Association with HSP90 Inhibits Cbl-Mediated Down-regulation of Mutant Epidermal Growth Factor Receptors

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Abstract
Activating mutations in the epidermal growth factor receptor (EGFR), localized in the activation loop within the kinase domain, have been discovered in non–small cell lung cancers (NSCLC). Most of these mutants are exquisitely sensitive to EGFR tyrosine kinase inhibitors, suggesting that they generate receptor dependence in the cancers that express them. 32D cells stably expressing EGFR-L858R but not wild-type EGFR exhibited ligand-independent receptor phosphorylation and viability. Ligand-induced receptor down-regulation (LIRD) was impaired in mutant-expressing cells. The EGFR mutants were constitutively associated with the E3 ubiquitin ligase Cbl but did not associate with the adaptor protein CIN85 on the addition of ligand. Inhibition of HSP90 activity with geldanamycin restored Cbl function as indicated by receptor ubiquitination and LIRD. These results suggest that EGFR mutants form defective endocytic complexes. In addition, HSP90 plays a role in maintaining the functional conformation of EGFR mutants and protecting activated receptors from LIRD. (Cancer Res 2006; 66(14): 6990-7)

Introduction
The epidermal growth factor receptor (EGFR) is a molecular sensor that perceives signals from outside of cells and generates intracellular signals for growth, proliferation, migration, and differentiation (1). In normal tissues, the temporal and spatial distribution of extracellular growth factors determines the quantity and quality of intracellular signals. However, if cells have abnormal sensor(s), they will exhibit uncontrolled proliferation and/or enhanced survival. Therefore, deregulation of EGFR signaling provides an opportunity for abnormal cell proliferation potentially leading to tumor development. Gene amplification and/or over-expression of EGFR have been reported in several tumors, including bladder, breast, head and neck, and kidney cancers, non–small cell lung cancer (NSCLC), and prostate cancer. EGFR vIII, a constitutively active mutant lacking a portion of the extracellular domain of the receptor, has been observed in glioblastomas (2). Similarly, the retroviral versions E1v-ErbB and S3v-ErbB lacking the ligand-binding domain and a COOH-terminal fragment show ligand-independent activation (3, 4).

In addition to amplification of signal transduction by the EGFR, maintenance of the active state of receptor as a result of impaired desensitization has been shown to contribute to oncogenicity (5, 6). Once activated by stimulation, EGFR initiates both positive and negative processes that convey signals to downstream pathways via the interaction with adaptor proteins (7) and remove receptors from the cell surface (8). In the latter case, the Cbl family of ubiquitin ligases plays a major role in ligand-dependent ubiquitination of many receptor tyrosine kinases (RTK; ref. 9). For the EGFR, Cbl ubiquitinates activated receptors, and this post-translational modification carries both internalization and degradation signals that control endocytosis and sorting of receptors for destruction in the lysosome (10). Cbl also promotes EGFR internalization via its interaction with the adaptor protein CIN85 (11), which constitutively interacts with endophilins, the regulatory component of clathrin-coated pits. Mutations that prevent interaction between Cbl and CIN85 have been shown to block receptor endocytosis and degradation without affecting Cbl-mediated active EGFR ubiquitination (11). Therefore, recognition and modification of activated EGFR by Cbl-CIN85 complex controls receptor levels, their intracellular trafficking, and the intensity of signaling output.

EGFR gene mutations have recently been identified in specific subsets of patients with NSCLC (12–14). The mutations are clinically relevant, as they are linked to tumor sensitivity to ATP-competitive, small-molecule EGFR inhibitors, such as gefitinib and erlotinib. These are localized in exons 18, 19, 20, and 21 of the EGFR gene, which encode the kinase domain. Although a secondary mutation responsible for drug resistance has also been predicted (15) and reported (16), the molecular mechanism of signaling by drug-sensitive and drug-resistant EGFRs is not entirely defined. As EGFRs prefer homodimerization or heterodimerization with other ErbB family members, it is not easy to address the sole and intrinsic function of these EGFR mutants. Therefore, to minimize background signaling caused by other ErbB receptors and ligands, we developed a “clean system” with 32D mouse hematopoietic cells. These are interleukin-3 (IL-3)–dependent cells, which do not express any of the ErbB coreceptors or ligands and have been used previously to study EGFR signaling (17–21) as well as that of other oncogenic tyrosine kinases (22–24).

Thus, we used 32D cells transfected with EGFR mutant vectors and human lung cancer cell lines harboring EGFR gene mutations in the activation loop. We report herein that EGFR mutants escaped ligand-induced receptor down-regulation (LIRD) and exhibited constitutive association with HSP90 and Cbl. The receptor mutants preferred the unphosphorylated form of Cbl and failed to recruit CIN85 to the endocytic complex on the addition of ligand. Inhibition of HSP90 permitted down-regulation of EGFR mutants in response to ligand. Taken together, these findings highlight a novel mechanism of increased signaling involving impaired receptor desensitization and maintenance of an active conformation as a result of binding to HSP90.
Impaired Down-regulation of Mutant EGF Receptors

Materials and Methods

Cell culture and reagents. The IL-3-dependent hematopoietic 32D cell line was obtained from Graham Carpenter (Vanderbilt University, Nashville, TN), maintained in RPMI 1640 containing 15% FCS and 10% WEHI-conditioned medium (as a source of IL-3), and supplemented with antibiotics. The human lung cancer cell line NCI H3255 was provided by Bruce Johnson (Dana-Farber Cancer Institute, Boston MA); the NCI H1975 line was a gift from Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). Both were maintained as described previously (25). Gefitinib was obtained from AstraZeneca Pharmaceuticals (Wilmington, DE); erlotinib was provided by Mark Sliwkowski (Genentech, South San Francisco, CA); cetuximab and its monovalent Fab fragments were a gift from Dan Hicklin (Imclone Systems, Inc., New York, NY); and PS-341 was provided by Millenium Pharmaceuticals (Cambridge, MA). Transforming growth factor-α (TGF-α) and geldanamycin were from R&D Systems (Minneapolis, MN) and Calbiochem (San Diego, CA), respectively.

Cell viability assay. Growth and viability of 32D cells (transfected with EGFRs) was analyzed with the trypan blue dye exclusion assay. At the indicated times, cells were harvested and resuspended in a trypan blue solution and counted using a hemocytometer. The cells with and without blue dye staining inside were recorded as dead and alive, respectively.

Western blot and immunoprecipitation. Cells were washed with PBS once, disrupted on ice for 30 minutes in NP-40 lysis buffer, and cleared by centrifugation. Protein concentration was determined with BCA reagent once, disrupted on ice for 30 minutes in NP-40 lysis buffer, and cleared by centrifugation. Protein concentration was determined with BCA reagent.

Figure 1. Ligand-independent phosphorylation of EGFR mutants is supersensitive to EGFR small-molecule inhibitors. A. 32D cells were stably transfected with WT or mutant (Q and R) EGFR expressing retroviral vectors. Cells were grown in serum-free or TGF-α (20 ng/ml)-containing medium with or without gefitinib (1 μM). Cell growth was monitored as indicated in Materials and Methods. Representative of three independent experiments using each cell line. Points, mean percentage of viable cells. B, cells were grown in the presence of various concentrations of gefitinib (0, 0.01, 0.1, and 1 μM) for 2 hours. Cell lysates were prepared, and Western blot analyses were done with the indicated antibodies. C, cells were treated with TGF-α (20 ng/ml) in the presence or absence of EGFR inhibitors (gefitinib, erlotinib, cetuximab, and monovalent Fab of cetuximab). Lysates were prepared, separated by SDS-PAGE, and then analyzed by Western blot.

3 hours while rocking. Precipitates were washed thrice with lysis buffer and once with PBS, resuspended in 2× Laemmli buffer, and resolved by SDS-PAGE followed by immunoblot analysis.

Plasmid construct and stable transfection. The pMSCVpuro-IRES-EGFP vector is derived from pMSCVpuro and pIRE2-EGFP vector (Clontech Laboratories, Inc., Mountain View, CA). A 1.6-kb XhoI/HpaI IRES-EGFP fragment from pIRE2-EGFP was cloned into pMSCVpuro vector (EcoRI site deleted) at same sites. A synthetic DNA fragment containing unique cloning sites was cloned into the resultant vector to generate pMSCVpuro-IRES-EGFP. Unique cloning sites in it were RggIII, XhoI, PmeI, BstBI, MluI, EcoRI, SwaI, NotI, and BamHI (in order), EGFR wild-type (WT) cDNA sequence (accession no. NM_005228) was introduced into pcDNA3.1 vector (Invitrogen, Carlsbad, CA) to add myc and His tags at the COOH terminal. Mutations were introduced in the EGFR sequence using QuikChange Site-Directed Mutagenesis kit (Strategene, La Jolla, CA) and using the following primers: for L858R (t2573g) sense, 5’-CACA-GATTGTTGGCGGTCCTGTTGGA-3’, and L858R (t2573g) antisense, 5’-CCCCAGCAGTTTGGCCCCCGAAAAATCTGTTG3’; for L861Q (t2582a) sense, 5’-GGGTTGGAAAGGGGAGGAAATTGCGGAA-3’, and L861Q (t2582a) antisense, 5’-TTCCGACCCCAAGCTTGTGTTGGCCAGCC-3’. Mutations were confirmed by sequencing (Vanderbilt DNA Sequencing Core Laboratory). WT EGFR and mutants were cut from pcDNA3.1 with PmeI and ligated into the pMSCVpuro-IRES-EGFP retrovirus vector. cDNA insert and correct orientation was confirmed by digestion with BglII. All constructs, including null vector, were stably transfected into 32D cells and sorted by fluorescence-activated cell sorting (FACS) based on green fluorescence expression. The expression of these receptors was regularly measured by Western blotting and FACS.

Xenograft studies. Six-week-old athymic BALB/c female mice [National Cancer Institute (NCI)-Frederick Cancer Center, Frederick, MD] were maintained in pressurized ventilated cages. Experiments were carried out under an Institutional Animal Care and Use Committee approved protocol, and institutional guidelines for the humane use of animals in research were followed. To generate xenograft tumor bearing mice, 5×10⁶ H1975 cells were mixed with Matrigel (Collaborative Research, Bedford, MA) and inoculated s.c. in the right flank via a 22-gauge needle. Mice were treated with 17-allylaminogeldanamycin (17-AAG) or the egg phospholipid vehicle only as control. 17-AAG was given thrice weekly using one of two schedules (Monday, Wednesday, and Friday).
Wednesday, and Friday) or (Monday, Tuesday, and Wednesday). Mice were weighed, and tumor volumes were calculated with the formula: \( \pi / 6 \times \text{larger diameter} \times \text{(smaller diameter)}^2 \). To analyze cellular markers, mice with established tumors were treated with one dose 17-AAG (50, 75, or 100 mg/kg) or vehicle only as control and then sacrificed 6 hours later. For immunoblotting, tumor tissue was homogenized in 2% SDS lysis buffer (pH 7.4).

**Results**

**EGFR mutants are more sensitive to small-molecule EGFR tyrosine kinase inhibitors.** We stably expressed retroviral vectors encoding EGFR-WT, L861Q, L858R, or null vector into 32D cells. These are mouse hematopoietic progenitors that lack endogenous ErbB receptors or ligands (17), making them ideal for biochemical characterization of the mutant receptors. Stable expression of each construct was confirmed by Western blot of green fluorescent protein–sorted cells (data not shown). Because the growth of 32D cells expressing EGFR is dependent on IL-3 and EGFR ligands (18), we examined if mutant receptor expression made IL-3 and/or EGFR ligands dispensable. Growth of 32D-EGFR-L861Q and 32D-EGFR-L858R cells in the presence of TGF-\( \alpha \) was more sensitive to the EGFR inhibitor gefitinib than that of 32D-EGFR-WT (Fig. 1A). We next examined the effect of the gefitinib and erlotinib, which reversibly inhibit ATP binding to the EGFR tyrosine kinase (26). In response to ligand stimulation, the phosphorylation on tyrosine residues of both mutant receptors was markedly more sensitive to gefitinib than WT EGFR (Fig. 1B).

As other oncogenic tyrosine kinase mutations identified in the activation loop confer an active conformation, we hypothesized that mutant EGFR display ligand-independent activation. L858R and L861Q were constitutively phosphorylated, whereas the WT EGFR required ligand to become phosphorylated (Fig. 1C). Cells were next stimulated with TGF-\( \alpha \) in the presence or absence of receptor inhibitors. Cetuximab partially reduced ligand-stimulated EGFR-WT but not mutant receptor phosphorylation. Monovalent Fab fragments of cetuximab had no effect. Gefitinib and erlotinib inhibited phosphorylation of all EGFRs regardless of TGF-\( \alpha \) stimulation (Fig. 1C).

**EGFR mutants generate ligand-independent cell survival.** In the absence of serum and IL-3, 32D cells rapidly undergo apoptosis. Therefore, we examined if mutant EGFRs will abrogate cell death under these conditions. Both 32D-EGFR-L861Q and 32D-EGFR-L858R cells but not cells expressing WT receptor proliferated for the first >24 hours following serum and IL-3 deprivation. However, all cells exhibited low viability after 3 days in culture (Fig. 2A), suggesting that survival signals generated by mutant EGFRs do not completely replace those provided by serum and IL-3. This difference in viability was more marked in serum-starved cells treated with TGF-\( \alpha \) (Fig. 2A), suggesting that the mutants are hyperresponsive to receptor ligands. In both 32D-EGFR-L861Q and 32D-EGFR-L858R cells, EGFR phosphorylation at Tyr1068 and AKT phosphorylation in Ser473 were detected in the presence or absence of ligand, whereas, in cells expressing EGFR-WT, these were phosphorylated only in the presence of TGF-\( \alpha \) (Fig. 2B).

**EGFR mutants avoid LIRD.** In response to ligand stimulation, the EGFR undergoes a process of desensitization involving endocytosis, down-regulation from the cell surface, and lysosomal degradation (27). Data shown in Figs. 1C and 2B indicated an atypical persistence of the EGFR mutants in ligand-stimulated cells. To extend these findings, we examined receptor levels in cells treated with TGF-\( \alpha \) for 1 to 6 hours. In 32D-EGFR-WT cells, receptor levels were markedly reduced 2 hours after ligand stimulation. However, 32D-EGFR-L861Q and 32D-EGFR-L858R cells maintained receptor levels on ligand stimulation (Fig. 3A). Similar results to these were observed in H3255 and H1975 human lung cancer cells. H3255 cells express L858R EGFR, and H1975 cells contain a receptor with two missense mutations, L858R and T790M (Fig. 3A, bottom).

To investigate mechanisms of receptor down-regulation, we examined the association with Cbl in EGFR immunoprecipitates. Similar results to these were observed in H3255 and H1975 human lung cancer cells. H3255 cells express L858R EGFR, and H1975 cells contain a receptor with two missense mutations, L858R and T790M (Fig. 3A, bottom).

To investigate mechanisms of receptor down-regulation, we examined the association with Cbl in EGFR immunoprecipitates. Similar to previous observations (28, 29), EGFR-WT showed ligand-dependent association with Cbl, whereas, in EGFR-L861Q and EGFR-L858R cells, the receptor-Cbl association was constitutive (Fig. 3B). Similarly, EGFR phosphorylation at Tyr1068, the Cbl-binding site, was constitutive in cells expressing mutant EGFR but...
ligand dependent in cells expressing WT receptor (Fig. 3B). This result raised the intriguing question about how the EGFR mutants can escape Cbl-mediated down-regulation despite being stably associated with the ubiquitin ligase. Thus, we examined the phosphorylated status of receptor-associated Cbl because this has been shown to negatively regulate Cbl function without affecting its binding to RTKs (30). In 32D-EGFR-WT cells, Cbl displayed ligand-dependent phosphorylation at Tyr774, whereas, in 32D-EGFR-L861Q and 32D-EGFR-L858R cells, Cbl showed constitutive phosphorylation with or without ligand stimulation (Fig. 3B). Interestingly, ligand addition reduced receptor-associated p-Cbl from EGFR-L861Q and EGFR-L858R but increased it from EGFR-WT. We confirmed this using P-Tyr immunoblots of EGFR pull-downs (120-kDa band in Fig. 3C).

These data suggest that mutant receptors prefer binding of unphosphorylated Cbl, whereas WT receptor recruits phosphorylated Cbl in response to the ligand. As COOH-terminal residues of Cbl, including Tyr774, link to binding of CIN85, we hypothesized that dephosphorylation of Cbl abrogates CIN85 binding to the EGFR-Cbl complex. Therefore, we next examined the association of CIN85 to the EGFR-Cbl complex. In contrast to EGFR-WT-Cbl-CIN85 complex formation by ligand stimulation, EGFR-L861Q and EGFR-L858R showed lack of association with CIN85 even in the presence of TGF-α (Fig. 3C). These data suggest that the loss of phosphorylation of Cbl results in failure of CIN85 recruitment to the mutant EGFR-Cbl complex.

HSP90 protects EGFR mutants from LIRD. Chaperone molecules, such as HSP90, contribute to the stability of oncogenic kinases. HSP90 helps the folding of nascent polypeptides as well as stabilizes mature kinases, such as HER-2 (ErbB2), Raf, and Src-family kinases. Thus, we proposed that stabilization by HSP90 may contribute to the resistance of EGFR mutants to LIRD. To test this speculation, we examined the association of receptors with HSP90. HSP90 was detected in immunoprecipitates of EGFR from 32D-EGFR-L861Q cells but not from 32D-EGFR-WT cells (Fig. 4A). This observation was similar to a recent report using lung cancer cells harboring EGFR mutations (25). To determine the dependence of the EGFR mutants on its association with the chaperone, we examined the effect of the HSP90 inhibitor geldanamycin on receptor stability. Geldanamycin binds to an NH2-terminal ATP-binding pocket of HSP90, thus inhibiting its function by preventing the folding of HSP90-dependent client proteins. On the addition of geldanamycin, receptor depletion was markedly faster in 32D-EGFR-L861Q and 32D-EGFR-L858R cells than 32D-EGFR-WT cells (Fig. 4B and C).

Inhibition of HSP90 enhances LIRD and ubiquitination of mutant EGFRs. Stabilization of conformationally labile kinases is a unique function of HSP90 (31). In addition to stable maturation of
the receptor mutants, we speculated that, by maintaining the EGFR mutants at the cell surface, HSP90 protected these receptors from LIRD. HER-2, another oncogenic kinase within the ErbB family of receptors, is known to associate with HSP90 via its kinase domain (32). If in fact HSP90 impaired Cbl-mediated receptor down-regulation, inhibition of HSP90 function would then restore ligand-induced receptor ubiquitination and down-regulation.

We therefore examined depletion of receptors using TGF-α in combination with the HSP90 inhibitor. On the addition of TGF-α, 32D-EGFR-WT cells exhibited receptor down-regulation, whereas EGFR levels in both 32D-EGFR-L861Q and 32D-EGFR-L858R cells were stable. A low dose of geldanamycin (0.1 μmol/L) had no appreciable effect against WT or the EGFR mutants. However, geldanamycin, in combination with TGF-α (20 ng/mL), enhanced down-regulation of both WT and the mutant receptors at 3 hours (Fig. 5A, top). Similar to these results in transfected 32D cells, combination treatment induced receptor down-regulation in both H1975 and H3255 NSCLC lines, whereas each single treatment showed little effect on EGFR protein steady-state levels (Fig. 5A, middle and bottom). These data suggest that geldanamycin-mediated inhibition of HSP90 function restores the sensitivity of EGFR mutants to LIRD. This was associated with restoration of ligand-induced receptor ubiquitination. TGF-α induced EGFR ubiquitination in 32D-EGFR-WT but not in 32D cells or NSCLC lines expressing mutant receptors. However, 32D-EGFR-L861Q, 32D-EGFR-L858R, H1975, and H3255 cells displayed EGFR ubiquitination only in response to geldanamycin alone or cotreatment of geldanamycin and TGF-α (Fig. 5B), implying that EGFR mutants interact with HSP90 not only for stabilization of their mature structure but also for prevention of ubiquitination in response to ligand.

**Inhibition of HSP90 destabilizes mutant EGFR in vivo.** Finally, we determined if blockade of HSP90 function with 17-AAG also inhibited the expression of mutant receptors in vivo. Nude mice bearing established gefitinib-resistant H1975 xenografts were treated with a single dose of 50 to 100 mg/kg of 17-AAG. Tumors were harvested 6 hours later followed by immunoblot analyses of tumor homogenates. There was a dose-dependent decrease in EGFR, HER-2, Raf-1, and cyclin D1 tumor content in response to treatment. Simultaneously, levels of phosphorylated Akt and mitogen-activated protein kinase (MAPK) were reduced, although total Akt and total MAPK were not altered. (Fig. 6A). H1975 xenografts are relatively homogeneous with cancer cells (data not shown). Therefore, although we cannot rule out a contribution from tumor/host stroma to the overall level of the proteins detected by immunoblot, we surmise that the majority of these signals are coming from lung cancer cells. Treatment with 17-AAG for 2 weeks inhibited tumor growth (Fig. 6B), suggesting that pharmacologic inhibition of HSP90 might be therapeutic against NSCLC harboring drug-resistant and sensitive-activating EGFR mutations.

**Discussion**

We used the L858R and L861Q EGFR mutants in this report. L858R is one of the two most common EGFR mutations detected in NSCLC (12). L861Q was identified by a mutagenesis screen in mice; this mutant EGFR exhibits increased tyrosine kinase activity and steady-state lower protein levels compared with WT receptor (33). The location of both L861Q and L858R is juxtaposed with each other in the activation loop, which provides the platform for kinase substrate(s) and modulates the catalytic activity of kinase by promoting a conformational change (34). Furthermore, oncogenic mutations in other RTKs involve an amino acid residue to the L861 of EGFR (35, 36). We observed ligand-independent phosphorylation of mutant EGFRs (Fig. 1), suggesting that the mutants turn on survival signal transduction pathways, such as Akt in a ligand-independent manner. However, the oncogenic signals generated by the EGFR mutants were not sufficient for sustained growth of 32D cells in the absence of serum with or without added receptor ligand (Fig. 2A). This result suggests that the 32D cells still require other growth factors, such as IL-3, for continuous proliferation and survival.

The EGFR mutants displayed ligand-independent phosphorylation at the Tyr1045, the Cbl-binding site, stable association with Cbl, and impaired ligand-mediated receptor ubiquitination and down-regulation. Previously reported mechanisms of impaired degradation of RTKs have mainly involved the sequestration of Cbl from activated EGFR (37, 38) rather than the constitutive association between both molecules. Thus, our data would imply an impairment in the processes that follow Cbl binding to mutant EGFRs. Eps15, Endophilin-2, CALM, Rabaptin-5, CIN85, and Hip1 are among several adaptor proteins involved in receptor down-regulation (39). CIN85 is recruited to the EGFR endocytic complex.
by Cbl (11, 40). In this study, we discovered that mutant receptors failed to recruit CIN85 to the EGFR-Cbl complex in response to TGF-α (Fig. 3C). This is the first report of impaired down-regulation of activated EGFR that is persistently associated with Cbl. The EGFR mutant-associated Cbl is mainly unphosphorylated, whereas Cbl bound to ligand-stimulated WT EGFR is tyrosine phosphorylated (Fig. 3B and C). Because the tyrosine-rich tail of Cbl plays a role in the recruitment of CIN85 to activated receptors (5), the preference of the mutants to bind unphosphorylated forms of Cbl may explain the defective recruitment of CIN85. The lack of receptor ubiquitination in TGF-α-stimulated 32D-EGFR-L861Q and 32D-EGFR-L858R cells suggests a mechanism inhibiting Cbl-mediated ubiquitination of the mutants. Several mechanisms have been proposed to explain negative regulation of Cbl function leading to receptor stabilization, such as dephosphorylation of Cbl by the SHP1 phosphatase, degradation of Cbl by AIP4, and sequestration of Cbl from activated receptors (30). Our data herein do not support any of these mechanisms.

Whereas, in normal tissues, RTKs achieve active conformation of their kinase domain on the binding of ligand or phosphorylation of tyrosine residue(s) in the activation loop (41), oncogenic mutations in the activation loop can trigger a conformational shift of the αC-helix toward an active state of the kinase domain without ligand stimulation (35, 36). Maintenance of this active conformation is required by oncogenic kinases to be protected from proteasomal degradation, which is designed for desensitization of signaling. Oncogenic kinases have been shown to adopt various mechanisms that spare them from these negative regulatory processes. One mechanism that alleviates the conformational instability of oncogenic proteins is their association with the HSP90 chaperone (31, 42, 43). Indeed, over 100 proteins, including signal transducers and transcription factors interact with HSP90 along with cochaperones (44, 45). For example, a point mutation within the kinase domain of the EGFR-homologous HER-2 receptor alters its conformation, resulting in its dissociation from HSP90. Conversely, substitution of one amino acid within the kinase domain of EGFR, such as D746G, confers binding of HSP90 (46). Shimamura et al. reported recently the association of EGFR mutants in human lung cancer cells with HSP90 (25). Other oncogenic kinases, such as Src, B-Raf, and Met are also associated with HSP90 (27, 47).

In this report, the EGFR mutants in 32D cells showed stable association with HSP90. Treatment with the HSP90 inhibitor geldanamycin induced degradation of the mutant receptors, which was much slower for WT EGFR (Fig. 4). The constitutive association of both mutant receptors with HSP90 but not the more geldanamycin-resistant WT EGFR suggests that the degradation on treatment with geldanamycin is HSP90 specific. Of note, other small molecules that also bind the HSP90 pocket

**Figure 5.** Inhibition of HSP90 enhances LIRD. A, 32D and cancer cells (H3255 and H1975) were subjected to indicated times of treatment with TGF-α (20 ng/ml), geldanamycin (0.1 μmol/L), or a mixture of both compounds at these doses. Cell lysates were prepared and analyzed by Western blot. B, cells were grown in medium containing PS-341 (100 nmol/L) for 2 hours and then treated with TGF-α, geldanamycin, or both together at the same concentrations used in (A) but for only 10 minutes. EGFR was precipitated with cetuximab as indicated in Materials and Methods, and immune complexes were analyzed by Western blot using ubiquitin (Ub) and EGFR antibodies.
Inhibition of HSP90 with geldanamycin reconstituted mutant (50), our result implies that Cbl and HSP90 bind independently.

Interestingly, EGFR-L861Q and EGFR-L858R showed constitutive levels of both mutant receptors are likely to be HSP90 specific. We surmise that the effects observed on the steady-state phosphorylation at Tyr 1045 in the COOH-terminal tail (49), whereas HSP90 binds to the receptor via its kinase domain (50), our result implies that Cbl and HSP90 bind independently. Inhibition of HSP90 with geldanamycin reconstituted mutant receptor ubiquitination and receptor degradation both in vitro and in vivo.

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The results also suggest that combinations of EGFR and HSP90 inhibitors are worth exploring against NSCLC containing EGFR mutations in the activation loop.

Acknowledgments

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