Mutation of Threonine 766 in the Epidermal Growth Factor Receptor Reveals a Hotspot for Resistance Formation against Selective Tyrosine Kinase Inhibitors*

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Small molecule inhibitors of protein tyrosine kinases such as STI571 represent a major new class of therapeutics for target-selective treatment of human cancer. Clinical resistance formation to the BCR-ABL inhibitor STI571 has been observed in patients with advanced chronic myeloid leukemia, and was frequently caused by a C to T single nucleotide change in the Ab1 kinase domain, which substituted Thr-315 by isoleucine and rendered BCR-ABL resistant to STI571 inhibition. The corresponding mutation in the epidermal growth factor receptor (EGFR) tyrosine kinase replaced Thr-766 of the EGFR by methionine and dramatically reduced the sensitivity of EGFR to inhibition by selective 4-anilinoquinazoline inhibitors such as PD153035. Inhibitor-resistant EGFR exhibited the same signaling capacity as wild-type receptor in vivo and provides a useful tool for analyzing EGFR-mediated signal transduction. Our data identify Thr-766 of the EGFR as a structural determinant that bears the potential to become a relevant feature in resistance formation during cancer therapy with EGFR-specific 4-anilinoquinazoline inhibitors.

Experimental Procedures

Cell Lines, Reagents, and Plasmids—CHO-K1 cells were from ATCC. Immortalized embryonic EF1.1/~ fibroblasts derived from EF1.1/~ mice were a generous gift from Maria Sibilia and Erwin Wagner (Vienna, Austria). Cell culture media and LipofectAMINE were purchased from Invitrogen. Radiochemicals were from Amersham Biosciences. PD153035, AG1478, and human recombinant EGF were from Calbiochem. All other reagents were obtained from Sigma.

Antibodies purchased were mouse monoclonal anti-HA antibody (Roche Molecular Biochemicals), polyclonal anti-EGFR antibody (Santa Cruz Biotechnology), mouse monoclonal anti-SHC antibody (BD Transduction Laboratories), rabbit polyclonal anti-Gab1 antibody (Upstate), mouse monoclonal anti-phospho-ERK1/2 antibody (Cell Signaling Technology), rabbit polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology) and rabbit polyclonal anti-c-Fos antibody (Santa Cruz Biotechnology). Rabbit polyclonal anti-SHC and mAb108.1 mouse monoclonal anti-EGFR antibodies have been described previously (10).

Human EGFR cDNA was either cloned in the expression vector pFRK5 or in the retroviral expression vector pLXSN, which allows moderate protein expression under transient plasmid transfection (11, 12). pcDNA3-HA-ERK2 has been described previously (10). Mutants of EGFR were generated using a mutagenesis kit according to the manufacturer’s instructions (Stratagene).

Cell Culture and Transfections—COS-7 and EF1.1/~ cells were

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† The abbreviations used are: CML, chronic myeloid leukemia; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated protein kinase; G6PDH, glucose-6-phosphate dehydrogenase; LPA, lyosphosphatidic acid; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; CHO, Chinese hamster ovary; mAb, monoclonal antibody.

‡ The advanced form of CML, also known as blast crisis, initially responded to STI571 but then became resistant to drug treatment (6). In a subset of these cases, resistance formation has been attributed to a single C to T nucleotide mutation that replaced Thr-315 of Ab1 by isoleucine and thereby rendered BCR-ABL insensitive to STI571 treatment (7). Thr-315 is located at a hydrophobic cavity near the nucleotide binding site of c-Ab1 and is critical for binding of the ATP-competitive inhibitor STI571 but not essential for positioning of ATP itself, explaining why the activity is preserved in the mutant kinase (8). These findings establish that even targeted intervention strategies can be prone to resistance formation and further raise the important question of whether similar mechanisms might generally apply to tyrosine kinase inhibitors for cancer therapy. Apart from STI571, the most advanced small molecule drugs for treatment of malignancies belong to the 4-anilinoquinazoline class of compounds and selectively target the epidermal growth factor receptor (EGFR) tyrosine kinase, which has been implicated in the progression of various tumors (3, 4, 9). Specific EGFR inhibitors such as the quinazolines derivatives ZD1839 (Iressa®) and OSI-774 (Tarceva®) are already in late stages of clinical development, but the structural determinants of the EGFR kinase domain required for quinazoline binding and potentially involved in resistance formation have not been analyzed yet. In this report, we demonstrate that mutations equivalent to those found in BCR-ABL from relapsed CML patients dramatically desensitize EGFR to inhibition by 4-anilinoquinazolines without affecting its kinase activity and signal characteristics in vivo.
cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. CHO-K1 cells were maintained in nutrient-mixture Ham’s F12 medium containing 10% fetal bovine serum. Retroviral infections of EF 1.1/H11002 cells were performed as described (13).

Polyclonal cell lines stably expressing wild-type or mutant EGFR were established by selection in G418-containing medium. For plasmid transfection experiments in 6-well dishes, COS-7 (CHO-K1) cells were seeded at 2.0 × 10^5 (3.0 × 10^5) per well 20 h before transfection. Cells

FIG. 1. **EGFR mutants resistant to the 4-anilinoquinazoline inhibitor PD153035.** A, residues surrounding Thr-315 in Abl aligned with the corresponding EGFR sequence. Thr-315 in Abl and the equivalent Thr-766 in EGFR are highlighted in gray. B, in vitro kinase assay. Wild-type EGFR and EGFR-T766M mutant were preincubated with the indicated concentrations of PD153035 and then subjected to in vitro kinase assays using G6PDH as a substrate. Kinase activities in the absence of inhibitor were set to 100%; activities measured with different PD153035 concentrations are expressed relative to this value. C, CHO-K1 cells were transiently transfected with either empty vector or pLXSN expression plasmids encoding EGFR, EGFR-T766I, or EGFR-T766M. Following serum starvation for 24 h, cells were preincubated with the indicated concentrations of PD153035 or an equal volume of Me2SO for 15 min prior to stimulation with 10 ng/ml EGF for 5 min. After cell lysis, EGFR was immunoprecipitated with mAb108.1. Following gel electrophoresis, tyrosine-phosphorylated EGFR was detected by immunoblotting with monoclonal anti-phosphotyrosine antibody (αPY, upper panels). In parallel, the amount of EGFR in immunoprecipitates was analyzed using polyclonal anti-EGFR antibody (lower panels). D, serum-starved CHO-K1 cells transiently expressing either wild-type EGFR or EGFR-T766V were preincubated with the indicated concentrations of PD153035 for 15 min prior to stimulation with 10 ng/ml EGF. After cell lysis, EGFR was immunoprecipitated and analyzed as described in panel C.
were incubated for 4 h in 1.0 ml of serum-free medium containing 9 μl (6 μCi) of LipofectAMINE (Invitrogen) and 1.5 μg (2 μCi) plasmid of DNA per well. The transfection mixtures were then either supplemented with 1 ml of medium containing 20% fetal bovine serum (COS-7) or replaced by fresh medium containing 10% fetal bovine serum (CHO-K1); 20 h later, cells were either lysed or serum-starved for a further 20 h in serum-free medium prior to stimulation and lysis.

**Cell Lysis, Immunoprecipitation, and Immunoblotting**—Serum-starved cells were treated with inhibitors and growth factors as indicated prior to cell lysis in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM sodium pyrophosphate plus additives (10 mM sodium fluoride, 1 mM orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Lysates were precleared by centrifugation and then immunoprecipitated with the respective antibodies and protein G-Sepharose for 3 h at 4°C. After three washes with lysis buffer without additives, bound proteins were eluted with SDS sample buffer, boiled for 3 min, and then resolved by SDS gel electrophoresis. After SDS-PAGE, proteins were transferred to nitrocellulose membrane and immunoblotted with the indicated antibodies. For analysis of c-Fos induction, SDS-containing lysis buffer to solubilize nuclear proteins was used (14).

**EGFR in Vitro Kinase Assay**—CHO-K1 cells were transiently transfected in 10-cm dishes with 12 μg of either pRK5-EGFR or pRK5-EGFR-T766M expression plasmid and 36 μl of LipofectAMINE. On the following day, dishes were stimulated with 100 ng/ml EGF for 5 min and then lysed with 950 μl of lysis buffer. After pre-clearing by centrifugation, aliquots of 140 μl of lysate were immunoprecipitated with mAb108.1 antibody. Beads were washed twice with 300 μl of lysis buffer without additives and twice with 200 μl of kinase buffer containing 20 mM Tris-Cl, pH 7.5, 3 mM MgCl₂, 3 mM MnCl₂, 100 μM orthovanadate, and 0.1 mM dithiothreitol. Precipitated EGFRs were then preincubated on ice for 15 min in kinase buffer supplemented with EGFR inhibitor as indicated. Kinase reactions were then started by the addition of 10 μM ATP, 2 μCi of [γ-32P]ATP and 3 μg of glucose-6-phosphate dehydrogenase (G6PDH) (Sigma) and performed for 5 min at room temperature (15). Reactions were stopped by the addition of 3× SDS sample buffer. After SDS-PAGE, proteins were transferred to nitrocellulose membrane and immunoblotted with the indicated antibodies. For analysis of c-Fos induction, SDS-containing lysis buffer to solubilize nuclear proteins was used (14).

**RESULTS**

**Kinase-active EGFR Mutants Resistant to Quinazoline Inhibitors**—Mutation of Thr-315 in Abl to isoleucine resulted in 100-fold less kinase activity resistant to STI571 (Fig. 1B). With 10 μM ATP present in the kinase reactions, the catalytic activity of the mutant kinase was about 50% when compared with wild-type EGFR (data not shown).

For further analysis of inhibitor sensitivity, we studied EGFR mutants in intact CHO-K1 cells that lack endogenous EGFR expression. In addition to the EGFR-T766M mutant, we also replaced Thr-766 of the EGFR by isoleucine to analyze the amino acid substitution equivalent to that found in STI571-insensitive BCR-ABL. Transiently expressed wild-type EGFR or the EGFR-T766M mutant were stimulated with the indicated concentrations of EGF for 5 min prior to cell lysis. EGFR was then immunoprecipitated and analyzed as described in panel A, C, after serum starvation, EGFR and EGFR-T766M-expressing EF1.1 fibroblasts were treated for the indicated times with 10 ng/ml EGF. Upon cell lysis, EGFR was isolated by immunoprecipitation and further analyzed as described in panel A.

**FIG. 2. Characterization of PD153035-insensitive EGFR-T766M in stably transfected fibroblasts. A,** control-infected EF1.1+/− fibroblasts devoid of EGFR expression or EF1.1−/− cells stably expressing either wild-type EGFR or the EGFR-T766M mutant were serum-starved for 24 h. Following preincubation with the indicated concentrations of PD153035 for 15 min, cells were stimulated for 5 min with 10 ng/ml EGF prior to lysis. EGFR was immunoprecipitated with mAb108.1 and analyzed for tyrosine phosphorylation by immunoblotting with monoclonal anti-phosphotyrosine antibody (αPY, upper panels). In parallel, the amount of EGFR in immunoprecipitates was analyzed using polyclonal anti-EGFR antibody (lower panels). B, serum-starved EF1.1 cells expressing either wild-type EGFR or the EGFR-T766M mutant were stimulated with the indicated concentrations of EGF for 5 min prior to cell lysis. EGFR was then immunoprecipitated and analyzed as described in panel A, C, after serum starvation, EGFR and EGFR-T766M-expressing EF1.1 fibroblasts were treated for the indicated times with 10 ng/ml EGF. Upon cell lysis, EGFR was isolated by immunoprecipitation and further analyzed as described in panel A.
Phosphorylation of wild-type EGFR and both mutants was similar, indicating that the replacement of Thr-766 by isoleucine or methionine had no significant effect on EGFR kinase activity in the context of cellular ATP concentrations (Fig. 1C). As further seen in Fig. 1C, pretreatment of cells with 10 nM PD153035 already strongly suppressed EGF-induced tyrosine phosphorylation of wild-type EGFR. In stark contrast, 1000-fold higher concentrations of PD153035 only partially reduced tyrosine phosphorylation of EGFR-T766I and had no inhibitory effect on the T766M mutant. Thus, even the modest threonine to isoleucine conversion at position 766 dramatically reduced the sensitivity of EGFR to PD153035 in intact cells. The longer side chain of methionine in position 766 conferred full resistance to mutant EGFR at all PD153035 concentrations tested. As evident for the T766M mutant, desensitization to PD153035 was even more pronounced under the physiologically relevant cellular conditions than observed in our in vitro kinase assay. Furthermore, similar results were obtained when the quinazoline AG1478 was used for EGFR inhibition (data not shown) (18), strongly suggesting that substitution of methionine or isoleucine for Thr-766 desensitized EGFR to specific 4-anilinoquinazoline inhibitors of its kinase activity (19). Moreover, the hydroxyl group of Thr-766 could establish hydrogen bonds contributing to high affinity inhibitor binding. To analyze this issue, we replaced Thr-766 by a valine residue of similar size and compared EGF-stimulated tyrosine phosphorylation of wild-type EGFR and the T766V mutant in transiently transfected CHO-K1 cells. As shown in Fig. 1D, the EGFR-T766V mutant exhibited an /10-fold reduced sensitivity to PD153035 inhibition compared with wild-type receptor, indicating that the hydroxyl group of Thr-766 is important for inhibitor binding but that its absence can only partially account for the dramatic EGFR resistance formation observed for the T766I and T766M mutants.

**Signaling Capacity of Drug-resistant EGFR**—Upon ligand-induced autophosphorylation, phosphotyrosine-dependent binding of the adaptor proteins SHC and Grb2 couples EGFR to Sos-mediated activation of Ras/mitogen-activated protein kinase (MAPK) signaling (20, 21). In addition, docking proteins such as Gab1 are tyrosine-phosphorylated upon EGFR activation and recruit additional signal transducers into receptor-proximal multiprotein complexes (22). To test whether these mitogenic signals can be mediated through the EGFR-T766M mutant generated by the equivalent C to T single nucleotide mutation as previously found in STI571-resistant BCR-ABL...
from advanced CML patients (7), we utilized retroviral gene transfer to stably express either wild-type EGFR or the T766M mutant in immortalized EF1.1−/− fibroblasts derived from EGFR knockout mice (23). By analyzing ligand-stimulated tyrosine phosphorylation of EGFR in the presence of different PD153035 concentrations, we first confirmed that mutation of Thr-766 to methionine rendered EGFR resistant to PD153035 inhibition in murine fibroblasts (Fig. 2A). The IC50 value was shifted from below 10 nM to more than 10 μM in agreement with our data from CHO-K1 cells presented above. Importantly, cellular tyrosine phosphorylation of both wild-type EGFR and the drug-resistant mutant was similar in response to low, non-saturating doses of EGF (Fig. 2B). Time course experiments further revealed comparable kinetics of EGFR down-regulation upon prolonged EGF treatment (Fig. 2C). Thus, wild-type EGFR and the PD153035-insensitive mutant showed the same activation and desensitization characteristics under physiologically relevant conditions in intact cells.

To characterize EGFR-proximal signaling in wild-type and mutant receptor-expressing cells, we examined EGFR-stimulated tyrosine phosphorylation of the adaptor proteins SHC and Gab1. These cellular tyrosine kinase substrates play essential roles in receptor tyrosine kinase-mediated signal transmission. As shown in Fig. 3, A and B, EGF-induced SHC and Gab1 tyrosine phosphorylation was abrogated when cells expressing wild-type EGFR were preincubated with 1 μM PD153035, whereas neither signaling event was affected in fibroblasts expressing the EGFR-T766M mutant upon inhibitor pretreatment. Moreover, similar sets of proteins, including the adaptor protein Grb2, were found to co-precipitate with SHC or Gab1 in both cell lines, demonstrating that the Thr-766 to methionine mutation did not alter EGFR-proximal signaling steps in intact cells (Fig. 3, A and B).

We next examined whether the EGFR-T766M could also trigger MAPK signal transduction and gene expression in a PD153035-insensitive manner. To assay for activation of ERK MAP kinases, lysates from EGF-treated cells were subjected to immunoblotting with antibody specific for dually phosphorylated ERK1 and ERK2. As shown in Fig. 3C, micromolar concentrations of PD153035 abrogated ERK phosphorylation in wild-type EGFR but not in EGFR-T766M-expressing fibroblasts. Moreover, although pretreatment with 100 nM PD153035 strongly suppressed autophosphorylation of wild-type EGFR (Fig. 2A), this inhibitor concentration was not yet sufficient to block EGF-induced ERK activation, indicating that the remaining residual EGFR activation is still sufficient to trigger the ERK pathway. Finally, EGF-induced expression of the immediate-early gene, c-fos, was investigated; we again found that the threonine to methionine mutation had conferred PD153035-resistance to this EGFR-mediated signaling event (Fig. 3D). Thus, our results show that the equivalent C to T nucleotide change as found in STI571-resistant BCR-ABL fully restores EGFR-mediated signaling in the presence of a selective 4-anilinoquinazoline inhibitor. These findings further demonstrate that none of the kinases downstream of EGFR were affected by the quinazoline compound PD153035, confirming the high specificity of this EGFR inhibitor.

Mitogenic Responses Mediated through Wild-type and Inhibitor-resistant EGFR—Targeted cancer therapy employing selective EGFR inhibitors primarily aims at suppressing tumor cell proliferation. To quantify how the anti-proliferative effect of a specific quinazoline inhibitor is diminished if EGFR had acquired drug resistance, we performed thymidine incorporation assays to measure the EGF-mediated proliferative responses in both wild-type and T766M mutant EGFR-expressing cell lines. PD153035 pretreatment inhibited EGF-stimulated DNA synthesis mediated through wild-type EGFR with an IC50 value of about 50 nM (Fig. 4), whereas half-maximal inhibition of EGF-triggered thymidine incorporation through the EGFR-T766M mutant occurred at about 50-fold higher PD153035 concentrations of around 2.5 μM. Because even up to 10 μM of the inhibitor were without effect on EGFR-T766M tyrosine phosphorylation in intact cells, this finding reveals that cellular targets of PD153035 distinct from EGFR are involved in EGF-triggered cell cycle progression. These unknown secondary targets are inhibited at much higher concentrations of PD153035, again demonstrating the high selectivity of this specific EGFR blocker and further implying that analogous EGFR mutations might render cancer patients resistant to treatment with selective EGFR blockers.
Quinazoline-resistant EGFR Mutant as a Tool for Target Validation—We reasoned that co-expression of inhibitor-resistant EGFR should complement for endogenous, inhibitor-sensitive EGFR upon PD153035 treatment and thereby provide a novel tool for chemical-genetic validation of EGFR function. To test this, we used the EGFR-dependent ERK MAPK activation upon stimulation of G protein-coupled receptors as a model system (10). Upon transient expression of hemagglutinin (HA) epitope-tagged ERK2, COS-7 cells were stimulated with either 10 μM LPA to activate its cognate G protein-coupled receptor or 1 ng/ml EGF. HA-ERK2 was then immunoprecipitated and analyzed by immunoblotting with activation-specific antibody recognizing dually phosphorylated ERK2. In agreement with published data (10), pretreatment of cells with 1 μM PD153035 strongly suppressed LPA-induced and blocked EGF-triggered HA-ERK2 activation (Fig. 5, upper two panels). Similar inhibition of LPA- and EGF-induced ERK2 activity by PD153035 was observed when wild-type EGFR was co-expressed (Fig. 5, middle two panels). But, in striking contrast, co-transfection of the PD153035-resistant EGFR-T766M mutant fully restored both LPA- and EGF-triggered HA-ERK2 activation in the presence of 1 μM PD153035 (Fig. 5, lower two panels). Thus, this result confirms that specific inhibition of EGFR by PD153035 suppresses LPA-stimulated ERK activation and establishes inhibitor-resistant EGFR as a useful tool for target validation in signal transduction analysis.

DISCUSSION

Our results identify Thr-766 as a critical structural determinant controlling inhibitor sensitivity of the EGFR. Importantly, introduction of bulkier hydrophobic side chains at this position fully preserved the cellular kinase activity of the EGFR in the presence of selective kinase inhibitors, indicating potential mechanisms of molecular resistance formation as previously found for BCR-ABL from STI571-treated CML patients. In addition to the Thr-315 to isoleucine substitution, 15/16. Fry, D. W., Kraker, A. J., McMichael, A., Ambroso, L. A., Nelson, J. M., Stein-Gerlach for critical reading of the manuscript.

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