Mutational Activation of ErbB2 Reveals a New Protein Kinase Autoinhibition Mechanism*1

Ying-Xin Fan1, Lily Wong1, Jinhui Ding5, Nikolay A. Spiridonov5, Richard C. Johnson*, and Gibbes R. Johnson†1,2

From the 1Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Food and Drug Administration and 2Bioinformatics Section, Laboratory of Neurogenetics, NIA, National Institutes of Health, Bethesda Maryland 20892 and 3Department of Neuroscience, Johns Hopkins University School of Medicine, Howard Hughes Medical Institute, Baltimore, Maryland 21205

Autoinhibition plays a key role in the control of protein kinase activity. ErbB2 is a unique receptor-tyrosine kinase that does not bind ligand but possesses an extracellular domain poised to engage other ErbBs. Little is known about the molecular mechanism for ErbB2 catalytic regulation. Here we show that ErbB2 kinase is strongly autoinhibited, and a loop connecting the αC helix and β4 sheet within the kinase domain plays a major role in the control of kinase activity. Mutations of two Gly residues at positions 776 and 778 in this loop dramatically increase ErbB2 catalytic activity. Kinetic analysis demonstrates that mutational activation is due to ~10- and ~7-fold increases in ATP binding affinity and turnover number, respectively. Expression of the activated ErbB2 mutants in cells resulted in elevated ligand-independent ErbB2 autophosphorylation, ErbB3 phosphorylation, and stimulation of mitogen-activated protein kinase. Molecular modeling suggests that the ErbB2 kinase domain is stabilized in an inactive state via a hydrophobic interaction between the αC-β4 and activation loops. Importantly, many ErbB2 human cancer mutations have been identified in the αC-β4 loop, including the activating G776S mutation studied here. Our findings reveal a new kinase regulatory mechanism in which the αC-β4 loop functions as an intramolecular switch that controls ErbB2 activity and suggests that loss of αC-β4 loop-mediated autoinhibition is involved in oncogenic activation of ErbB2.

Protein-tyrosine kinases (PTKs)3 play a central role in cellular signaling. The appropriate regulation of the catalytic activity of PTKs is crucial to many physiological processes, and abnormal kinase activation can have severe consequences leading to cell transformation and cancer (1). In quiescent cells PTKs are maintained in an inactive state by a variety of autoinhibition mechanisms. Upon stimulation, the autoinhibition is relieved, leading to kinase activation. Several PTK activation mechanisms have been recognized, and a common PTK activation mechanism occurs via phosphorylation of tyrosine(s) in a segment referred to as the activation loop (2, 3).

ErbB2 (HER2, neu) is a member of the ErbB family of receptor PTKs which also includes epidermal growth factor receptor (EGFR, ErbB1, HER1), ErbB3 (HER3), and ErbB4 (HER4). ErbB receptors consist of an extracellular domain (ECD), a transmembrane helix, an intracellular PTK domain, and a carboxyl tail harboring tyrosine phosphorylation sites, which upon phosphorylation provide docking sites for signal transducers. ErbB receptors play important roles in the control of cell proliferation, migration, survival, and differentiation of target cells (4, 5). Overexpression or dysregulation of ErbB receptor activity, especially EGFR and ErbB2, has been implicated in various human cancers (6, 7). Accordingly, these receptors have been intensely studied to understand their importance in cancer biology and as therapeutic targets. ErbB2 overexpression is observed in 20–30% of human breast cancers and is associated with a more aggressive course and a poor prognosis (8). A humanized anti-ErbB2 monoclonal antibody, Herceptin, has been approved for the clinical treatment of ErbB2-overexpressing breast cancer, and several small molecular ErbB2 kinase inhibitors are currently in development (6).

Engagement of ligand by receptor PTKs is a major mechanism of activation. However, a distinguishing characteristic of ErbB2 is that, unlike other ErbBs, it lacks the ability to bind a specific ligand. The crystallographic studies have revealed that the ECDs of EGFR (9, 10), ErbB3 (HER3), and ErbB4 (HER4). ErbB receptors consist of an extracellular domain (ECD), a transmembrane helix, an intracellular PTK domain, and a carboxyl tail harboring tyrosine phosphorylation sites, which upon phosphorylation provide docking sites for signal transducers. ErbB receptors play important roles in the control of cell proliferation, migration, survival, and differentiation of target cells (4, 5). Overexpression or dysregulation of ErbB receptor activity, especially EGFR and ErbB2, has been implicated in various human cancers (6, 7). Accordingly, these receptors have been intensely studied to understand their importance in cancer biology and as therapeutic targets. ErbB2 overexpression is observed in 20–30% of human breast cancers and is associated with a more aggressive course and a poor prognosis (8). A humanized anti-ErbB2 monoclonal antibody, Herceptin, has been approved for the clinical treatment of ErbB2-overexpressing breast cancer, and several small molecular ErbB2 kinase inhibitors are currently in development (6).

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 15 and 25.

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, Bldg. 29A, Rm. 3B-20, 8800 Rockville Pike, Bethesda, MD 20892. E-mail: gibbes.johnson@fda.hhs.gov.

3 The abbreviations used are: PTK, protein-tyrosine kinase; EGFR, epidermal growth factor (EGF) receptor; Ab, antibody; ECD, extracellular domain; Gab1, Grb2-associated binding protein 1; WT, wild type; GA, geldanamycin; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; HRG-β1, heregulin-β1; Ni2+–NTA, nickel-nitrilotriacetic acid; CV, column volume.

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Protein-tyrosine kinases (PTKs)3 play a central role in cellular signaling. The appropriate regulation of the catalytic activity of PTKs is crucial to many physiological processes, and abnormal kinase activation can have severe consequences leading to cell transformation and cancer (1). In quiescent cells PTKs are maintained in an inactive state by a variety of autoinhibition mechanisms. Upon stimulation, the autoinhibition is relieved, leading to kinase activation. Several PTK activation mechanisms have been recognized, and a common PTK activation mechanism occurs via phosphorylation of tyrosine(s) in a segment referred to as the activation loop (2, 3).

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Like other receptor PTKs, the kinase activity of ErbB2 plays an important role in ErbB2-mediated physiological events. Mice that express only a kinase-inactive ErbB2 die at midgestation, suggesting that the catalytic activity of ErbB2 is absolutely required for embryonic development (17). However, few studies have been focused on the enzymatic properties of ErbB2 kinase (18–20). To date, the kinase domain structure of ErbB2 has not been determined. The molecular mechanism by which the ErbB2 kinase activity is controlled and activated remains to be understood. In the present work we demonstrated that ErbB2 kinase is autoinhibited by a loop between the αC helix and β4 sheet in its kinase domain. Mutations in this loop can release the autoinhibition interaction that leads to ErbB2 kinase activation. Our steady-state kinetic investigation revealed that the mutations in the αC-β4 loop significantly increase the binding affinity for ATP and catalytic rate, and constitutive signaling was observed when these activated ErbB2s were expressed in cells. Our findings identify a new kinase regulation mechanism in which the αC-β4 loop functions as an intramolecular switch that regulates ErbB2 activity. Furthermore, because almost all ErbB2 kinase domain mutations in human cancers have been localized to the αC-β4 loop, it appears likely that loss of this αC-β4 loop-mediated autoinhibition is involved in oncogenic activation of ErbB2.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Antibodies (Abs) against MAPK and phosphotyrosine were obtained from BD Transduction Laboratories, and Abs against ErbB2 and Myc were obtained from Neomarkers. Abs against ErbB3 and Hsp90 were obtained from Santa Cruz Biotechnology. Abs against phosphorylated ErbB2 and ErbB3 were from Cell Signaling. Abs for phospho-MAPK and HA were obtained from New England Biolabs and Roche Applied Science, respectively. Geldanamycin was from Alexis Biochemicals, and heregulin-β1 (HRG-β1) was generously provided by Stewart Thompson at Berlex Biosciences. Peptides were prepared as described previously (21).

**Mammalian Expression Constructs**—ErbB cDNAs were subcloned into pcDNA 3.1/Myc-his (Invitrogen) using standard techniques. Point mutations were produced using the QuikChange kit (Stratagene), and chimeric EGFR/ErbB2-KD and ErbB2/EGFR-KD constructs were prepared using splicing by overlap extension (22). The identities of expression constructs were confirmed by DNA sequencing.

**Cell Culture, Transfections, and Generation of Stable Cell Lines**—32D cell lines expressing EGFR, ErbB2, and ErbB3 were generated as described previously and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% medium conditioned by WEHI-3B cells (23). To generate cells expressing wild type or mutant ErbB2 along with ErbB3, 32D-E3 cells, which express only ErbB3 (24), were transfected with 20 μg of plasmid DNA by electroporation (960 microfarads, 0.25 kV) using a Bio-Rad Gene Pulser II, and stable transfectants were generated in G418. Fluorescence-activated cell sorting analysis (BD Biosciences FACS-Star) of stable transfectants was performed with ErbB2 monoclonal Ab (Ab-2, Neomarker) and visualized using a fluorescent isothiocyanate-conjugated goat anti-mouse secondary antibody. Cos7 cells were cultured and transfected as previously described (25), and 293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and transfected using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer.

**Purification of ErbB2s**—Myc/hexahistidine-tagged full-length human EGFR or ErbB2 were purified by Co2⁺-based immobilized metal affinity chromatography from stable 32D cell lines as described previously (21). cDNA encoding Myc/hexahistidine-tagged human EGFR (681–1186) and ErbB2-(712–1255) were subcloned into pENTR/dTOPO (Invitrogen), and baculoviruses were produced using the baculovirus expression system in SF9 cells (Invitrogen). Cells were harvested 3 days after infection by centrifugation at 4000 x g and extracted in 5 ml of lysis buffer/g of cell pellet (20 mM Hepes, pH 8.0, 200 mM NaCl, 1% Tween 20, 10% glycerol, 10 mM β-mercaptoethanol, 5 mM β-glycerophosphate, 0.1 mM PMSF phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM sodium orthovanadate, and 5 mM imidazole) at 4 °C for 1 h. The cell lysate was centrifuged at 22,000 x g for 1 h at 4 °C, and the supernatant was incubated with Ni2⁺-NTA resin (Qiagen) at 4 °C for 1 h. A 0.4-ml column volume (CV) of Ni2⁺-NTA resin was used per 1 liter of SF9 culture. The Ni2⁺-NTA-bound proteins were washed in series with the following solutions: 3 x 5 CV of 5 mM imidazole in wash buffer (20 mM Hepes, pH 8.0, at 4 °C, 100 mM KCl, 20% glycerol, 10 mM β-mercaptoethanol, 0.1% Tween 20), 1 x 5 CV of wash buffer supplemented with 1 mM KCl, 2 x 5 CV 15 mM imidazole in wash buffer. Recombinant proteins were then eluted with 3 CV of 100 mM imidazole in wash buffer. Protein was quantified by Coomassie Blue staining of SDS-PAGE using bovine serum albumin as a standard.

**Kinase Assays**—All ErbB kinase reactions were performed in 25 μl of Hepes, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 50 μM sodium orthovanadate, 0.5 mM dithiothreitol, 0.1% Tween 20, 40 μg/ml bovine serum albumin, 200 μM ATP with 1.25 mCi/ml [γ³²P]ATP (Amershams Biosciences), and 0.1 μM receptor kinases in a total volume of 40 μl at 25 °C. Reactions were stopped by the addition of 8.5% H₃PO₄. Terminated reaction mixtures were transferred to P-30 Filtermats (Wallac) and washed 3 x with 0.85% H₃PO₄ and 3 x with water, and bound radioactivity was quantified with a Trilux Microbeta scintillation counter (PerkinElmer Life Sciences). The steady-state kinetic parameters were determined conventionally for two-substrate enzymes by keeping one substrate constant at saturation level and varying the concentration of the other. Initial rates were determined from the linear range of the reaction (<5% substrate converted). Four replicates were measured in all experiments, and steady-state kinetic parameters, Kₘ and k_cat were calculated using the Enzyme Kinetic Module of Sigma Plot (Jandel Scientific). The errors for k_cat/K_m were calculated with the formula,

\[
(k_{cat}/K_m) \sqrt{((S.E.1/k_{cat})^2 + (S.E.2/K_m)^2) (Eq. 1)}
\]

where S.E.₁ and S.E.₂ are standard error for k_cat and K_m, respectively.

**Structural Modeling and Analysis**—Sequence alignment of ErbB2 and EGFR kinase domains was performed using ClustalX
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(26) and manually adjusted to keep the overall secondary structures. The structure of ErbB2 was modeled and refined according to the crystal structure of EGFR (Protein Data Bank code 2G57) using NEST in Jackal package (27). PyMol was used for model display and figure generation.

RESULTS

ErbB2 Kinase Has a Lower Constitutive Catalytic Activity Relative to EGFR—In an attempt to measure ErbB2 kinase activity in cancer cells, we surprisingly found that no significant in vitro kinase activity was detected in ErbB2 immunoprecipitants derived from T47D breast cancer cells, whereas EGFR and ErbB4 kinase activity was easily detected (data not shown). We tried numerous lysis and assay conditions (i.e. cell lines, detergents, antibodies, and substrates), but the measured ErbB2 kinase activity was always much lower than EGFR and ErbB4. Similar results have been reported by others (28). To better understand the basis for the low ErbB2 catalytic activity, we generated human ErbB2 or EGFR constructs tagged with an Myc epitope and hexahistidine at the C terminus. The complete sequences of these cDNA constructs were confirmed, and the receptors were capable of ligand-dependent signaling when expressed in ErbB-null myeloid 32D cells as previously described (23). These receptors were expressed in Cos7 cells and immunopurified by anti-Myc antibody. As shown in Fig. 1A, whereas the protein levels in the immunoprecipitants were comparable as indicated by anti-Myc Western blotting, the autokinase activity of ErbB2 (lane 4) was very low relative to EGFR (lane 2). No significant phosphorylation was detected for kinase-inactive receptors EGFR K721M (lane 1) and ErbB2 K753M (lane 3). The catalytic activities of the immunopurified ErbB2 and EGFR were also examined using a synthetic peptide Gab1 Y627, which contains a sequence identical to 17 amino acids surrounding a major tyrosine phosphorylation site in Gab1 at position 627 (21). ErbB2 kinase activity for Gab1 Y627 was ∼34-fold lower than that of EGFR (Fig. 1B). We also found that the activity of ErbB4 immunoprecipitated from Cos7 cells was very similar to EGFR but was much higher than ErbB2 (data not shown).

To study whether the ECD or transmembrane region inhibits the catalytic activity of the ErbB2 kinase, we engineered chimeric receptors by reciprocally switching EGFR and ErbB2 kinase domains. The swapped kinase domains of EGFR and ErbB2 contain amino acids 686–953 and 718–985, respectively, and these chimeras were designated as EGFR/ErbB2-KD and ErbB2/EGFR-KD. As shown in Fig. 1A, EGFR/ErbB2-KD had a much lower autokinase activity than ErbB2/EGFR-KD (lane 5 and 6). The EGFR/ErbB2-KD activity toward Gab1 Y627 peptide was also ∼8-fold lower than ErbB2/EGFR-KD (Fig. 2B). We also generated truncated versions of the receptors, namely EGFR-(681–1186) and ErbB2-(712–1255), which lack the ECD and transmembrane region. Consistent with the full-length and chimeric receptor results, EGFR-(681–1186) possessed much higher autokinase and Gab1 peptide activities relative to ErbB2-(712–1255).

To rule out the possibility that the observed low activity of ErbB2 is due to the Cos7 expression system or to the bound antibody in the immunoprecipitants, we purified soluble full-length EGFR or ErbB2 from stable 32D cell lines as previously described (21). Unlike EGFR in immunoprecipitants, soluble EGFR can be activated by EGF ∼5-fold upon 1 μM EGF stimulation in an in vitro kinase reaction, whereas as expected, the ErbB2 activity was not affected by EGFR. Consistent with the results from immunoprecipitation experiments, the activity of the soluble ErbB2 preparation was also very low and was ∼7 and 1.4% of EGFR activities in the absence and presence of 1 μM EGF, respectively (Fig. 1C). The truncated versions of the receptors, ErbB2-(712–1255) and EGFR-(681–1186), were cloned into baculovirus and expressed in Sf9 cells. The proteins were purified using the Ni2+-NTA affinity method as described under “Experimental Procedures.” No significant protein impurities were detected in the purified ErbB kinases by either Coomassie Blue (See Fig. 2C) or silver staining (data not shown). Again, as shown in Fig. 1D, the ErbB2-(712–1255) kinase activity was ∼10-fold lower than its EGFR counterpart.

To rule out that the activity difference between EGFR and ErbB2 is due to the assay conditions, we tested the ErbB2 kinase activities toward a number of peptide substrates, including a peptide whose sequence was optimized for ErbB2 using a degenerate peptide library (19) in the presence of Mg2+, Mn2+, or both. Under all conditions tested, ErbB2 kinase activity was
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Mutations in Kinase Domain Increase ErbB2 Catalytic Activity and Reveal Autoinhibition—To identify particular residues responsible for the low ErbB2 kinase activity, we aligned the ErbB2 kinase domain sequence with EGFR and ErbB4 (supplemental Fig. 1S). The sequence of ErbB2 kinase domain shares ~83 and 77% identity with EGFR and ErbB4, respectively. In the same region, EGFR and ErbB4 share 79% identity. The residues conserved throughout all protein kinases are unaltered in ErbB2. However, some residues identical in EGFR and ErbB4 are altered in ErbB2. Taking into consideration that the orientation of αC helix is critical for the catalytic activity of all protein kinases (2, 29), we speculated that the altered residues in ErbB2 within the loop located between the αC helix and β4 sheet (designated αC-β4 loop) may play a negative regulatory role and render the ErbB2 kinase in a low activity or inactive state. In this loop, five of the eight residues in ErbB2 (Gly-776, Gly-778, Ser-779, Tyr-781, and Ser-783) differ from those in EGFR. We generated full-length and truncated mutant ErbB2s in which single or multiple residues were changed to their counterparts in EGFR. The mutant with all five changes was designated 5M, and the mutant containing S779N, Y781H, and S783C was termed 3M. As shown in Fig. 2A, G776S mutation increased the full-length ErbB2 kinase activity ~3-fold, whereas G778D, G776S/G778D, and 5M produced more dramatic increases (~10–11-fold). The activities of these 3 mutants reached up to 80% that of EGFR. In contrast, virtually no activation was observed for 3M. Very similar activations were found for ErbB2-(712–1255) carrying these mutations (Fig. 2B). The sequence alignment also revealed, in addition to the αC-β4 loop, considerable variations in the ErbB2 kinase domain that differ from EGFR and ErbB4. These differences include two continuous segments 813–817 (NRGRL) and 963–965 (SEC), located within the αα-E and αH-αl loops, respectively, and a valine at 839 just before the catalytic loop and a threonine at 875 in the activation loop. However, changing these residues resulted in little or no increase in the ErbB2 kinase activity (Fig. 2B).

Furthermore, we purified truncated versions of activated ErbB2 mutants from Sf9 cells and measured their kinase activities. The kinase activities of these purified kinases (Fig. 2D) were very similar to those achieved with the immunopurified proteins from Cos7 cells (Fig. 2B). These data demonstrate that the residues within the αC-β4 loop in the N-lobe of the ErbB2 kinase domain, and especially Gly-778, function to restrain ErbB2 kinase activity.

Activation of ErbB2 by Mutation Is Not Due to Hsp90 Release—Previous studies have demonstrated that the ErbB2 αC-β4 loop is required for Hsp90 binding and geldanamycin (GA)-induced degradation of ErbB2. Mutations in this loop disrupt Hsp90 association and confer GA resistance (30–32). To investigate whether Hsp90 association directly inhibits ErbB2 kinase, we expressed EGFR or various versions of ErbB2 in Cos7 cells and treated the cells with 1 μM GA. Consistent with the published data (31, 32), we found that receptors containing the ErbB2 kinase domain bound Hsp90 (Fig. 3, lanes 4, 7, and 10), and GA treatment decreased the Hsp90 association (lanes 5, 8, and 11). Conversely, no Hsp90 binding to EGFR was detected (lanes 1–3). In an alternative approach, the immunocomplexes from untreated cell were treated with 1 μM GA in vitro. As shown in Fig. 3A (lanes 6, 9, and 12), ErbB2s and Hsp90 interaction was diminished by GA. Our results demonstrated that release of Hsp90 by either approach did not yield a significant increase in ErbB2 kinase activity (Fig. 3A, bar graph). We analyzed the immunocomplexes by SDS-PAGE and silver staining and found that only a small percentage (<5%) of ErbB2 in the immunocomplex had bound Hsp90 (data not shown). We also tested the effects of point mutations on the interaction of ErbB2 with Hsp90. As shown in Fig. 3B, all mutants had decreased ErbB2-Hsp90 interaction, consistent with previous work (31). However, the activation induced by these mutations is unlikely due to the decrease in the Hsp90 binding because the ErbB2 mutants purified from Sf9 cells, in which no Hsp90 association was detected by silver staining, had similar activation profiles (Fig. 2C and D). These results demonstrate that Hsp90 association was not responsible for the low ErbB2 kinase activity, and activation of ErbB2 by αC-β4 loop mutations is not due to Hsp90 release.

FIGURE 2. Mutations in ErbB2 kinase domain activate its catalytic activity. A, the full-length EGFR, WT, or mutant ErbB2s expressed in Cos7 cells were immunoprecipitated and subjected to kinase assay as in Fig. 18. B, kinase activity of truncated versions of receptor kinases were measured as in A. C, truncated EGFR and ErbB2s expressed in Sf9 cells were purified and analyzed by SDS-PAGE and Coomassie Blue staining. D, specific kinase activities of kinases purified from Sf9 cells were measured. Data represent the means ± S.E. of duplicate experiments.
Activated ErbB2s Have Increased ATP Binding Affinity and Turnover Number, but Their Affinity to Peptide Substrates Is Not Significantly Changed—To elucidate the enzymatic mechanism of mutation-induced ErbB2 activation, we performed steady-state kinetic studies using the kinases purified from Sf9 cells. These studies provide measurements of relative substrate affinity ($K_m$) and catalytic rate ($k_{cat}$, turnover number). The combined parameter, $k_{cat}/K_m$, which is referred to as specificity constant, provides the best measure of catalytic efficiency comparisons between enzymes. The apparent $K_m$ and $k_{cat}$ values of EGFR, wild type (WT), and mutant ErbB2s for ATP were determined by measuring the initial velocities at a fixed Gab1 Y627 peptide concentration of 1.2 mM. The Lineweaver-Burk plots for ATP of various kinases are shown in Fig. 4A, and the obtained kinetic parameters are summarized in Table 1. $K_m$ and $k_{cat}$ of WT ErbB2 for ATP were 20.8 $\mu$M and 1.4 min$^{-1}$, respectively. Compared with EGFR kinase, the ErbB2 $K_m$ for ATP was ~10-fold higher, whereas the $k_{cat}$ was ~5-fold lower. As a result, the overall catalytic efficiency of ErbB2, as indicated by $k_{cat}/K_m$, was strikingly lower (~50-fold) than that of EGFR. This analysis clearly showed that the poor catalytic ability of ErbB2 kinase resulted from both low ATP binding affinity and low turnover number ($k_{cat}$). G776S in ErbB2 had a small effect on the $K_m$ for ATP, whereas G778D, G776S/G778D, and 5M decreased the $K_m$ values by 4.1-, 8.6-, and 9.6-fold, respectively. The $k_{cat}$ values of G776S, G778D, G776S/G778D, and 5M were increased 2.1-, 6.0-, 7.0-, and 5.3-fold, respectively (Fig. 4A and Table 1). The increases in $k_{cat}$ and the concomitant decreases in $K_m$ yielded striking increases in the ErbB2 catalytic efficiency ($k_{cat}/K_m$). Compared with WT ErbB2, the increases in $k_{cat}/K_m$ values of G778D, G776S/G778D, and 5M were ~23-, 62- and 53-fold, respectively. The kinetic parameters of these mutants were close to these for the EGFR kinase.

We also examined the steady-state enzymatic parameters of EGFR and ErbB2 kinases for the peptide substrate Gab1 Y627. The kinetic parameters for these peptides were measured by keeping ATP concentration at a saturation level (200 $\mu$M). To eliminate the contribution of autophosphorylation, which affected the overall velocity to a varying extent depending upon the peptide concentration, we first incubated the receptor kinases with the kinase reaction buffer for 10 min to saturate the autophosphorylation and then initiated the peptide reaction. The initial rates were determined from the linear range of the reaction (supplemental Fig. S2). Fig. 4B contains Lineweaver-Burk plots of Gab1 Y627 phosphorylation catalyzed by EGFR, WT, G776S, and 5M ErbB2 kinases. As summarized in Table 2, relative to EGFR kinase, WT ErbB2 kinase possessed ~7.2-fold lower $k_{cat}/K_m$ values. The G776S, G778D, G776S/G778D, and 5M ErbB2 mutations increased $k_{cat}/K_m$ values for the peptide ~4–13-fold. Increased $k_{cat}$ values were mostly

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**TABLE 1**

Enzymatic parameters for ATP

| Kinases | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ |
|---------|------------|------------------------|--------------|
| EGFR    | 2.1 ± 0.1  | 6.9 ± 0.1              | 3286 ± 164   |
| WT ErbB2| 20.8 ± 2.3 | 1.4 ± 0.2              | 67 ± 12      |
| G776S   | 17.4 ± 1.8 | 2.9 ± 0.2              | 167 ± 21     |
| G778D   | 5.4 ± 0.4  | 8.5 ± 0.3              | 1574 ± 129   |
| G776S/G778D | 2.3 ± 0.2 | 9.7 ± 0.4              | 4217 ± 406   |
| 5M      | 2.1 ± 0.1  | 7.5 ± 0.2              | 3571 ± 195   |

**TABLE 2**

Enzymatic parameters for Gab1 Y627 peptide

| Kinases | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ |
|---------|------------|------------------------|--------------|
| EGFR    | 2.1 ± 0.1  | 6.9 ± 0.1              | 3286 ± 164   |
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| G776S   | 17.4 ± 1.8 | 2.9 ± 0.2              | 167 ± 21     |
| G778D   | 5.4 ± 0.4  | 8.5 ± 0.3              | 1574 ± 129   |
| G776S/G778D | 2.3 ± 0.2 | 9.7 ± 0.4              | 4217 ± 406   |
| 5M      | 2.1 ± 0.1  | 7.5 ± 0.2              | 3571 ± 195   |
responsible for the enhanced catalytic efficiencies of these ErbB2 mutants. In contrast to the results for ATP, the effects of the mutations on peptide binding affinities were relatively modest (less than 2-fold). In summary, these kinetic results demonstrate that the αC-β4 loop mutations relieve the ErbB2 kinase autoinhibition, resulting in increased ATP binding affinity and catalysis rate without significant change in the peptide substrate binding.

**Mutational Activation of ErbB2 Results in Elevated Cell Signaling**—To test the cellular signaling capacity of the ErbB2 mutants, we transiently expressed WT or mutant ErbB2s along with ErbB3 and epitope-tagged MAPK (HA-MAPK) in 293 cells and measured phosphorylation of the receptors and MAPK activation (Fig. 5A). Overall receptor phosphoryrosine in addition to phosphorylation at specific sites in ErbB2 (Tyr(P)-1221/1222; pErbB2) and ErbB3 (Tyr(P)-1289; pErbB3) were analyzed in lysates from the cells. Consistent with our in vitro kinase results, the levels of total phosphotyrosine, pErB2, and pErbB3 were markedly higher in the cells expressing mutant ErbB2s compared with the WT-expressing cells. Relative to WT ErbB2, expression of the ErbB2 mutants also resulted in MAPK activation.

It was not possible to evaluate the ligand-induced cell signaling capacity of the ErbB2 mutants in 293 cells because HRG-β1 (neuregulin) stimulation in mock-transfected control cells resulted in MAPK activation due to low levels of endogenous ErbBs (data not shown). The 32D murine hematopoietic cell line that does not express endogenous ErbB receptors or ligands has been proved to be a powerful tool in the study of ErB signaling (23, 33). The 32D-E3 cells stably express only ErbB3 (24). To further study the signaling potential of the activating ErbB2 mutants, we generated cell lines stably expressing WT or mutant ErbB2 in a 32D-E3 background. These cell lines express comparable levels of the receptors as determined by fluorescence-activated cell sorter analysis (data not shown) and Western blotting (Fig. 5B). We specifically immunoprecipitated ErbB2 and ErbB3 with Myc and ErbB3 antibodies, respectively, before the immunoblotting analysis. As shown in Fig. 5B, consistent with the results from the 293 cells, the basal phosphorylation levels of ErbB2 and ErbB3 in both cell lysates and the immunoprecipitants were significantly higher in the cells expressing the ErbB2 mutants relative to WT ErbB2-expressing cells. Upon HRG-β1 stimulation, pErB3 and pErbB2 in the WT ErbB2-expressing cells increased dramatically. Conversely, the HRG-β1 stimulation did not further increase the phosphorylation of either receptor in the cells expressing ErbB2 mutants.

**DISCUSSION**

PTK activity in normal resting cells is tightly controlled by multiple layers of autoinhibitory mechanisms. The loss of kinase regulation can result in disastrous consequences such as cancer (1, 3). Engagement of ligand by receptor PTKs is a major mechanism of activation. However, ErbB2 lacks the ability to bind a specific ligand, and its ECD has a conformation that resembles the ligand-bound active form of EGFR and appears poised to engage another ErbB. Thus, unlike the EGFR and ErbB4, the structure of the ErbB2 ECD does not provide a barrier that can prevent ErbB2 kinase activation. An alternative inhibitory mechanism is required to maintain control of ErbB2 kinase activity. In this work we clearly define that the catalytic activity of ErbB2 kinase is strongly autoinhibited relative to EGFR and ErbB4. Our most important finding is that ErbB2 catalytic activity is controlled by an autoinhibition mechanism existing within the kinase domain itself. Compared with EGFR kinase, ErbB2 kinase has a ~7-fold lower catalytic rate constant and a ~12-fold lower binding constant for ATP. Our results

**TABLE 2**

| Kinases | $K_{in}$ | $k_{cat}$ | $k_{cat}/K_{in}$ |
|---------|---------|-----------|------------------|
| EGFR    | 91.2 ± 9.7 | 7.7 ± 0.2 | 84.4 ± 9.2      |
| WT ErbB2| 128.3 ± 20.9 | 1.5 ± 0.1 | 74.1 ± 2.1      |
| G776S   | 74.4 ± 9.9  | 3.3 ± 0.1 | 44.4 ± 6.1      |
| G778D   | 95.4 ± 6.9  | 7.3 ± 0.2 | 67.5 ± 5.9      |
| G776S/G778D | 108.5 ± 6.5 | 13.2 ± 0.2 | 121.7 ± 7.5    |
| 5M      | 75.5 ± 6.8  | 11.7 ± 0.3 | 155.0 ± 14.3    |

Results represent means ± S.E. of four replicates.

**FIGURE 5.** Kinase domain mutations activate ErbB2 cell signaling. A, WT or mutant ErbB2s was transiently expressed in 293 cells along with ErbB3 and HA-MAPK. Lysates of the cells were analyzed by Western blotting (WB) for phosphotyrosine (pY) and phosphorylation at specific sites in ErbB2 (Tyr(P)-1221/1222; pErbB2) or ErbB3 (Tyr(P)-1289; pErbB3). To monitor MAPK activation, cell lysates were subjected to immunoprecipitation with anti-HA antibody and MAPK activation was monitored using an Ab specific for phosphorylated form of MAPK (pMAPK). B, 32D-E3 cells stably expressing WT or mutant ErbB2 were serum-starved for 3 h and treated with 100 ng/ml HRG-β1 at 37 °C for 5 min. ErbB2s and ErbB3 were immunoprecipitated with anti-Myc and anti-ErbB3 Abs, respectively. Cell lysates and immunoprecipitants were subjected to Western blotting analysis with indicated antibodies.

No basal level of phosphorylated MAPK (p-MAPK) was detected in the WT ErbB2-expressing cells, whereas marked levels of pMAPK were observed in the cells expressing mutant ErbB2s. In contrast to ligand-independent phosphorylation of either ErbB2 or ErbB3 in cells expressing ErbB2 mutants, MAPK activation was further increased upon HRG-β1 stimulation. No significant differences in the magnitude of MAPK activation were found between WT and mutant ErbB2s in the HRG-β1-treated cells. Taken together, these results demonstrate that expression of the activating ErbB2 αC-β4 loop mutants results in enhanced cell signaling.
Autoinhibition Mechanism of ErbB2 Kinase

![Diagram of ErbB2 kinase structure](image)

**FIGURE 6. Structural modeling of ErbB2 kinase domain.** A, ErbB2 kinase structure modeled upon the inactive form of EGFR kinase (Protein Data Bank code 2G57) (42). Important structure elements and residues depicted are αC helix (salmon), αC-β4 loop (yellow), β4 strand (cyan), activation loop (green), Lys-753 (blue), and Glu-770 (pink). Lys-753 and Glu-770 do not form a salt bridge in this inactive form. B, detailed view of the hydrophobic interaction between αC-β4 and activation loops. Residues forming the αC-β4 loop hydrophobic patch are shown as yellow spheres, with Gly-776 and Gly-778 shown as red spheres. The activation loop hydrophobic patch residues are shown as green sticks, and important residue side chains are labeled.

indicate that the autoinhibited conformation of ErbB2 restricts access of ATP to the active site and is not in an optimal state for catalysis.

ErbB2-containing heterodimers mediate stronger cellular signals than other ErbB combinations. It has been demonstrated that unlike ErbB4, ErbB2 presence is sufficient to mediate EGF-induced MAPK and Akt activation by a kinase-inactive EGFR (23). Several reasons may be attributable to the high ErbB2 signaling potency. First, as a preferred dimerization partner, ErbB2 enhances binding affinity of other ErbBs for their ligands (34, 35). Second, the intracellular C-terminal region of ErbB2 is rich in high affinity sites for phosphotyrosine-binding proteins relative to other ErbBs (36). Our previous work demonstrated that the phosphorylation sites in this region of ErbB2 are phosphorylated more efficiently by EGFR kinase than the corresponding sites in other ErbBs (37). Third, unlike EGFR, whose ligand-induced down-regulation is mediated by c-Cbl and is a fast process, ErbB2 and ErbB2-containing dimers undergo slow endocytosis, resulting in relatively prolonged and enhanced cellular signals (38). Finally, Src has been found to be activated by ErbB2 and to synergistically modulate ErbB2-mediated cell signaling by providing an additional kinase activity (39).

We found that mutation of residues in the αC-β4 loop resulted in a dramatic increase in ErbB2 kinase activity. This striking finding indicates that this loop is responsible for the low ErbB2 kinase activity and plays a critical role in ErbB2 control. Mutations in the αC-β4 loop activated ErbB2 kinase by releasing the autoinhibition. These activated forms of ErbB2 have increased ATP binding affinity and catalysis rate. The overall catalytic efficiency of the activated mutants, as indicated by the specificity constant (kcat/Km) for ATP, increased up to 63-fold compared with the WT ErbB2. These results provide evidence that the αC-β4 loop functions as a switch in ErbB2 regulation, and the two glycines at positions 776 and 778 are the key residues in this switch. We propose that the αC-β4 loop regulates ErbB2 kinase activity by orienting the αC helix to a conformation, which restricts ATP binding and catalysis.

The two key elements for kinase regulation, namely the activation loop and αC helix, are structurally coupled (2). For most PTKs, the catalytic activity is stimulated via phosphorylation of the activation loop. In these situations the activation loop phosphorylation is accompanied by a conformational change of the αC helix to an orientation ideal for catalysis. In some instances, such as cyclin-dependent kinases, the kinase activation requires cyclin binding, which induces αC helix conformation change and activation loop phosphorylation (40). Previous work (41) and our results4 suggest that phosphorylation of ErbB2 Tyr877 in the activation loop does not stimulate ErbB2 activation and is not a reason for the mutational activation observed here. In the case of ErbB2, the kinase activity appears to be regulated primarily by orientation of the αC helix.

To analyze the structural basis of the ErbB2 kinase autoinhibition, we modeled the ErbB2 kinase domain structure upon a recently published structure of the inactive EGFR (Protein Data Bank code 2G57) (42). The inactive form of EGFR was chosen as a template for ErbB2 modeling because of the low ErbB2 activity. As shown in Fig. 6, the αC-β4 loop in the N-lobe of the ErbB2 kinase forms a hydrophobic patch that interacts with another hydrophobic strip formed by residues in the activation loop centered by Phe-864. This phenylalanine is a part of the DFG motif which is conserved in the C-lobe of all protein kinases. In this proposed structure there would be no salt bridge formed between Lys-753 and the Glu-770 in the αC helix which is requisite for ATP binding and catalysis. Swapping of the small neutral Gly-778, which is centered in the αC-β4 loop hydrophobic patch, with a negative aspartic acid decreases the strength of the hydrophobic interaction. This change presumably induces conformational changes of both the αC helix and activation loop and, therefore, activates the kinase. The decreased hydrophobic interaction may also increase the flexibility of the kinase active site, which is essential for enzyme functions (43). Furthermore, as suggested by others (31, 32), the electrostatic interactions induced by the aspartic acid may also play a role in the kinase activation.

To test the hypothesis that mutations of Ser-744 and Asp-746 to glycines in the EGFR αC-β4 loop would repress EGFR kinase activity, we generated and purified this mutant from Sf9 cells and evaluated its activity in vitro. However, this mutation did not significantly alter the catalytic activity (data not shown). We also analyzed an ErbB2 L866R mutation, which is analo-

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4 Y. X. Fan and G. R. Johnson, unpublished results.
gous to the activating EGFR L834R frequently found in lung cancer patients. In the EGFR this Leu stabilizes the EGFR in an autoinhibited state which is lost upon mutation to Arg (42). Surprisingly, the L866R mutation in neither WT nor G776S/G778D ErbB2 was activating but led to a moderate decrease in the ErbB2 kinase activity (data not shown). These results indicate that the catalytic activity of the ErbB kinases is controlled by many elements in a delicate and comprehensive manner. These findings also provide evidence that the mechanism for regulation of EGFR catalytic activity is different from that for ErbB2.

A recent structural study found an asymmetrical dimer of EGFR kinase in which the C-lobe of one monomer allosterically activates the other monomer through a conformational change of the αC helix in a manner that is similar to activation of cyclin-dependent kinases by cyclins (42). ErbB2 kinase may be activated by a similar mechanism upon the formation of dimers. However, compared with EGFR kinase, the inactive state of ErbB2 is stabilized by a stronger hydrophobic interaction between the αC-β4 and activation loops, and the free energy (∆G) required for ErbB2 activation is higher. EGFR kinase is able to be activated by many factors, such as certain divalent cations (Mn²⁺), (NH₄)₄SO₄ (44), or the crystal lattice force (42). Under our assay conditions a significant population of the EGFR kinase in which the C-lobe of one monomer allosterically activates the other monomer through a conformational change of the αC helix is involved. EGFR kinase appears to exist in the active form, whereas related closely with the ErbB2 kinase activity (data not shown). These results indicate that the catalytic activity of the ErbB kinases is controlled by many elements in a delicate and comprehensive manner. These findings also provide evidence that the mechanism for regulation of EGFR catalytic activity is different from that for ErbB2.

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