-cbl became associated with a tyrosine-phosphorylated protein complex (Fig. 2A). This observation indicates that homodimers ErbB-2 can recruit c-Cbl with no involvement of other ErbB family members. Nevertheless, experiments performed with 32D cells co-expressing ErbB-2 and ErbB-1 at comparable levels implied that phosphorylation of c-Cbl, which was induced by two ErbB-2-activating mAbs, N28 and N29, was significantly weaker than that induced by EGF (Fig. 2B). In conclusion, homodimers
of Neu/ErbB-2 that are induced by either a transforming mutation or bivalent antibodies can directly interact with c-Cbl, although this interaction appears significantly weaker than that exhibited by EGF-induced homodimers of ErbB-1.

We have previously reported that the oncogenic form of Neu is constitutively associated with the p85 regulatory subunit of PI 3-kinase (22). Similarly, c-Cbl was shown to form a phosphotyrosine-dependent physical complex with this Src homology-2 (SH2) domain-containing protein in EGF-stimulated cells (53, 54). These observations, when combined with our conclusion that c-Cbl can directly interact with Neu, predict the existence of a ternary complex. Indeed, complexes of Neu-Cbl, Neu-p85 and p85-Cbl were detectable by using a co-immunoprecipitation assay in Neu-transformed cells. Interestingly, similar complexes apparently exist in v-Src-transformed fibroblasts but not in the relevant parental NIH-3T3 cells (Fig. 3).

Experiments that are not presented suggest that the physical association observed in v-Src-transformed cells was due to tyrosine phosphorylation of the endogenous murine ErbB-2 by v-Src. Taken together, the results shown in Figs. 1–3 suggest that in addition to activation of positive effectors, like PI 3-kinase and Shc (see below), the oncogenic form of Neu recruits the negative regulator c-Cbl.

c-Cbl Suppresses Signaling by an Oncogenic Neu—Because overexpression of c-Cbl can enhance endocytic removal of ErbB-1/EGF receptors and subsequently accelerates their degradation (32, 33), we examined whether it similarly down-regulates the oncogenic Neu receptor. Chinese hamster ovary (CHO) cells were transfected with an expression vector encoding the oncogenic Neu protein, and thereafter the surface of cell monolayers was labeled with biotin. The rate of turnover of the surface-associated Neu protein was then traced by immunoprecipitation followed by blotting with streptavidin-conjugated peroxidase. In the absence of an overexpressed c-Cbl protein, Neu molecules remained at the cell surface for more than 3 h. However, when we co-expressed Neu and c-Cbl the receptor almost completely disappeared from the cell surface (Fig. 4A). Lack of expression of Neu at the cell surface was not a result of reduced transcription because the intracellular pool of Neu was hardly influenced by c-Cbl (Fig. 4A, middle panel). This conclusion was also supported by analysis of neu transcript levels by using semi-quantitative polymerase chain reaction (data not shown). Thus, c-Cbl primarily affects Neu molecules residing at the cell surface. Because high expression of c-Cbl led to close to complete disappearance of Neu from the cell surface, we used lower amounts of the expression plasmids and tested Neu-mediated signaling. As expected, c-Cbl-induced receptor down-regulation was accompanied by a decrease in receptor signaling: a marked decrement in both phosphorylation of Neu and its association with Shc molecules was observed upon gradual overexpression of c-Cbl (Fig. 4B). Likewise, by using an antibody specific to the active form of MAPK, we found that over-regulation of PI 3-kinase (22). Similarly, c-Cbl was shown to form a phosphotyrosine-dependent physical complex with this Src homology-2 (SH2) domain-containing protein in EGF-stimulated cells (53, 54). These observations, when combined with our conclusion that c-Cbl can directly interact with Neu, predict the existence of a ternary complex. Indeed, complexes of Neu-Cbl, Neu-p85 and p85-Cbl were detectable by using a co-immunoprecipitation assay in Neu-transformed cells. Interestingly, similar complexes apparently exist in v-Src-transformed fibroblasts but not in the relevant parental NIH-3T3 cells (Fig. 3).

Experiments that are not presented suggest that the physical association observed in v-Src-transformed cells was due to tyrosine phosphorylation of the endogenous murine ErbB-2 by v-Src. Taken together, the results shown in Figs. 1–3 suggest that in addition to activation of positive effectors, like PI 3-kinase and Shc (see below), the oncogenic form of Neu recruits the negative regulator c-Cbl.
expression of Neu stimulated MAPK activity, but moderate ectopic expression of c-Cbl inhibited the effect of Neu on MAPK (Fig. 4B). In accordance with the ability of c-Cbl to enhance ubiquitination of ErbB-1, we found that covalent attachment of a co-expressed hemagglutinin (HA)-tagged ubiquitin to Neu was significantly enhanced in the presence of an overexpressed c-Cbl (Fig. 4C). Although c-Cbl-induced poly-ubiquitination of Neu was associated with enhanced degradation of the receptor, immunoblotting analysis indicated that only a small portion of Neu underwent poly-ubiquitination (Fig. 4C, lower panel). This observation is consistent with observations made with other c-Cbl-coupled receptors (32, 33, 35, 36) and implies that poly-ubiquitination is coupled to rapid degradation of surface Neu molecules.

The SH2 domain of c-Cbl mediates its interaction with cytoplasmic as well as membranal protein kinases (e.g. ZAP-70, and the receptors for platelet-derived growth factor and EGF, reviewed in Ref. 50). By using a mutant form of c-Cbl, denoted Cbl-C, whose SH2 domain was deleted, we learned that this domain was essential for Cbl-induced down-regulation of Neu. Thus, Cbl-C exhibited an impaired ability to induce down-regulation of cell surface Neu molecules labeled with biotin, and it also failed to suppress Shc association with the oncprotein (Fig. 5A). Likewise, a mutant form of the transforming Neu receptor, which lacks the whole carboxyl-terminal auto-phosphorylation domain, but retains the tyrosine residue most distal to the kinase domain (mutant denoted P1 (46)), remained on the cell surface even when c-Cbl was overexpressed (Fig. 5B). Finally, we tested the oncogenic counterpart of c-Cbl, v-Cbl. This oncoprotein contains the SH2 domain but lacks the RING finger, and therefore it acts as a dominant negative mutant of c-Cbl (32, 55). Evidently, v-Cbl was unable to down-regulate surface-exposed Neu molecules, and it could not suppress Shc recruitment by Neu (Fig. 5A). Furthermore, when v-Cbl was co-expressed together with c-Cbl, it interfered with the action of c-Cbl and restored cell surface receptor levels, as well as recruitment of Shc (Fig. 5A, lane labeled c-Cbl+v-Cbl). Taken together, these results raise the possibility that the SH2 domain of c-Cbl directly interacts with the carboxyl-terminal portion of Neu, thus leading to down-regulation of Neu signaling.

Fig. 4. Overexpression of c-Cbl down-regulates oncogenic Neu and inhibits its signaling. A, CHO cells were transiently transfected with expression vectors encoding either an oncogenic Neu alone or Neu and c-Cbl, as indicated. Two days post-transfection, cell monolayers were placed on ice, and their surface proteins were labeled with biotin. After removal of the excess unbound biotin reagent, cells were incubated at 37°C for the indicated time intervals. To determine the level of residual surface receptors, cell lysates were subjected to immunoprecipitation (IP) with an anti-Neu antibody, fractionated by electrophoresis, and probed with an HRP-conjugated streptavidin. The expression levels of both Neu and the hemagglutinin-tagged c-Cbl were monitored by immunoblotting (IB) of whole cell extracts with antibodies directed to either Neu or HA, as indicated (lower panels). B, CHO cells were transiently transfected with increasing concentrations of a c-cbl-encoding plasmid (indicated by a slope), along with a plasmid driving the expression of neu, or an empty control plasmid (labeled −). Two days later, cells were subjected to immunoprecipitation and immunoblotting with the indicated antibodies. To monitor co-immunoprecipitation and expression levels of Neu, c-Cbl (either an ectopic HA-tagged protein or both the endogenous and exogenous Cbl proteins), and MAPK, cell lysates were subjected to immunoprecipitation (IP) and/or immunoblotting (IB) with the indicated antibodies. An antibody to phosphotyrosine (P-Tyr) was used to detect tyrosine phosphorylation of Neu, and an antibody to the doubly phosphorylated form of MAPK detected the active form of Erk proteins. Note the appearance of both p66 and p52 isoforms of Shc. C, CHO cells were transiently transfected with a plasmid encoding an HA-tagged ubiquitin and a neu expression vector in the presence or absence of a c-cbl plasmid, as indicated. The covalent attachment of HA-tagged ubiquitin molecules to Neu was detected by immunoprecipitation with an anti-Neu antibody followed by immunoblotting with an anti-HA antibody. Note partial disappearance of Neu in c-Cbl overexpressing cells (lower panel).
down-regulation of Neu is suppressed and the oncogenic receptor retains its signaling ability.

The serum response element (SRE) that lies in the enhancer/promoter region of the fos gene integrates signaling events initiated at the cell surface by a variety of hormones, growth factors, and certain stress conditions (reviewed in Ref. 56). Signaling by Neu is funneled to the SRE through activation from the serum response element.

The major conclusion derived from the results we presented in this report is that coupling of c-Cbl to Neu/ErbB-2 occurs but only at very low efficiency. To detect this weak interaction we had to use the most active form of Neu/ErbB-2, namely an oncogenic Neu/ErbB-2 gene was originally isolated from the B104 cell line derived from a carcinogen-induced rat neuro/glioblastoma (57). To test the effects of c-Cbl on the oncogenic action of the neu-encoded protein, we infected B104 cells with a recombinant retrovirus encoding c-Cbl. Infection resulted in a 5-fold increase in c-Cbl expression, as determined by immunoblotting (Fig. 7A). Concomitant with its overexpression, c-Cbl physically associated with the transforming Neu protein (Fig. 7A). Consistent with a negative effect of c-Cbl, labeling of the cell surface with biotin and subsequent immunoprecipitation of Neu revealed its chronic down-regulation from the surface of c-Cbl-overexpressing B104 cells. Infection with a control virus, encoding only a selectable gene marker, did not affect Neu expression at the cell surface (Fig. 7A). To examine the down-regulatory function of c-Cbl in vivo, we injected the c-Cbl-infected cells as well as their control infected cells into athymic mice. This led to the appearance of tumors of variable size already within the 1st week after injection. Western blot analysis and quantification of the c-Cbl protein in individual tumors, which were scored 24 days after injection, revealed an inverse relationship between tumor volume and the expression levels of c-Cbl (Fig. 7B). By contrast, no similar correlation was observed when individual clones of the B104-Puro control subline were grown as tumors in mice. Thus, in combination with the biochemical data we presented, the inverse relationship between c-Cbl expression and tumor size suggests that overexpression of c-Cbl can inhibit tumor growth driven by the neu oncogene.

**DISCUSSION**

The major conclusion derived from the results we presented in this report is that coupling of c-Cbl to Neu/ErbB-2 occurs but only at very low efficiency. To detect this weak interaction we had to use the most active form of Neu/ErbB-2, namely an oncogenic rodent form carrying a mutation at the transmembrane domain (7). This form of Neu exists in homodimers (9), and therefore it displays constitutively high tyrosine kinase activity (8, 58). Previous works that addressed the interaction between ErbB-2 and c-Cbl by using natural and artificial li-
plotted data were subjected to linear regression analysis. The open triangles.

Closed circles.

The activity of Neu/ErbB-2 as shown below. (iii) Because Neu/ErbB-2 has no endogenous ligand (63), homodimers of ErbB-2 may not form under physiological conditions. However, an oncogenic mutation (9), overexpression at the cell surface (64), and aggregation by a bivalent antibody (59) or by a synthetic intracellular ligand (41) lead to homodimer formation. In some of these cases coupling to c-Cbl has been demonstrated and associated with the mechanism of immunotherapy. Thus, coupling of Neu to c-Cbl may play a role in restricting transformation by an active Neu or by an overexpressed human ErbB-2.

Alternatively, the importance of the relatively weak interactions between ErbB-2 and c-Cbl, as compared with the respective coupling of ErbB-1 to c-Cbl (Fig. 2B), may be exemplified from an evolutionary perspective. Unlike mammals, which express four different ErbB proteins, invertebrates such as the nematode Caenorhabditis elegans and the fruit fly Drosophila express only a single ErbB ortholog (65). Nevertheless, both invertebrate forms of the ErbB receptors can recruit c-Cbl, and the latter acts as their negative regulator (66–68). Along with the gene duplication events that gave rise to the four ErbB proteins, each receptor acquired a set of docking sites capable of recruiting partly overlapping repertoires of signaling proteins (69). This generated a layered signaling network whose ability to diversify growth factor signals is enormously high (70). Interestingly, however, diversity is not limited to positive signaling pathways, but it also affects negative regulatory pathways. Examples include the Ras-specific GTPase-activating protein and certain tyrosine phosphatases. Moreover, the four ErbB proteins display remarkably different negative regulation through endocytosis and degradation (47, 71). Thus, whereas ErbB-1 is targeted primarily to lysosomal degradation, and the neuregulin receptor ErbB-3 is constitutively recycled (72), ErbB-2 is a slowly internalizing receptor that can inhibit the rate of down-regulation of ErbB-1 (61). Because c-Cbl can control sorting to lysosomal degradation or to recycling (32), and it may also affect the rate of receptor internalization (36), earlier conclusions that ErbB-2 is uncoupled to c-Cbl seemed an attractive explanation to the rather unique endocytic behavior of this receptor.

Nevertheless, several observations cast some doubt on the lack of Cbl-ErbB-2 interactions. Thus, the rates of internalization and degradation of the oncogenic Neu protein are remarkably high (8). In addition, antibodies to Neu/ErbB-2 can enhance endocytosis and degradation of the wild type receptor through a pathway that resembles that taken by a ligand-activated ErbB-1 (8, 73). In fact, neuregulin can enhance polyubiquitination of ErbB-2 upon binding to its own direct receptor (44). Consistently, Neu recruits the clathrin-associated adaptin molecule (AP-2), and it undergoes endocytosis through clathrin-coated pits (43). Thus, our present conclusion that c-Cbl can recruit a fully active ErbB-2, albeit at low efficiency, reconciles earlier observations (40–42) with the endocytic behavior of ErbB-2. Accordingly, when ErbB-2 is stimulated it forms a physical complex with c-Cbl, similar to the association that occurs between c-Cbl and ErbB-1 (33, 38). It is likely that the variant SH2 domain of c-Cbl binds to one of two potential docking sites of c-Cbl (consensus sequence: pYXXXp) located at the carboxy-terminal domain of ErbB-2 (Fig. 5). It is relevant that analysis of a series of ErbB-1/ErbB-2 chimeric proteins attributed the relatively slow rate of ErbB-2 endocytosis to the latter domain of the oncoprotein (74). Consistent with direct interactions between c-Cbl and the C-tail of ErbB-2, mutagenesis of one of the two potential docking sites of ErbB-2 rendered the receptor refractory to mAb-induced down-regulation (59).
Moreover, EGF and mAb-induced down-regulation of this mutant form of ErbB-2 (tyrosine 1112 replaced by a phenylalanine) is significantly slower than the rate displayed by the wild type receptor. Despite these observations, the involvement of indirect mechanisms cannot be excluded. For example, the c-Cbl binds Grb-2, Shc, and PI 3-kinase may indirectly link the ubiquitin ligase to ErbB-2. Consistent with this alternative model, the fully active form of ErbB-2 exists in a physical complex with PI 3-kinase and Shc (Figs. 3–5). Although we have previously reported that PI 3-kinase activity associates with Neu, this receptor lacks the tyrosine-based consensus sequence for p85 binding (pXXM) and may not directly bind the receptor (22). On the other hand, a specific tyrosine located at the carboxyl-terminal domain of c-Cbl has been identified as a p85-binding site, which may mediate the interaction between p85 and ErbB-1 (54, 75). We therefore infer that c-Cbl may similarly couple p85 to Neu.

The relatively weak coupling of c-Cbl to ErbB-2 may explain, in part, why this receptor is transforming when overexpressed (60, 76). Consistent with this proposition, an endocytosis-defective mutant of ErbB-1 is more mitogenic and transforming than the wild type receptor (77). Indeed, a correlation exists between c-Cbl coupling, rate of ErbB endocytosis, and relatively weak mitogenesis (47). Similarly, superior proliferative activity, achieved by heterodimer formation between ErbB-2 and the kinase-defective ErbB-3 receptor, has been attributed to the escape of the ErbB-2/ErbB-3 complex from c-Cbl-induced degradation (48). These considerations are relevant to human cancers; the ErbB-2/ErbB-3 complex is thought to act as a major proliferative engine of carcinomas, especially when ErbB-2 is overexpressed (78). In addition, ErbB-2 appears to act as a ligand-less co-receptor capable of amplifying signaling by other ErbB proteins (63). Thus, inefficient clearance of ErbB-2 from the cell surface and its slow intracellular degradation may render this oncoprotein available for repeated engagements in heterodimer formation. Blocking the tyrosine kinase activity of ErbB-2 by using specific tyrophostins, antisense-based reduction or ErbB-2 expression was markedly reduced in the lymph nodes of mice following their infection with a retrovirus that causes a murine acquired immunodeficiency syndrome (79). Taken together, the data we presented suggest that tethering oncogenic growth factor receptors like Neu/ErbB-2 to the endocytic degradation pathway may be an applicable approach for the suppression of transforming signaling pathways.
43. Gilboa, L., Ben-Levy, R., Yarden, Y., and Henis, Y. (1995) *J. Biol. Chem.* **270**, 7061–7067
44. Magnifico, A., Tagliabue, E., Ardini, E., Casalini, P., Colnaghi, M. I., and Menard, S. (1998) *FEBS Lett.* **422**, 129–131
45. Minnaugh, E. G., Chavany, C., and Neckers, L. (1996) *J. Biol. Chem.* **271**, 22796–22801
46. Ben-Levy, R., Paterson, H. F., Marshall, C. J., and Yarden, Y. (1994) *EMBO J.* **13**, 3302–3311
47. Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1996) *EMBO J.* **15**, 2452–2467
48. Waterman, H., Alroy, I., Strano, S., Seger, R., and Yarden, Y. (1999) *EMBO J.* **18**, 3348–3358
49. Johansen, F. E., and Prywes, R. (1994) *Mol. Cell. Biol.* **14**, 5920–5928
50. Hill, C. S., and Treisman, R. (1995) *Cell* **80**, 199–211
51. Thien, C. B. F., and Langdon, W. (1998) *Immunol. Cell Biol.* **76**, 473–482
52. Schubert, D., Heinemann, S., Carlisle, W., Tarkias, H., Kimes, B., Patrick, J., and Steinbach, J. H. (1974) *Nature* **249**, 224–227
53. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) *Mol. Cell. Biol.* **16**, 5276–5287
54. Fukazawa, T., Miyake, S., Band, V., and Band, H. (1996) *J. Biol. Chem.* **271**, 14554–14559
55. Sorkin, A., Di Fiore, P. P., and Carpenter, G. (1993) *Oncogene* **8**, 3021–3028
56. Hudziak, R. M., Schlessinger, J., and Ullrich, A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5394–5398
57. Klapper, L. N., Waterman, H., Sela, M., and Yarden, Y. (2000) *Cancer Res.* **60**, 3584–3589
58. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. (1987) *Science* **237**, 178–182
59. Worleylake, R., Opresko, L. K., and Wiley, H. S. (1999) *J. Biol. Chem.* **274**, 8865–8874
60. Lenerfer, A. E., Pinkas Kramarski, R., van de Poll, M. L., van Vught, M. J., Klapper, L. N., Tzahar, E., Waterman, H., Sela, M., van Zelen, E. J., and Yarden, Y. (1996) *EMBO J.* **17**, 3385–3397
61. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) *Mol. Cell. Biol.* **16**, 5276–5287
62. Waterman, H., Sabanai, I., Geiger, B., and Yarden, Y. (1998) *J. Biol. Chem.* **273**, 13819–13827
63. Carraway, K. L., and Cantley, L. C. (1994) *Cell* **78**, 5–8
64. Burden, S., and Yarden, Y. (1997) *Neuron* **18**, 847–855
65. Feshchenko, E. A., Langdon, W. Y., and Tsygankov, A. Y. (1998) *J. Biol. Chem.* **273**, 8323–8331
66. Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1990) *Science* **247**, 962–964
67. Trebak, M., Lambert, C. A., Rahmouni, S., Debrus, S., Defresne, M. P., Regnier, D., Boniver, J., and Moutschen, M. (1998) *Cell Immunol.* **188**, 151–157