ARTICLE P 14-18

Structural mechanism of inhibitor-resistance by ERK2 mutations

Yun Seong Park[†], Myeongbin Kim[†] and Seong Eon Ryu^{*}

Department of Bioengineering, College of Engineering, Hanyang University, Seoul 04673, Republic of Korea

^{*}Correspondence: ryuse@hanyang.ac.kr

[†]These authors contributed equally to this work.

Extracellular signal-regulated kinase (ERK) is a serine-threonine kinase that is involved in the regulation of cellular signals. ERK inhibitors have been developed to treat cancers with B-Raf proto-oncogene mutations. However, the use of these inhibitors in disease settings induces ERK mutations resistant to the inhibitors, which poses major difficulties in effective cancer treatment. Here, we present the crystal structures of the ERK Y36H and G37C mutants that occur in cancer cells resistant to ERK inhibitors. The structures revealed mechanisms by which these mutations confer inhibitor-resistance to ERK. The Y36H mutant structure revealed a resistance mechanism that involves rotations of the His36 residue in the Gly-rich loop and the Tyr64 residue in the helix C, which blocks the entrance of inhibitors to the ATP-binding pocket. Furthermore, the G37C mutant structure exhibited that the mutation-induced rigidity in dihedral angles plays a major role in inducing inhibitor-resistance. Detailed structural information on the resistance mechanism suggests strategy for designing of novel inhibitors that can circumvent mutation-induced inhibitor resistance.

INTRODUCTION

Extracellular signal-regulated kinase (ERK) is a key enzyme in the mitogen-activated protein kinase (MAPK) pathway. ERK remains inactive until two amino residues within the activation loop are phosphorylated by mitogen-activated kinase (MEK) in response to extracellular growth factors. The ERK signaling pathway is involved in regulating cell proliferation, differentiation, and survival (Chaikwad et al., 2014; Goetz et al., 2014; Tong and Seeliger, 2015; Jaiswal et al., 2018; Pegram et al., 2019; Smorodinsky-Atias et al., 2020). It has been found that the tumor is resistant to ERK inhibitor treatment when the patient is continuously administered a small molecule inhibitor. It has recently been shown to induce targeted resistance to inhibitors in cancer cells in vitro as well as in vivo. And in subsequent studies, a wider variety of inhibitor-resistant mutations were identified (Smorodinsky-Atias et al., 2020).

ERK inhibition has been found to be effective in overcoming acquired resistance to BRAF/MEK inhibitors and has been used, but even if patients succeed in cancer treatment based on ERK inhibitors, patients relapse within 12 months due to acquired resistance. The patient's drug-resistant ERK showed reactivation of the MEK/ERK signaling and sustained ERK activation involving multiple mechanisms (Jaiswal et al., 2018). There have been many attempts to overcome or manage ERK resistance. Nevertheless, although inhibitors targeting ERK have had dramatic success in cancer treatment, reactivation of ERK signaling due to tolerance presents a difficult problem for many patients (Garraway and Jänne, 2012). In addition, the mechanism of obtaining ERK inhibitor resistance and signal reactivation is still unknown (Pegram et al., 2019). Therefore, understanding the

regulatory mechanisms of ERK and the effect of ERK mutations on inhibitor binding is an important goal.

A significant number of ERK mutations have been reported due to the continued use of ERK inhibitors against cancer cells (Jaiswal et al., 2018; Smorodinsky-Atias et al., 2020). Since relatively little research has been conducted with respect to ERK mutations, many of their properties remain to be identified (Smorodinsky-Atias et al., 2020). For most mutations leading to inhibitor-resistances, the mechanism of action is unknown; in most cases, the effect on the structure and catalytic properties of ERKs has not been established (Goetz et al., 2014). In particular, mutations occurring at Tyr36 and Gly37, which reside in the Gly-rich loop, exhibit resistance to the representative ERK2 inhibitors, Vertex-11e and SCH772984 (Brenan et al., 2016; Smorodinsky-Atias et al., 2020). To understand structural mechanism of mutation-induced inhibitor resistance, we determined the crystal structures of the human ERK2 Y36H and G37C mutant proteins. The structures revealed an inhibitor-resistance mechanism that involved the conformational rearrangement of the Gly-rich loop and adjacent helix C, and the change in loop flexibility due to the restriction of dihedral angles. This information can be exploited for designing novel inhibitors that can circumvent mutation-induced resistance.

RESULTS AND DISCUSSION

Mutation-induced loop conformation change

Structures of the inhibitor-resistant mutants Y36H and G37C of human ERK2 were determined by the molecular replacement method using the wild type rat ERK2 structure as the target. The diffraction data collection of crystals from the mutants are

presented in Table 1. The structures were refined to 2.2 and 2.4 Å resolutions for the Y36H and G37C mutants, respectively (Table 1). The overall structure of the mutants was similar to that of the wild-type (Figure 1). However, significant structural differences were observed in the Gly-rich loop where residues 36 and 37 reside (Figure 1 and 2).

The structure of the Y36H mutant exhibited conformational changes in the Gly-rich loop (Figure 1A). In the wild type ERK2 structure, the hydroxyphenyl ring of Tyr36 interacts with

TABLE 1 | Data collection and refinement statistics

	Y36H	G37C
Space group	P2 ₁	P2 ₁
Resolution range (Å)	30.06 – 2.28 (2.36 – 2.28)	29.97 – 2.48 (2.57 – 2.48)
Unit cell dimension a, b, c (Å)	48.86, 70.61, 60.91	48.82, 70.54, 60.24
α , β , γ (°)	90.00, 109.65, 90.00	90.00, 109.40, 90.00
Multiplicity	2.8 (2.1)	2.9 (2.6)
Completeness (%)	94.9 (85.0)	94.4 (96.9)
I/ σ I	11.0 (3.7)	8.8 (2.7)
R _{merge} (%)	7.3 (17.4)	9.7 (23.7)
R _{work} / R _{free} (%)	17.8 / 23.9	18.4 / 26.6
RMS in bonds (Å) / angles (°)	0.008 / 0.94	0.009 / 0.99
Average B factor (Å ²)	33.0	37.7
Number of non-H atoms macromolecules / solvent	2832 / 102	2852 / 71
Ramachandran favored (%)	95.07	92.51
Ramachandran allowed (%)	4.93	7.49
Ramachandran outliers (%)	0	0

FIGURE 1 | Structure of ERK2 wild type and mutants. (A) Superposition of the wild type (PDB ID: 5UMO) and Y36H mutant structures that are in gray and green, respectively. (B) Superposition of the wild type (PDB ID: 5UMO) and G37C mutant structures that are in gray and magenta, respectively.

that of Tyr64 in the helix C, forming a π - π interaction (Figure 1A). The Y36H mutation, involving a side chain change from hydroxyphenyl to imidazole, disrupted the π - π interaction, thereby resulting in the rotation of the imidazole side chain of His36 away from the hydroxyphenyl of Tyr64. The side chain of Tyr64 rotated and filled the empty space created by the rotation of the imidazole side chain of mutated His36 in the ERK2 Y36H mutant.

The G37C mutation introduced a thioethyl side chain in the residue, restricting the main chain dihedral angles of the residue in the favored regions of the Ramachandran plot. In the wild type structure with the Gly37 residue, the dihedral angles of the residue were 107.20° and 167.10° that were located in the unfavored region (Figure 3). Cys37 would not be stable with the original dihedral angles, and the G37C mutant structure exhibited dihedral angles of -94.63° and 163.21° that were within the allowed regions (Figure 3). The change in dihedral angles in the residue 37 affected the conformation of adjacent regions, leading to rearrangement of the entire Gly-rich loop (Figure 1B). Further, the thioethyl side chain of Cys37 pointed toward the inhibitor-binding pocket (see below). The loop conformation-rearrangement also slightly shifted the position of

FIGURE 2 | Electron density map of ERK2 mutants. The 2Fo-Fc maps are displayed as superposed with final models. (A) The Y36H mutant. (B) The G37C mutant.

FIGURE 3 | Mutation-induced dihedral angle switch. (A) The dihedral angle position of Gly37 (black-circled blue-triangular point) in the wild type ERK2 structure (PDB ID: 5UMO) is located in the unfavored region of the Ramachandran plot. (B) The dihedral angle position of Cys37 (black-circled blue-rectangular point) in the ERK2 G37C mutant structure is located in the favored region of the Ramachandran plot.

FIGURE 4 | Inhibitor structure. (A) Vertex-11e. The inhibitor parts are labeled as the core and extension 1 (ext1) regions. (B) SCH772984. The inhibitor parts are labeled as the core, extension 1 (ext1), and extension 2 (ext2) regions.

FIGURE 5 | Superposition of the ERK2 mutant structures with the Vertex-11e-complexed wild type ERK2 structure (PDB ID: 6OPK). (A) The Y36H mutant is in green and the Vertex-11e-complexed wild type ERK2 is in orange. (B) The G37C mutant is in magenta and the Vertex-11e-complexed wild type ERK2 is in orange.

the hydroxyphenyl side chain of Tyr36, leading to a tighter π - π interaction with the side chain of Tyr64 than that of the wild-type structure.

Inhibitor resistance mechanism

Vertex-11e and SCH772984 are representative ERK2 inhibitors that are highly potent and orally bioavailable with K_i values of the low nanomolar range (Aronov et al., 2009; Morris et al., 2013) (Figure 4). However, when ERK2 acquires inhibitor-resistant mutations, such as Y36H and G37C, the inhibitors no longer exhibit enzyme inhibition. Both the inhibitors bind to the ATP-binding pocket of ERK2 (Chaikuad et al., 2014; Pegram et al., 2019). In the ERK2-inhibitor complex structure, the core regions of both the inhibitors (Figure 4) were bound to the central region of the ATP-binding pocket where the adenosine base of the ATP molecule is positioned, and extension 1 and 2 regions interact with peripheral regions of the pocket. The tip of the Gly-rich loop, involving residues 36 and 37, provides inhibitor interactions in the peripheral regions.

The superposition of the ERK2-Vertex-11e complex with the Y36H mutant indicated that the rotated imidazole side chain of His36 overlaps with the extension 1 region of the inhibitor (Figure 5A), inhibiting the binding of the inhibitor to the Y36H mutant.

FIGURE 6 | Superposition of the ERK2 mutant structures with the SCH772984-complexed wild type ERK2 structure (PDB ID: 6OPI). (A) The Y36H mutant is in green and the SCH772984-complexed wild type ERK2 is in yellow. (B) The G37C mutant is in magenta and the SCH772984-complexed wild type ERK2 is in yellow.

The superposition with the G37C mutant also exhibited collision of the thioethyl side chain of Cys37 to the extension 1 region of the inhibitor (Figure 5B). In the ERK2-SCH772984 complex structure, the extended piperazine-phenyl-pyrimidine (PPP) motif (extension 2 region in Figure 4) replaces the hydroxyphenyl side chain of Tyr36 to form π - π interaction with the side chain of Tyr64 (Figure 6A).

However, in the Y36H mutant structure, the side chain of Tyr64 rotated to the empty space created due to the rearrangement of the mutated His36, and the superposition of the ERK2-SCH772984 complex structure with the Y36H mutant structure exhibited an overlap of the PPP motif with the side chain of Tyr64. Consequently, the entrance of the PPP motif for the π - π interaction was blocked. In the G37C mutant structure, the thioethyl side chain of Cys37 overlapped with the extension 1 region of SCH772984, inhibiting the binding of the inhibitor to the G37C mutant (Figure 6B). The lack of main chain flexibility due to addition of a side chain in residue 37 also likely contributed to the inability of inhibitor binding because the binding of the inhibitors to the ATP-binding pocket requires an induced fit of the Gly-rich loop conformation (Zhang et al., 2012).

Implications for potent inhibitor design

Structures of the Y36H and G37C mutants revealed mechanisms for the inhibitor-resistance of the ERK2 mutations. In the case of the Y36H mutation, the rearranged side chain of the C-helix Tyr64 blocked access of the extension 2 region of SCH772984 (Figure 6). Therefore, we consider that shortening of the extension 2 region, for example, deletion of the terminal ring system, would help avoid blockage of the SCH772984 entrance to the ATP-binding pocket. Because the hydroxyphenyl side chain of Tyr64 is a prominent moiety in the peripheral entrance of the ATP-binding pocket, a shortened extension 2 region may yield an inhibitor with high potency and low possibility of inducing inhibitor-resistance.

The G37C mutation induced rigidity in the Gly-rich loop due to restriction of the dihedral angles. This rigidity affected the induced fit of the loop for optimal inhibitor interactions. In addition, the thioethyl side chain of the mutated Cys37 partially overlapped with extension 1 regions of both Vertex-11e and SCH772984. Therefore, structural alternatives to the extension 1 region can provide optimal interactions with the wild type and mutated residues in the Gly-rich loop and holds implications in designing novel inhibitors. With the guidance of structural information of inhibitor-resistance, the rational design in combination with chemical library search can yield potent inhibitors that have less possibility of resistant mutant induction.

METHODS

Site-directed mutagenesis

The expression vector pET-28a encoding the wild-type human ERK2 full-length domain was used as a template for the polymerase chain reaction during mutagenesis. Mutations were introduced using a QuikChange site-directed mutagenesis kit (Stratagene) and respective primers. The histidine substitution at Tyr36 was introduced using primers 5'-GCGAGGGCGCCACGGCATGGTGTG-3', and 5'-CACACCATGCCGTGGGC GCCCTCGC-3' to produce the full-length ERK2 Y36H mutant. The cysteine substitutions at Gly37 was introduced using primers 5'-GAGGGCGCCTACTGCATGGTGTGCTC-3', and 5'-GAGCACCATGCGAGTAGGCGCCCTC-3', to produce the full-length ERK2 G37C mutant.

Expression and purification

The recombinant vectors encoding wild type and mutated (Y36H and G37C) ERK2's were used to transform an E.coli strain BL21 (DE3) competent cell. Protein overexpression in transformed cells were induced with 0.1 mM IPTG for 16 h at 18°C. The cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C, washed with distilled water, then centrifuged again at 6,000 rpm for 10 min and stored at -74°C. Harvested cells were suspended in a buffer containing 50 mM Tris-Cl (pH 7.5), 500 mM NaCl, and 0.05% (v/v) β -mercaptoethanol, and sonicated on ice. Cell lysate was separated to soluble and insoluble fractions

by centrifugation at 16500 rpm for 1 h at 4°C. The His-tagged proteins were purified by affinity column (Ni-NTA, Qiagen) equilibrated with an equilibration buffer containing 50 mM Tris-Cl (pH 7.5) and 200 mM NaCl. The sample-loaded column was washed by a wash buffer containing 50 mM Tris-Cl (pH 7.5) and 1.0 M NaCl. After the column was washed, proteins were eluted with an elution buffer containing 50 mM Tris-Cl (pH 7.5), 200 mM NaCl, 500 mM imidazole, and 5 mM β -mercaptoethanol. Next, the proteins were further purified by the Sephacryl S-100 gel filtration chromatography with a final buffer containing 20 mM MOPS (pH 6.7), 150 mM NaCl and 5 mM β -mercaptoethanol. Purified proteins were concentrated to 30 mg/mL and stored at -74°C.

Crystallization and diffraction data collection

All crystallization tests were performed at 18°C and 4°C using the sitting drop vapor diffusion method in 96-well sitting drop plates, and appropriate temperatures were selected. Crystals were grown in a 1:1 ratio mixture of reservoir buffer and concentrated protein (30 mg/mL) to a final volume of 1 μ L. Crystallization trials were performed by using the Mosquito Crystallization system (TTP Labtech) with Index (Hampton research) and Morpheus[®] (Molecular Dimensions) crystal screen kits. Best crystals were obtained from 100 mM Tris-Cl (pH 8.5), 1.35 M ammonium sulfate, and 13% glycerol (Y36H); 100 mM HEPES/MOPS (pH 7.5), 20% PEG 500MME, 10% PEG 20000, 25 mM DL-glutamic acid monohydrate, 25 mM DL-alanine, 25 mM DL-lysine, and 25 mM glycine (G37C). X-ray diffraction data of crystals were collected in Pohang Light Source beamlines 7A and 11C (Park et al., 2017). The crystals of ERK2 Y36H were flash-frozen using a cryo solution containing 100 mM Tris-Cl (pH 8.5), 1.35 M ammonium sulfate, and 20% glycerol. In contrast, cryo solution for ERK2 G37C was 100 mM HEPES/MOPS (pH 7.5), 20% PEG 500MME, 10% PEG 20000, 25 mM DL-glutamic acid monohydrate, 25 mM DL-alanine, 25 mM DL-lysine, and 25 mM glycine. Crystals were flash-frozen in liquid nitrogen.

Structure determination

Diffraction data were processed using the program HKL2000 (Otwinowski and Minor, 1997). Data processing and merging produced 2.2 and 2.4 Å resolution data sets for the Y36H and G37C mutant crystals, respectively. The crystal structures were determined by the molecular replacement method using the wild type ERK2 structure (PDB code: 5UMO) as the target. For the molecular replacement solution, the program PHENIX (Liebschner et al., 2019) was used. The programs COOT (Emsley et al., 2010) and PHENIX were used for model building and refinement, respectively. R_{work} and R_{free} for the final model of the Y36H mutant structure were 17.8 and 23.9%, respectively, and R_{work} and R_{free} for the final model of the G37C mutant structure were 18.0 and 26.7%, respectively.

DATA DEPOSITION

Structural coordinates of human ERK2 Y36H and G37C were deposited in Protein Data Bank with codes of 7E73 and 7E75, respectively.

ACKNOWLEDGEMENTS

We thank the staff members of beamline 5C at the Pohang Accelerator Laboratory (PAL) for diffraction data collection. This work was supported by the biomedical technology development project, National Research Foundation, KOREA [NRF-2015M3A9B5030302].

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

Original Submission: Feb 22, 2021

Revised Version Received: Mar 16, 2021

Accepted: Mar 16, 2021

REFERENCES

- Aronov, A.M., Tang, Q., Martinez-Botella, G., Bemis, G.W., Cao, J., Chen, G., Ewing, N.P., Ford, P.J., Germann, U.A., Green, J., Hale, M.R., Jacobs, M., Janetka, J.W., Maltais, F., Markland, W., et al. (2009). Structure-guided design of potent and selective pyrimidylpyrrole inhibitors of extracellular signal-regulated kinase (ERK) using conformational control. *J Med Chem* **52**, 6362-6368.
- Brenan, L., Andreev, A., Cohen, O., Pantel, S., Kamburov, A., Cacchiarelli, D., Persky, N.S., Zhu, C., Bagul, M., Goetz, E.M., Burgin, A.B., Garraway, L.A., Getz, G., Mikkelsen, T.S., Piccioni, F., et al. (2016). Phenotypic characterization of a comprehensive set of MAPK1/ERK2 missense mutants. *Cell Rep* **17**, 1171-1183.
- Chaikuad, A., Tacconi, E.M., Zimmer, J., Liang, Y., Gray, N.S., Tarsounas, M., and Knapp, S. (2014). A unique inhibitor binding site in ERK1/2 is associated with slow binding kinetics. *Nat Chem Biol* **10**, 853-860.
- Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486-501.
- Garraway, L.A., and Jänne, P.A. (2012). Circumventing cancer drug resistance in the era of personalized medicine. *Cancer Discov* **2**, 214-226.
- Goetz, E.M., Ghandi, M., Treacy, D.J., Wagle, N., and Garraway, L.A. (2014). ERK mutations confer resistance to mitogen-activated protein kinase pathway inhibitors. *Cancer Res* **74**, 7079-7089.
- Jaiswal, B.S., Durinck, S., Stawiski, E.W., Yin, J., Wang, W., Lin, E., Moffat, J., Martin, S.E., Modrusan, Z., and Seshagiri, S. (2018). ERK mutations and amplification confer resistance to ERK-inhibitor therapy. *Clin Cancer Res* **24**, 4044-4055.
- Liebschner, D., Afonine, P.V., Baker, M.L., Bunkóczi, G., Chen, V.B., Croll, T.I., Hintze, B., Hung, L.W., Jain, S., McCoy, A.J., Moriarty, N.W., Oeffner, R.D., Poon, B.K., Prisant, M.G., Read, R.J., et al. (2019). Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. *Acta Crystallogr D Struct Biol* **75**, 861-877.
- Morris, E.J., Jha, S., Restaino, C.R., Dayananth, P., Zhu, H., Cooper, A., Carr, D., Deng, Y., Jin, W., Black, S., Long, B., Liu, J., Dinunzio, E., Windsor, W., Zhang, R., et al. (2013). Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. *Cancer Discov* **3**, 742-750.
- Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* **276**, 307-326.
- Park, S.Y., Ha, S.C., and Kim, Y.G. (2017). The protein crystallography beamlines at the Pohang Light Source II. *Biodesign* **5**, 30-34.
- Pegram, L.M., Liddle, J.C., Xiao, Y., Hoh, M., Rudolph, J., Iverson, D.B., Vigers, G.P., Smith, D., Zhang, H., Wang, W., Moffat, J.G., and Ahn, N.G. (2019). Activation loop dynamics are controlled by conformation-selective inhibitors of ERK2. *Proc Natl Acad Sci U S A* **116**, 15463-15468.
- Smorodinsky-Atias, K., Soudah, N., and Engelberg, D. (2020). Mutations that confer drug-resistance, oncogenicity and intrinsic activity on the ERK MAP kinases-current state of the art. *Cells* **9**, 129.
- Tong, M., and Seeliger, M.A. (2015). Targeting conformational plasticity of protein kinases. *ACS Chem Biol* **10**, 190-200.
- Zhang, J., Shapiro, P., and Pozharski, E. (2012). Structure of extracellular signal-regulated kinase 2 in complex with ATP and ADP. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **68**, 1434-1439.