Acquired Substrate Preference for GAB1 Protein Bestows Transforming Activity to ERBB2 Kinase Lung Cancer Mutants

Ying-Xin Fan, Lily Wong, Michael P. Marino, Wu Ou, Yi Shen, Wen Jin Wu, Kwok-Kin Wong, Jakob Reiser, and Gibbes R. Johnson

From the Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Division of Monoclonal Antibodies, Center for Drug Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892 and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

Background: Activating ERBB2 mutants drive tumor formation.
Results: Oncogenic ERBB2 has a striking substrate preference for GAB1 in vitro, and GAB1 hyper-phosphorylation is required for mutant ERBB2-induced cell signaling and transformation.
Conclusion: Acquired substrate preference for GAB1 is critical to the ERBB2 mutant-mediated oncogenesis.
Significance: Understanding the activation mechanism of mutant ERBB2s may lead to the development of therapies targeted against these oncogenic kinases.

Activating mutations in the αC-β4 loop of the ERBB2 kinase domain, such as ERBB2YVMA and ERBB2G776V, have been identified in human lung cancers and found to drive tumor formation. Here we observe that the docking protein GAB1 is hyper-phosphorylated in carcinomas from transgenic mice and in cell lines expressing these ERBB2 cancer mutants. Using dominant negative GAB1 mutants lacking canonical tyrosine residues for SHP2 and P13K interactions or lentiviral shRNA that targets GAB1, we demonstrate that GAB1 phosphorylation is required for ERBB2 mutant-induced cell signaling, cell transformation, and tumorigenesis. An enzyme kinetic analysis comparing ERBB2YVMA to wild type using physiologically relevant peptide substrates reveals that ERBB2YVMA kinase adopts a striking preference for GAB1 phosphorylation sites as evidenced by ~150-fold increases in the specificity constants ($k_{cat}/K_m$) for several GAB1 peptides, and this change in substrate selectivity was predominantly attributed to the peptide binding affinities as reflected by the apparent $K_m$ values. Furthermore, we demonstrate that ERBB2YVMA phosphorylates GAB1 protein ~70-fold faster than wild type ERBB2 in vitro. Notably, the mutation does not significantly alter the $K_m$ for ATP or sensitivity to lapatinib, suggesting that, unlike EGFR lung cancer mutants, the ATP binding cleft of the kinase is not significantly changed. Taken together, our results indicate that the acquired substrate preference for GAB1 is critical for the ERBB2 mutant-induced oncogenesis.

Protein kinases are key players in cellular signaling, and their activity is strictly controlled by multiple layers of autoinhibition under normal physiological conditions (1–3). Mutations can cause relief of autoinhibitory constraints and constitutive activation of kinases. Many human cancers arise from and are addicted to constitutively activated protein kinases (1, 4). The development of cancer therapies targeting mutated kinases requires detailed characterization of their specific roles in the process of tumorigenesis and investigation of the underlying activation mechanisms.

ERBB2 (also known as Her2/Neu) is a member of the ERBB receptor-tyrosine kinase family that also includes EGF receptor (EGFR,3 ERBB1/Her1), ERBB3 (Her3), and ERBB4 (Her4). A unique characteristic of ERBB2 is that it does not have a specific ligand and has been considered a preferred dimerization partner for other ERBBs. Overexpression or dysregulation of ERBB2 kinase activity has been found in various human cancers, and accordingly, the receptor has been extensively studied as a therapeutic target (5, 6). An ERBB2 monoclonal antibody, Herceptin, and the tyrosine kinase inhibitor, lapatinib, have been approved for the clinical treatment of ERBB2-overexpressing breast cancer, and many inhibitors are currently in development (6). ERBB2 kinase mutations in the αC-β4 region have been identified in non-small cell lung cancers (7, 8). Our previous work has demonstrated that the unique glycine-rich αC-β4 loop plays a critical autoinhibitory role in control of the ERBB2 kinase activity (9), and this observation has been verified by a recent crystallographic structure (10). An earlier work revealed that the most common oncogenic ERBB2 mutation with an in-frame duplication of four residues YVMA before Gly-776 (ERBB2YVMA) has increased autokinase activity and is more potent than wild type (WT) kinase in cell signaling and tumorigenicity (11). Using a transgenic animal model, ERBB2YVMA has been demonstrated to drive rapid development of lung tumors, which have striking histological and radiologic phenotypic similarities to human bronchogenic adenocarcinomas (12).

This article contains supplemental Table S1 and Figs. 1 and 2.

1 To whom correspondence may be addressed: Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Food and Drug Administration, Bldg. 29A, Rm. 3B-20, 8800 Rockville Pike, Bethesda, MD 20892. E-mail: ying-xin.fan@fda.hhs.gov.

2 To whom correspondence may be addressed: Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892. E-mail: gibbes.johnson@fda.hhs.gov.

3 The abbreviation used is: EGFR, EGF receptor.
Adaptor/docking proteins are required for transmitting key downstream cellular signals from receptor kinases and contribute to the specificity and amplification of the signaling pathways by selectively recruiting and activating signaling proteins (13). GAB1 is the prototype of a docking protein family that also includes Gab2 and Gab3 (for reviews, see Refs. 14-16) and contains a pleckstrin homology domain, several proline-rich motifs, and conserved tyrosine residues that can be phosphorylated by activated receptor-tyrosine kinases. These Gab1 phosphotyrosines selectively bind Src homology 2 domain containing signaling proteins such as Shp2 and P13-kinase (P13K), and Gab1 is a critical mediator of Egrf- and Erbb2-mediated signaling (17-20). Egrf- or Erbb2-mediated Akt and Mapk signaling pathways are impaired in GAB1-deficient cells, and Gab1 has been identified as a direct substrate for Egrf and other receptor kinases (21, 22). Our previous work demonstrated that ligand-activated Egrf has an increased preference for Gab1 versus receptor autophosphorylation sites by selectively decreasing the \( K_m \) for the major Gab1 phosphorylation site (23).

The aim of our work is to understand the molecular mechanism for oncogenic signaling by Erbb2 lung cancer mutants and the role of Gab1 in this pathological process. We found that Gab1 phosphorylation is required for mutant Erbb2-induced activation of oncogenic signaling pathways, cell transformation, and tumorigenesis. To directly assess the activation mechanism, we performed a systematic enzymatic study using physiologically relevant peptide substrates corresponding to major phosphorylation sites derived from Gab1 and Erbb receptors. The results demonstrate that the oncogenic Erbb2 not only possesses dramatically increased catalytic activity relative to the WT kinase but has adopted a striking preference for Gab1 via increased binding affinity for phosphorylation sites in Gab1.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Beas2b and H1781 cells were obtained from the American Type Culture Collection, and T47D Tet-off cell line was from Clontech. Antibodies used included: Mapk (transduction laboratories), pmapk (New England Biolabs); monoclonal Gab1 (abgent); polyclonal Gab1 (Upstate biotechnology); Erbb2 (neomarkers); Myc (santa cruz), perb2 Tyr-1221/1222, pGab1 Tyr-627, Akt, pAkt Ser-473, pAkt Thr-308, and p85 of P13K (cell signaling); pGab1 Tyr-627 for immunostaining (abnova); M2 flag (sigma). Laptinib and ppi were obtained from Chemie tek and Calbiochem, respectively. Peptides were prepared as described previously (23). Myc/hexahistidine-tagged WT and mutant human Erbb2 kinase domains spanning residues 712-1029 were expressed in S9 cells and purified with Ni2+NTA affinity method as described previously (9).

**retroviruses and Lentiviruses**—To generate retroviruses encoding Myc-tagged WT or mutant Erbb2s, cDNAs were subcloned into RevTet-off vectors, and retroviruses were produced in P167 cells. The pSLIK-Neo/Rei pitt lentivector plasmids encoding Myc-tagged Erbb2 or Flag-tagged Gab1 and the pNL(CMV)/CMV/WPREΔU3 lentivector plasmid encoding Flag-tagged Gab1 were constructed as described previously (24, 25). Mutations were introduced using the QuickChange kit (Stratagene). Identities of all expression constructs were confirmed by DNA sequencing. Gab1 and Erbb3 lentiviral shRNAs were purchased from Santa cruz.

**transduction and generation of Stable cell lines**—Beas2b cells were grown in DMEM containing 10% FBS. H1781 and T47D Tet-off cells were cultured as instructed by the providers. To generate T47D cells stably expressing Erbb2s, cells were infected with WT or mutant Erbb2 retroviruses, selected with 200 \( \mu \)g/ml hygromycin, and expression was induced by removal of doxycycline for 18 h. To generate Beas2b cells stably expressing Erbb2s, cells were transduced by WT or mutant Erbb2 lentiviruses and selected with 800 \( \mu \)g/ml G418 for 2 weeks, and expression was induced with 0.1 \( \mu \)g/ml doxycycline. Expression of WT or mutant Gab1s in Beas2b or T47D cells was achieved by transduction with CMV-driven lentiviruses, and expression of Gab1s in H1781 was achieved by transduction with doxycycline-inducible lentiviruses followed by 800 \( \mu \)g/ml G418 selection and 0.1 \( \mu \)g/ml doxycycline induction. Stable Gab1 knockdown cell lines were generated by shRNA lentivirus transduction followed by selection in 4 \( \mu \)g/ml Puromycin.

**Immunoprecipitation and Immunoblot analysis**—Cells were serum-starved overnight and lysed in 50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 1 mm Na3VO4, 2 \( \mu \)g/ml aprotinin, 2 \( \mu \)g/ml leupeptin, 100 \( \mu \)g/ml phenylmethylsulfonyl fluoride, 1 mm dithiothreitol, 20 mm p-nitrophenyl phosphate, and 1% Triton X-100. Cell lysates were incubated with Gab1 monoclonal antibody for 1 h at 4 °C followed by 1 h of incubation with protein G-Sepharose. Immunoprecipitated proteins were separated on 8% SDS-polyacrylamide gels and transferred to PVDF membranes. Immunoblot analysis was performed as described previously (23).

**Immunohistochemical Staining**—Analyses were performed on formalin-fixed paraffin sections of lung tissues of age-matched normal or transgenic mice that express Erbb2YVMA (17). Slides were deparaffinized in xylene, rehydrated sequentially in ethanol, treated with target retrieval solution (Dako), quenched in hydrogen peroxide (3%), blocked in 10% normal murine serum, and incubated overnight at 4 °C with primary antibody against pGab1 Tyr-627 or normal rabbit IgG. Slides were developed using avidin-biotin peroxidase complex (Vector) and AEC substrate (invitrogen).

**Cell proliferation and Viability assay**—H1781 cells were seeded in triplicate at a density of 2 \( \times \) 104/well in 6-well plates in complete medium, and the number of cells was subsequently counted with Bio-Rad CT10 automated cell counter. For T47D viability study, cells were plated in five replicates at a density of 8 \( \times \) 103/well in 96-well plates in complete growth medium and changed to serum-free medium the next day, and survival cells were monitored with Cell Titer AQ kit (Promega). Soft Agar colony formation assay—0.5 ml of 0.5% agarose (Invitrogen) was solidified in the bottom of each well of six well plates. Beas2b (5 \( \times \) 104) cells in growth medium were mixed with 0.3% agarose in four replicates and laid on the bottom agar. Colonies measuring \( \geq 50 \mu \)m were counted under a microscope with a grid in the eyepiece. Eight randomly selected fields were counted from each well after 14 days.
Mouse Tumorigenicity Assay—Cells at ∼80% confluence were harvested by trypsinization and resuspended in serum-free medium, and 5 x 10⁶ cells in 0.1 ml were injected subcutaneously into 4-week-old female athymic nude mice (NCI, National Institutes of Health). Tumor formation was monitored twice a week, and the size of each tumor was measured with a digital caliper. The volume of the tumors was calculated by the formula: volume = width² x length/2.

Kinase Assays—Reactions and steady-state kinetic parameter measurements were performed as described previously (9, 26). The kinetic parameters and standard errors were obtained using Enzyme Kinetics of Sigma Plot.

Statistical Analyses—Means, standard deviations, and p values from t test were obtained using Sigma Plot software.

RESULTS

Oncogenic ERBB2 Mutant-induced GAB1 Phosphorylation Correlates with Activation of AKT and MAPK—Previous work has demonstrated that expression of ERBB2YVMA in human BEAS2B bronchial epithelial cells potently activates AKT and MAPK signaling pathways and induces cellular transformation (11). We stably transduced BEAS2B cells with lentiviral vectors that express green fluorescent protein (GFP), myc-tagged WT, or mutant ERBB2s from a doxycycline-inducible promoter. In addition to ERBB2YVMA and ERBB2G776VC identified in cancer, we also included the activated ERBB2G776S/G778D described in our previous work (9). The cell lysates were analyzed using specific antibodies against major phosphorylation sites in ERBB2 (Tyr-1221/1222) and GAB1 (Tyr-627). As shown in Fig. 1, A, left, and B, the expression levels of all myc-tagged ERBB2s were comparable, whereas the phosphorylation levels of ERBB2YVMA, ERBB2G776VC, and ERBB2G776S/G778D were significantly higher than WT receptor. GAB1 phosphorylation was elevated in cells expressing ERBB2YVMA or ERBB2G776VC, accompanied by markedly reduced mobility of the GAB1 bands in SDS-PAGE. Interestingly, GAB1 phosphorylation in ERBB2G776S/G778D expressing cells was significantly lower than in cells expressing the two cancer mutants. Correlated to the hyper-phosphorylation of GAB1, expression of ERBB2YVMA and ERBB2G776VC resulted in increased levels of activated AKT and MAPK. These results indicate that the oncogenic ERBB2 mutants are much more potent at inducing constitutive GAB1 phosphorylation and subsequent signaling than WT receptor and the activated ERBB2G776S/G778D.
Interactions of phosphorylated GAB1 with PI3K and SHP2 are required for receptor kinase-mediated activation of AKT and MAPK, respectively (17, 18, 27). Phosphorylation of Tyr-627 and 659 in GAB1 is essential for SHP2 association and MAPK activation (27, 28), whereas phosphorylation of Tyr-447, -472, and -589 is required for binding to the p85 subunit of PI3K and AKT signaling (17). Accordingly, we immunoprecipitated GAB1 from cell lysates and analyzed for the presence of PI3K and SHP2. As shown in Fig. 1, A, left, bottom, and D, expression of ERBB2YVMA or ERBB2G776VC in BEAS2B cells resulted in significantly increased phosphorylation of GAB1 and association of SHP2 and PI3K with GAB1, relative to WT ERBB2-expressing cells. In the ERBB2G776S/G778D cells, the increase in association of GAB1 with either SHP2 or PI3K is modest. Immunoprecipitation of SHP2 in the reciprocal experiment confirmed these findings (data not shown).

Next, we characterized signaling in T47D breast carcinoma cells that stably express WT or ERBB2YVMA (Fig. 1, A, middle, and C). Similar to that observed in BEAS-2B cells, phosphorylation levels of GAB1, AKT, and MAPK were much higher in T47D/ERBB2YVMA cells relative to WT. Correlated with its phosphorylation, GAB1 association with either SHP2 or PI3K also dramatically increased in T47D/ERBB2YVMA cells (Fig. 1, A, middle, bottom, and D). Furthermore, we detected high levels of GAB1, AKT, and MAPK phosphorylation as well as GAB1 interaction with SHP2 and PI3K in H1781 lung cancer cells, which harbor the homozygous oncogenic ERBB2G776VC mutation (Fig. 1A, right). These results demonstrate that signaling by the oncogenic ERBB2 mutants results in constitutive hyperphosphorylation of GAB1, association of SHP2 and PI3K with GAB1, and concomitant activation of AKT and MAPK. It is noteworthy that the levels of ERBB3 protein and phosphorylation in these three cell lines were very different. In BEAS2B cells, ERBB3 protein level was very low, and phosphorylation was undetectable. T47D cells had a moderate level of ERBB3, and ERBB2YVMA induced higher ERBB3 phosphorylation than WT kinase. Relative to BEAS-2B and T47D cells, H1781 cells had the highest level of ERBB3 protein and phosphorylation.

To extend these observations, we performed immunohistochemical staining for phosphorylated GAB1 Tyr-627 on serial sections of lung tissues from transgenic mice expressing ERBB2YVMA. In contrast to histology of the normal lung tissues, intrabronchial carcinomas were clearly identified in the transgenic mice (Fig. 2, top). Positive staining for phospho-GAB1 Tyr-627 was observed in the carcinoma cells of the tumors, whereas the control lung tissues were negative (Fig. 2, middle). The specificity of the immunostaining was confirmed using a normal rabbit IgG (Fig. 2, bottom panels). These results demonstrate that GAB1 is highly phosphorylated in ERBB2YVMA-driven lung carcinomas, coincident with the previously documented activation of AKT and MAPK in carcinomas of the transgenic mice (12).

GAB1 Mediates AKT and MAPK Activation by ERBB2 Lung Cancer Mutants—To investigate whether GAB1 phosphorylation is required for ERBB2 lung cancer mutant-induced signaling, we generated FLAG-tagged GAB1 mutants in which tyrosines responsible for binding of SHP2, PI3K, or both were replaced with phenylalanines, and mutant proteins were designated GAB1–1F (Y627F/Y659F), GAB1–2F (Y627F/Y659F), GAB1–3F (Y447F/Y472F/Y589F), and GAB1–5F (Y447F/Y472F/Y589F/Y627F/Y659F). Expression of GAB1–1F and -2F blocked the phosphorylation of endogenous GAB1 at Tyr-627 and, as expected, substantially suppressed phosphorylation of MAPK in BEAS2B/YVMA, T47D/YVMA, or H1781 cells. However, expression of GAB1 lacking PI3K sites (GAB1–3F and -5F) inhibited AKT activation differentially in these cells (Fig. 3). Whereas GAB1–3F and -5F blocked AKT activation to a barely detectable level in BEAS2B/YVMA and to a low level in T47D/YVMA cells (~30%), neither GAB1–3F nor -5F impaired AKT activation in H1781 cells (Fig. 3). It should be noted that the use of phospho-GAB1 Tyr-627 antibody in these experiments demonstrated that a high level of phosphorylation occurred at this site in GAB1–3F as the Tyr-627 site is retained in GAB1–3F.

To further examine the role of GAB1 in mutant ERBB2-mediated signaling, we generated stable GAB1 shRNA cell lines in the BEAS2B/YVMA, T47D/YVMA, and H1781 cell backgrounds. As shown in Fig. 4A, GAB1 shRNA specifically reduced endogenous GAB1 protein, whereas control shRNA had no effect, and ERBB2, ERBB3, AKT, and MAPK protein levels were not changed. Consistent with the results obtained from the GAB1 dominant negative mutants, GAB1 knockdown by shRNA significantly inhibited both AKT and MAPK signaling in BEAS2B and T47D cells expressing ERBB2YVMA (Fig. 4, A and B). However, in H1781 cells, GAB1 knockdown only suppressed MAPK activation without an effect on AKT signaling. Nevertheless, these data demonstrate that tyrosine phosphorylation of GAB1 plays an important role in mutant ERBB2-mediated AKT and/or MAPK signaling pathways.

ERBB3 Knockdown Reduces AKT Activation in H1781 Cells—The inability of either dominant negative GAB1 mutants lacking PI3K docking sites (GAB1–3F or -5F) or GAB1 knockdown to affect AKT activation in H1781 cells may be related to the high expression and phosphorylation level of ERBB3. The catalytically inactive ERBB3 receptor contains at least six canonical PI3K binding sites in its C terminus and thus may provide a redundant pathway for ERBB2G776VC- or G778D-induced AKT stimulation (17, 29). To test this hypothesis, we knocked down endogenous ERBB3 in H1781 using lentiviral shRNA in the absence or...
presence of GAB-5F expression. As shown in Fig. 5, ERBB3 protein was specifically reduced, and AKT activation was suppressed independent of the expression of GAB1-5F. The results demonstrate that rather than GAB1, ERBB3 plays a predominant role in AKT activation in H1781 cells.

**GAB1 Is Critical for Mutant ERBB2-induced Cell Transformation, Proliferation, and Survival**—Two hallmarks of the oncogenic transformation of cells are morphology change and acquisition of anchorage-independent growth. As shown in Fig. 6A, top, BEAS2B cells expressing ERBB2YVMA or ERBB2G776VC underwent striking morphological transformation. The cells expressing WT ERBB2 or GFP displayed a normal epithelial cell morphology, which is flat and non-refractile. In contrast, cells expressing either ERBB2YVMA or ERBB2G776VC had a typical sharply elongated, spindle-shaped, and highly refractile appearance of transformed cells. To determine whether GAB1 is involved in the mutant ERBB2-induced morphological change, we assessed the effects of expression of WT or mutant GAB1s on the cells. As shown in Fig. 6A, bottom, expression of WT GAB1 had no obvious effects on the transformed morphology of BEAS2B/YVMA cells. Conversely, expression of either GAB1-2F, -3F, or -5F blocked the ERBB2YVMA-mediated morphological change.

Next, we determined the role of GAB1 in ERBB2YVMA-induced BEAS2B cell transformation using an anchorage-inde-
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expression of GAB1–2F, -3F, or -5F markedly delayed tumor formation and significantly inhibited tumor growth (Fig. 6C).

We also investigated the role of GAB1 in proliferation of H1781 cells and survival of T47D/YVMA cells in serum-free medium. As shown in Fig. 6D, H1781 cells stably expressing GAB1–2F or -5F grew at considerably slower rates, in contrast to cells expressing GAB1-3F, which proliferated at a rate similar to WT GAB1 or GFP cells. Serum starvation resulted in a rapid decline in the viability of T47D cells expressing WT ERBB2 or empty vector, whereas T47D/YVMA cells survived in serum-free medium up to 7 days (Fig. 6E). Expression of GAB1–2F, -3F, or -5F all inhibited ERBB2YVMA-induced T47D cell survival in the absence of serum (Fig. 6E), indicating that GAB1-dependent signals are important for ERBB2YVMA-promoted survival of T47D cells. Together, these results demonstrate that GAB1 phosphorylation is critical for oncogenic mutant ERBB2-induced transformation of BEAS2B cells, proliferation of H1781 cells, and survival of T47D in serum-free medium.

**ERBB2YVMA Acquires a Substrate Preference for GAB1 Phosphorylation Sites**—To elucidate the mechanism of mutant ERBB2-mediated GAB1 hyperphosphorylation, we performed steady-state kinetic studies, which provide measurements of Michaelis (Km) and catalytic rate (kcat) constants. The specificity constant, kcat/Km, serves as the best parameter for comparisons of catalytic efficiencies between enzymes. We determined the kinetic parameters for the WT and mutant kinases toward peptides containing sequences for the major phosphorylation sites of GAB1, ERBB2, and ERBB3. Fig. 7A contains representative Lineweaver-Burk plots of GAB1 Tyr-627 peptide phosphorylation catalyzed by WT and ERBB2YVMA kinases, and the obtained kinetic parameters for all tested peptide substrates are summarized in Table 1. As illustrated in Fig. 7C, relative to WT kinase, the kcat values of ERBB2YVMA for all tested peptides were increased ~12-fold. However, the mutation differentially decreased the Km values for the peptides (Fig. 7C, Table 1). The Km values of ERBB2YVMA for ERBB peptides were modestly decreased by ~2–3-fold relative to WT, but strikingly, the Km for GAB1 Tyr-447, Tyr-627, and Tyr-659 peptides were more dramatically decreased (>10-fold). The decreased Km and concomitantly increased kcat yielded striking increases in catalytic efficiency of ERBB2YVMA for GAB1 peptides as indicated by the specificity constants (Fig. 7C, bottom; Table 1). The kcat/Km values for all 3 GAB1 peptides were increased >150-fold relative to the WT kinase. ERBB2YVMA had an extremely high kcat/Km value of 2438 min⁻¹ mm⁻¹ for GAB1 Tyr-627 peptide, which was ~29-fold higher than the best ERBB peptide substrate. In contrast to the findings with the peptides, Km values of ERBB2YVMA for ATP were only modestly altered relative to WT (~2-fold) (Fig. 7B and Table 2). Taken together, these results demonstrate that the YVMA insertion not only increases the catalytic activity of the kinase but, more importantly, results in an alteration in substrate specificity and a preference for GAB1 phosphorylation sites.

To validate that ERBB2YVMA has a remarkable preference for GAB1 phosphorylation sites within the GAB1 protein, we expressed and purified GST-tagged full-length and truncated GAB1 Tyr-627 was more significantly changed by WT GAB1 expression, whereas expression of GAB1–2F, -3F, or -5F markedly delayed tumor formation and significantly inhibited tumor growth (Fig. 6C).

pendent colony formation assay. Two weeks after cells were seeded in soft agar, colonies larger than ~50 μm were counted (Fig. 6B). Consistent with a previous report (11), cells expressing ERBB2YVMA and ERBB2G776VC formed many more colonies than WT ERBB2- or GFP-expressing cells. However, the colony-forming activity of BEAS2B/YVMA cells was suppressed dramatically by expression of either GAB1–2F, -3F, or -5F. Finally, we evaluated the role of GAB1 in ERBB2YVMA-driven tumorigenicity *in vivo*. BEAS2B cells expressing WT or mutant ERBB2s or BEAS2B/YVMA cells also expressing either WT or mutant GAB1s were implanted in athymic nude mice. As depicted in Fig. 6C, tumors were observed in mice injected with BEAS2B/YVMA 12 days after inoculation and grew to large sizes in 3 weeks, but no tumor was observed in mice injected with cells expressing WT ERBB2 or GFP during the same time frame (data not shown). Tumor formation and growth was not

![FIGURE 6. Expression of GAB1 mutants lacking SHP2 or PI3K docking sites inhibits mutant ERBB2-induced cell transformation, proliferation, and survival.](image)

expression of GAB1–2F, -3F, or -5F markedly delayed tumor formation of animals injected with ERBB2YVMA cells were provided in supplemental data. Each data point of tumor sizes expressing WT or mutant GAB1s. Phase contrast photographs of cells were taken at 10 x magnification. Indicated BEAS2B cells were seeded in soft agarose, and colonies measuring ≥50 μm were counted at day 14. Bars represent the mean ± S.D. of five replicates. C, indicated BEAS2B cells (5 × 10⁴) were injected subcutaneously in nude mice, tumor volumes were measured at the indicated time points, and results represent the mean ± S.D. for six animals. *p values for each data point of tumor sizes expressing WT or mutant GAB1 relative to tumor size from animals injected with ERBB2YVMA cells were provided in supplemental Table S1. D, H1781 cells expressing WT or mutant GAB1s were seeded in triplicate in 6-well plates in complete medium, and cells were counted at day 14. Results represent the mean ± S.D. for five replicates. E, T47D cells expressing the indicated proteins were plated in five replicates in 96-well plates in complete growth medium and serum-starved starting the next day. Cell survival was monitored with Cell Titer AQ kit (Promega) at day 7, and results represent the mean ± S.D. for five replicates. All p values were calculated with comparison to control (GFP). *p < 0.01; **p < 0.001; ***p < 0.0001.
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FIGURE 7. ERBB2YVMA has increased catalytic activity and substrate preference toward GAB1 tyrosine phosphorylation sites. A, shown are Lineweaver-Burk plots for GAB1 Tyr-627 peptide using 0.1 μM purified WT or ERBB2YVMA. Initial velocities were measured and analyzed using 100 μM ATP, and data points represent the mean ± S.E. (n = 4) from a representative experiment. B, shown are Lineweaver-Burk plots for ATP using 1.2 mM GAB1 Tyr-627 peptide. Data points represent mean ± S.E. (n = 4). C, shown is a comparison of kinetic parameters of WT and ERBB2YVMA for various peptides derived from major phosphorylation sites in GAB1, ERBB2, and ERBB3. D, purified GST-tagged GAB1 (1 μM) or truncated GAB1556–694 (2 μM) were phosphorylated by 150 nM WT, 150 nM ERBB2G776S/G778D, or 50 nM ERBB2YVMA in the presence of 100 μM [γ-32P]ATP, and reactions were terminated at indicated times by the addition of 0.1 mM EDTA and subjected to SDS-PAGE, Coomassie staining (GAB1 and GAB1556–694), and autoradiography (pGAB1 and pGAB1556–694). E, individual GAB1 protein bands were excised, and incorporated 32P was quantified using a scintillation counter. Activity was calculated using the slope of time course and is presented as relative activity to WT kinase.

TABLE 2

Enzymatic parameters for ATP. Results represent mean ± S.E. (n = 4)

| Kinases     | Km (μM) | kcat (min⁻¹) | kcat/Km (min⁻¹ μM⁻¹) |
|-------------|---------|---------------|-----------------------|
| WT          | 4.3 ± 0.4 | 25 ± 0.1 | 587 ± 66 |
| YVMA        | 7.8 ± 0.5 | 25.7 ± 0.7 | 3295 ± 257 |

TABLE 1

Enzymatic parameters of WT and mutant ERBB2 kinases for peptides derived from ERBB2, ERBB3, and GAB1 major phosphorylation sites. Phosphoacceptor tyrosines are in bold and underlined, and results represent mean ± S.E. (n = 4)

| Peptide/Sequence | Enzyme | Km (μM) | kcat (min⁻¹) | kcat/Km (min⁻¹ μM⁻¹) |
|------------------|--------|---------|---------------|-----------------------|
| ERBB2 Tyr-1139   | WT     | 398 ± 27 | 0.82 ± 0.02 | 2.1 ± 0.2 |
| ERBB2 Tyr-1180   | YVMA   | 149 ± 14 | 9.8 ± 0.3 | 65.8 ± 6.5 |
| ERBB3 Tyr-1178   | YVMA   | 515 ± 55 | 0.91 ± 0.1 | 1.8 ± 0.3 |
| ERBB3 Tyr-1180   | WT     | 149 ± 5 | 9.6 ± 0.1 | 64.4 ± 2.6 |
| ERBB3 Tyr-1178   | YVMA   | 371 ± 35 | 1.3 ± 0.1 | 3.5 ± 0.3 |
| ERBB3 Tyr-1309   | YVMA   | 117 ± 5 | 13 ± 0.1 | 111 ± 6 |
| ERBB3 Tyr-1309   | WT     | 633 ± 61 | 1.5 ± 0.1 | 2.4 ± 0.2 |
| GAB1 Tyr-447     | YVMA   | 191 ± 5 | 16.3 ± 0.3 | 85.3 ± 3.3 |
| GAB1 Tyr-627     | WT     | 326 ± 23 | 0.94 ± 0.03 | 2.9 ± 0.2 |
| GAB1 Tyr-627     | YVMA   | 32.4 ± 2.4 | 12.5 ± 0.3 | 386 ± 30 |
| GAB1 Tyr-627     | WT     | 176 ± 18 | 2.8 ± 0.1 | 15.9 ± 1.7 |
| GAB1 Tyr-627     | YVMA   | 12.8 ± 0.9 | 31.2 ± 0.4 | 2438 ± 149 |
| GAB1 Tyr-659     | WT     | 378 ± 31 | 0.99 ± 0.03 | 2.6 ± 0.2 |
| GAB1 Tyr-659     | YVMA   | 35.8 ± 2.4 | 15.8 ± 0.3 | 441 ± 31 |
and H1781 cells (Figs. 3 and 4). Dominant negative GAB1 mutants lacking P13K binding sites or GAB1 knockdown inhibited ERBB2YVMA-mediated AKT activation in BEAS2B and T47D cells but had no obvious effect in H1781 cells (Figs. 3 and 4). However, knockdown of ERBB3 significantly reduces AKT activation in H1781 cells, indicating that ERBB3, rather than GAB1, plays a predominant role in AKT activation in H1781 cells (Fig. 5). Furthermore, expression of dominant negative GAB1s also inhibited ERBB2YVMA-driven BEAS2B cell morphology change, colony formation in soft agar, and tumor formation in nude mice and ERBB2YVMA-mediated survival of T47D cells in serum-free medium (Fig. 6). Taken together, our results provide compelling evidence that oncogenic ERBB2-mediated hyperphosphorylation of GAB1 plays a major role in the transforming activity of these mutants.

Despite the dramatic benefits from ERBB2-targeted therapies, the outcomes of these therapies are uniformly limited by the development of drug resistance (33, 34). Two major possible mechanisms are acquired secondary mutation of the target kinase and induced amplification of other receptor kinases, such as c-MET (35) and IGF-1 receptor (36). GAB1 has been demonstrated to play essential roles in transformation elicited by these receptors and, more interestingly, to serve as a bridge mediating the cross-talk between ERBB2 and other receptor-signaling pathways. Therefore, GAB1-targeted anti-cancer therapies could be more efficacious by blocking oncogenic signaling mediated by individual receptor kinases and their cross-talk. Strategies to target GAB1 and other docking proteins have been proposed (15, 37).

The catalytic activity of all protein kinases is regulated by two key coupled structural elements, namely the activation loop and αC helix (2). Compelling evidence has revealed that, in contrast with most receptor-tyrosine kinases, phosphorylation of the activation loop is not required for activation of ERBB kinases (9, 38). Structural studies have provided evidence that EGFR (39) and ERBB4 (40) kinases are activated through an allosteric mechanism via formation of an asymmetric dimer. Upon dimer formation, the helix αC of one kinase domain is restrained to the catalytically competent conformation by the C-lobe of the other. The proper orientation of αC helix for kinase activation in EGFR and ERBB4 is highly controlled through hydrogen bonds and hydrophobic interactions of residues in the αC-β4 loop with residues in the activation loop and the C-lobe (10). Conversely, the unique Gly-rich αC-β4 region in ERBB2 fails to form these interactions and imparts greater flexibility to the enzyme active site. Compiled evidence has demonstrated that changes in flexibility or slight conformational adjustment within the enzyme active site can result in dramatic functional changes (41, 42). Indeed, our previous work revealed that ERBB2 has much lower intrinsic catalytic activity relative to EGFR and ERBB4 kinases (9). The αC-β4 loop mutations in human lung cancer might introduce interactions, which stabilize the αC-helix in an active orientation.

The catalytic specificity of protein kinases toward their physiological targets is believed to be critical for fidelity in signaling (43). A systematic enzyme kinetic analysis comparing ERBB2YVMA to WT kinases with peptide substrates containing major physiological phosphorylation sites is a powerful method to determine whether the gain-of-function oncogenic mutations alter substrate specificity of the kinase and, therefore, provides important insights into the molecular basis of mutant kinase-induced signaling. An important finding in the work presented here is that the YVMA insertion resulted in significantly greater relative increases in specificity constants for GAB1 peptides compared with ERBB2 and ERBB3 phosphorylation site peptides. Therefore, ERBB2YVMA adopts a strikingly increased preference for GAB1 phosphorylation sites. The alteration of the specificity is predominantly attributed to the increased substrate binding affinity as reflected by the decreased $K_m$ values. It should be noted that ERBB2YVMA may also have an acquired preference for other physiological substrates that we did not test in our current steady-state kinetic studies using peptides. The concept of altered substrate specificity is illustrated in Fig. 7C in which the kinetic parameters, $K_m$, $k_{cat}$, and $k_{cat}/K_m$ of WT and mutant ERBB2 kinases for a number of physiologically relevant peptides were compared. The concomitant effects of the decreased $K_m$ and the increased $k_{cat}$ yield ~150-fold increases in the specificity constants ($k_{cat}/K_m$) for several GAB1 peptides. These kinetic findings with peptide substrates were verified by our observation that ERBB2YVMA phosphorylated purified GAB1 proteins at an ~70-fold faster rate relative to WT ERBB2 (Fig. 7, D and E). A decrease in the $K_m$ value for specific phosphorylation sites may play a more important role in pathological signaling in cancer cells in which the concentrations of these substrate targets are much lower than the $K_m$. In our previous work we found that G776S/G778D mutations in the αC-β4 loop also dramatically activate the ERBB2 kinase. However, even though the G776S/G778D mutation is localized to the same region, the activation mechanism is very different from that of ERBB2YVMA. In contrast to the YVMA insertion, G776S/G778D does not significantly alter the $K_m$ values for the peptide substrates. Even though ERBB2G776S/G778D has a $k_{cat}$ value similar to ERBB2YVMA, expression of this mutant led to only slight increases in GAB1 phosphorylation and MAPK and AKT activation in BEAS2B cells (Fig. 1). Thus, mutational activation per se, as in the case of ERBB2G776S/G778D, need not result in a change in substrate specificity of the kinase. Collectively, our results demonstrate that (i) the αC-β4 loop plays a vital role of in the regulation of ERBB2 kinase activity and alterations in this region can result in different activated states of the kinase, and (ii) the acquisition of the substrate preference for GAB1 via decreased $K_m$ values is one of the primary mechanisms for transforming activity of ERBB2 mutations in lung cancer.

Another noteworthy finding from our enzyme kinetic analyses is that the $K_m$ value of ERBB2YVMA for ATP is not significantly altered compared with WT ERBB2 (Fig. 7B and Table 2). Furthermore, GAB1 phosphorylation by ERBB2YVMA or by ligand-activated WT ERBB2 in cells was equally responsive to lapatinib, an ATP competitive inhibitor for the ERBB2 kinase (supplemental Fig. S1). These results are consistent with earlier findings (11) demonstrating that cellular signaling by WT or ERBB2YVMA are similarly sensitive to lapatinib. A number of activating EGFR mutations have also been identified in non-small cell lung cancers (44–46), and the most frequent mutants identified in lung cancer are an ATP binding loop deletion (Del
746–750) and an activation loop point mutation (L858R). A comparison of oncogenic mutation in ERBB2 to EGFR and other protein kinases is shown in supplemental Fig. S2. The EGFR mutations lead to constitutive kinase activation and, importantly, confer exquisite sensitivity to the small molecule kinase inhibitors, gefitinib and erlotinib (44–47). These activated EGFR mutants have been found to have compromised affinity for ATP relative to the WT EGFR kinase (48, 49). Of interest, EGFR Y780M, were also identified in non-small cell lung cancers (50). These results suggest that, in contrast to what was postulated previously, the insertions in the α-C-β4 loop region in both EGFR and ERBB2 kinases do not alter the ATP binding cleft dramatically (51). The “Achilles heel” for the α-C-β4 loop insertion mutants of ERBB receptor kinases may not exist in the ATP binding region. Therefore, treatment of lung cancers harboring ERBB2 mutations may benefit from alternative inhibition strategies aimed at targeting other important structural elements such as the peptide binding site.

Acknowledgments—We thank Drs. Yan Wang, Kula Jha, Kurt Stromberg, and Yetao Jin for help in the immunohistochemical analysis and critical reading of the manuscript.

REFERENCES

1. Blume-Jensen, P., and Hunter, T. (2001) Oncogenic kinase signalling. Nature 411, 355–365
2. Huse, M., and Kurian, J. (2002) The conformational plasticity of protein kinases. Cell 109, 275–282
3. Lemmon, M. A., and Schlessinger, J. (2010) Cell signaling by receptor-tyrosine kinases. Cell 141, 1117–1134
4. Weinstein, I. B., and Joe, A. (2008) Oncogene addiction. Cancer Res. 68, 3077–3080
5. Citri, A., and Yarden, Y. (2006) EGFR-ERBB signaling. Towards the systems level. Nat. Rev. Mol. Cell Biol. 7, 505–516
6. Hynes, N. E., and Lane, H. A. (2005) ERBB receptors and cancer. The molecular mechanisms. Nat. Rev. Mol. Cell Biol. 6, 341–354
7. Shigematsu, H., Takahashi, T., Nomura, M., Majmudar, K., Suzuki, M., Lee, H., Wistuba, I. I., Fong, K. M., Toyooka, S., Shimizu, N., Fujisawa, T., Minna, J. D., and Gazdar, A. F. (2005) Somatic mutations of the HER2 kinase domain in lung adenocarcinomas. Cancer Res. 65, 1642–1646
8. Stephens, P., Hunter, C., Bignell, G., Edkins, S., Davies, H., Teague, I., Stevens, C., O’Meara, S., Smith, R., Parker, A., Barthorpe, A., Bow, M., Brackenbury, L., Butler, A., Clarke, O., Cole, J., Dicks, E., Dike, A., Drozd, A., Edwards, K., Forbes, S., Foster, R., Gray, K., Greenman, C., Halliday, K., Hills, K., Kosmidou, V., Lugg, R., Menzies, A., Perry, J., Petty, R., Raine, K., Ratford, L., Shepherd, R., Small, A., Stephens, T., Tofts, C., Varian, J., West, S., Widas, S., Yates, A., Brasseur, F., Cooper, C. S., Flanagan, A. M., Knowles, M., Leung, S. Y., Louis, D. N., Looijenga, L. H., Malkowicz, B., Pierotti, M. A., Teh, B., Chenex-Trench, G., Weber, B. L., Yuen, S. T., Harris, G., Goldstraw, P., Nicholson, A. G., Futreal, P. A., Wooster, R., and Stratton, M. R. (2004) Lung cancer. Intraocular ERBB2 kinase mutations in tumours. Nature 431, 525–526
9. Fan, Y. X., Wong, L., Ding, J., Spiriidonov, N. A., Johnson, R. C., and Johnson, G. R. (2008) Mutational activation of ErbB2 reveals a new protein kinase autoinhibition mechanism. J. Biol. Chem. 283, 1588–1596
10. Aertgeerts, K., Skene, R., Yano, J., Sang, B. C., Zou, H., Snell, G., Jennings, A., Iwamoto, K., Habuka, N., Hirokawa, A., Ishikawa, T., Tanaka, T., Miki, H., Ohta, Y., and Sugabe, S. (2011) Structural analysis of the mechanism of inhibition and allosteric activation of the kinase domain of HER2. J. Biol. Chem. 286, 18756–18765
11. Wang, S. E., Narasanna, A., Perez-Torres, M., Xiang, B., Wu, F. Y., Yang, S., Carpenter, G., Gazdar, A. F., Muthuswamy, S. K., and Arteaga, C. L. (2006) HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. Cancer Cell 10, 25–38
12. Perera, S. A., Li, D., Shimamura, T., Raso, M. G., Ji, H., Chen, L., Borgman, C. I., Zaghloul, S., Brandstetter, K. A., Kubo, S., Takahashi, M., Chirieac, L. R., Pader, R. F., Bronson, R. T., Shapiro, G. I., Greulich, H., Meyerson, M., Guertler, U., Chesa, P. G., Solca, F., Wistuba, I. I., and Wong, K. K. (2009) HER2VYMA drives rapid development of adenocarcinomas in mice that are sensitive to BIBW2992 and rapamycin combination therapy. Proc. Natl. Acad. Sci. U.S.A. 106, 476–479
13. Gu, H., and Neel, B. G. (2003) The “Gab” in signal transduction. Trends Cell Biol. 13, 122–130
14. Wöhlrle, F. U., Daly, R. J., and Brummer, T. (2009) Function, regulation and pathological roles of the Gab/DO5 docking proteins. Cell Commun. Signal 7, 525–534
15. Mattoon, D. R., Lamotte, B., Lax, I., and Schlessinger, J. (2004) The docking protein Gab1 is the primary mediator of EGF-stimulated activation of the PI-3K/Akt cell survival pathway. BMC Biol. 2, 24
16. Yamasaki, S., Nishida, K., Yoshida, Y., Itoh, M., Hibi, M., and Hirano, T. (2003) Gab1 is required for EGFR receptor signaling and the transformation by activated ErbB2. Oncogene 22, 1546–1556
17. Itoh, M., Yoshida, Y., Nishida, K., Namimatsu, M., Hibi, M., and Hirano, T. (2000) Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. Mol. Cell. Biol. 20, 3695–3704
18. Deb, T. B., Wong, L., Salomon, D. S., Zhou, G., Dixon, J. E., Gutkind, J. S., Thompson, S. A., and Johnson, G. R. (1998) A common requirement for the catalytic activity and both SH2 domains of SHP-2 in mitogen-activated protein (MAP) kinase activation by the ErbB family of receptors. A specific role for SHP-2 in map but not e-Jun-amino-terminal kinase activation. J. Biol. Chem. 273, 16643–16646
19. Holgado-Madurga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J. (1996) A Grb2-associated docking protein in EGFR and insulin receptor signalling. Nature 379, 560–564
20. Lehr, S., Koziak, J., Herken, A., Klein, E., Siethoff, C., Knebel, B., Noelle, V., Brüning, J. C., Klein, H. W., Meyer, H. E., Krone, W., and Müller-Wieland, D. (1999) Identification of tyrosine phosphorylation sites in human Gab-1 protein by EGFR receptor kinase in vitro. Biochemistry 38, 151–159
21. Fan, Y. X., Wong, L., Deb, T. B., and Johnson, G. R. (2004) Ligand regulates epidermal growth factor receptor kinase specificity. Activation increases preference for Gab1 and SHC versus autophosphorylation sites. J. Biol. Chem. 279, 38143–38150
22. Pluta, K., Luce, M. J., Bao, L., Agha-Mohammadi, S., and Reiser, J. (2005) Tight control of transgene expression by lentivirus vectors containing second-generation tetracycline-responsive promoters. J. Gene Med. 7, 803–817
23. Ou, W., Marino, M. P., Suzuki, A., Joshi, B. H., Husain, S. R., Maisner, A., Galanis, E., Puri, R., and Reiser, J. (2012) Specific targeting of human IL-13 receptor α2-positive cells with lentiviral vectors displaying IL-13. Hum. Gene Ther. Methods 23, 137–147
24. Fan, Y. X., Wong, L., and Johnson, G. R. (2005) EGFR kinase possesses a broad specificity for ErbB phosphorylation sites, and ligand increases catalytic-centre activity without affecting substrate binding affinity. Biochem. J. 392, 417–423
25. Cunnick, J. M., Dorsey, J. F., Munoz-Antonia, T., Mei, L., and Wu, J. (2000) Requirement of SHP2 binding to Grb2-associated binder-1 for mitogen-activated protein kinase activation in response to lysophosphatidic acid and epidermal growth factor. J. Biol. Chem. 275, 13842–13848
26. Cunnick, J. M., Mei, L., Doupnik, C. A., and Wu, J. (2001) Phosphotyrosines 627 and 659 of Gab1 constitute a bisphosphoryl tyrosine-based activation motif (BTAM) conferring binding and activation of SHP2.
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J. Biol. Chem. 276, 24380–24387

29. Sithanandam, G., Smith, G. T., Fields, J. R., Fornwald, L. W., and Anderson, L. M. (2005) Alternate paths from epidermal growth factor receptor to Akt in malignant versus nontransformed lung epithelial cells. ErbB3 versus Gab1. Am. J. Respir. Cell Mol. Biol. 33, 490–499

30. Hoenen, A., Martin, D., Clement, P. M., Cools, J., and Gutkind, J. S. (2013) Role of Grb2-associated binder 1 (GAB1) in epidermal growth factor receptor (EGFR)-induced signaling in head and neck squamous cell carcinoma. Int. J. Cancer 132, 1042–1050

31. Felici, A., Giubellino, A., and Bottaro, D. P. (2010) Gab1 mediates hepatocyte growth factor-stimulated mitogenesis and morphogenesis in multi-potent myeloid cells. J. Cell. Biochem. 111, 310–321

32. Mood, K., Saucier, C., Bong, Y. S., Lee, H. S., Park, M., and Daar, I. O. (2011) Gab1 is required for cell cycle transition, cell proliferation, and transformation induced by an oncogenic met receptor. Mol. Biol. Cell 17, 3717–3728

33. Mohd Sharial, M. S., Crown, J., and Hennessy, B. T. (2012) Overcoming resistance and restoring sensitivity to HER2-targeted therapies in breast cancer. Ann. Oncol. 23, 3007–3016

34. Vu, T., and Clare, F. X. (2012) Trastuzumab. Updated mechanisms of action and resistance in breast cancer. Front. Oncol. 2, 62

35. Shattuck, D. L., Miller, J. K., Carraway, K. L., 3rd, and Sweeney, C. (2008) Overcoming resistance to trastuzumab (Herceptin) in breast cancer cells. Cancer Res. 68, 1471–1477

36. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D., and Pollak, M. (2001) Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). J. Natl. Cancer Inst. 93, 1852–1857

37. Yart, A., Mayieux, P., and Raynal, P. (2003) Gab1, SHP-2 and other novel regulators of Ras. Targets for anticancer drug discovery? Curr. Cancer Drug Targets 3, 177–192

38. Segatto, O., Lonardo, F., Pierce, J. H., Bottaro, D. P., and Di Fiore, P. P. (1990) The role of autophosphorylation in modulation of erbB-2 transforming function. New Biol. 2, 187–195

39. Zhang, X., Guersako, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 125, 1137–1149

40. Qi, C., Tarrant, M. K., Choi, S. H., Sathyamurthy, A., Bose, R., Banjade, S., Pal, A., Bornmann, W. G., Lemmon, M. A., Cole, P. A., and Leahy, D. J. (2008) Mechanism of activation and inhibition of the HER4/ErbB4 kinase. Structure 16, 460–467

41. Fan, Y. X., Ju, M., Zhou, J. M., and Tsou, C. L. (1996) Activation of chicken liver dihydrofolate reductase by urea and guanidine hydrochloride is accompanied by conformational change at the active site. Biochem. J. 315, 97–102

42. Tsou, C. L. (1993) Conformational flexibility of enzyme active sites. Science 262, 380–381

43. Songyang, Z., Carraway, K. L., 3rd, Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., and Eng, C. (1995) Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. Nature 373, 536–539

44. Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., Okimoto, R. A., Brannigan, B. W., Harris, P. L., Haserlat, S. M., Supko, J. G., Haluska, F. G., Louis, D. N., Christiani, D. C., Settleman, J., and Haber, D. A. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. 350, 2129–2139

45. Paez, J. G., Jänne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M. J., Sellers, W. R., Johnson, B. E., and Meyerson, M. (2004) EGFR mutations in lung cancer. Correlation with clinical response to gefitinib therapy. Science 304, 1497–1500

46. Pao, W., Miller, V., Zakowski, M., Doherty, J., Politi, K., Sarkaria, I., Singh, B., Heelan, R., Rusch, V., Fulton, L., Mardis, E., Kupper, D., Wilson, R., Kris, M., and Varmus, H. (2004) EGFR receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc. Natl. Acad. Sci. U.S.A. 101, 13306–13311

47. Sordella, R., Bell, D. W., Haber, D. A., and Settleman, J. (2004) Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 305, 1163–1167

48. Yun, C. H., Mengwasser, K. E., Toms, A. V., Woo, M. S., Greulich, H., Wong, K. K., Meyerson, M., and Eck, M. J. (2008) The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc. Natl. Acad. Sci. U.S.A. 105, 2070–2075

49. Qi, C., Tarrant, M. K., Boronina, T., Longo, P. A., Kavran, J. M., Cole, R. N., Cole, P. A., and Leahy, D. J. (2009) In vitro enzymatic characterization of near full-length EGFR in activated and inhibited states. Biochemistry 48, 6624–6632

50. Greulich, H., Chen, T. H., Feng, W., Jänne, P. A., Alvarez, J. V., Zappaterra, M., Bulmer, S. E., Frank, D. A., Hahn, W. C., Sellers, W. R., and Meyerson, M. (2005) Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. PLoS Med. 2, e13

51. Gazdar, A. F., Shigematsu, H., Herz, J., and Minna, J. D. (2004) Mutations and addiction to EGFR. The Achilles “heal” of lung cancers? Trends Mol. Med. 10, 481–486