Structural Basis of RIP1 Inhibition by Necrostatins

Tian Xie,1,4 Wei Peng,1,4 Yexing Liu,1,4 Chuangye Yan,1 Jenny Maki,2 Alexei Degterev,2 Junying Yuan,3 and Yigong Shi1,*

1Ministry of Education Protein Science Laboratory, State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua-Peking Joint Center for Life Sciences, Center for Structural Biology, School of Life Sciences and School of Medicine, Tsinghua University, Beijing 100084, China
2Department of Biochemistry, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA
3Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
4These authors contributed equally to this work
*Correspondence: shi-lab@tsinghua.edu.cn
http://dx.doi.org/10.1016/j.str.2013.01.016

SUMMARY

Necroptosis is a cellular mechanism that mediates necrotic cell death. The receptor-interacting serine/threonine protein kinase 1 (RIP1) is an essential upstream signaling molecule in tumor-necrosis-factor-α-induced necroptosis. Necrostatins, a series of small-molecule inhibitors, suppress necroptosis by specifically inhibiting RIP1 kinase activity. Both RIP1 structure and the mechanisms by which necrostatins inhibit RIP1 remain unknown. Here, we report the crystal structures of the RIP1 kinase domain individually bound to necrostatin-1 analog, necrostatin-3 analog, and necrostatin-4. Necrostatin, caged in a hydrophobic pocket between the N- and C-lobes of the kinase domain, stabilizes RIP1 in an inactive conformation through interactions with highly conserved amino acids in the activation loop and the surrounding structural elements. Structural comparison of RIP1 with the inhibitor-bound oncogenic kinase B-RAF reveals partially overlapping binding sites for necrostatin and for the anticancer compound PLX4032. Our study provides a structural basis for RIP1 inhibition by necrostatins and offers insights into potential structure-based drug design.

INTRODUCTION

Necroptosis, also known as programmed necrosis, is a cellular mechanism that mediates necrotic cell death (Christofferson and Yuan, 2010; Vandenabeele et al., 2010). Necroptosis may be activated in response to the stimulation of death receptors by their cognate ligands in the absence of caspase activity, the essential mediators of apoptosis. Necrotic cell death is characterized by typical morphological features of necrosis, including early plasma membrane permeabilization, swollen organelles, dilated nuclear membrane, and condensed chromatin (Vandenabeele et al., 2010). Necroptosis has been implicated in the pathology of a number of diseases, such as ischemic injury, neurodegeneration, and viral infection (Vandenabeele et al., 2010). Inhibition of necroptosis represents an attractive therapeutic strategy for preserving cell viability and functions in these diseases.

The receptor-interacting serine/threonine protein kinase 1 (RIP1) contains an N-terminal kinase domain, an RIP homotypic interaction motif (Sun et al., 2002), and a C-terminal death interaction motif (Sun et al., 2002), and a C-terminal death domain (Stanger et al., 1995). Activation of tumor necrosis factor receptor 1 (TNFR1) by TNFα may lead to multiple downstream signaling events, including NF-κB activation, apoptosis, and necroptosis. The Ser/Thr kinase activity of RIP1 is essential for necroptosis and ripoptosome-mediated caspase-dependent apoptosis (Feoktistova et al., 2011; Tenev et al., 2011) but is dispensable for NF-κB activation, which relies on the ubiquitination of the central intermediate domain of RIP1. During necroptosis, RIP1 kinase activity is involved in mediating the transition from receptor-anchored complex I to cytosolic complex II, which is defined by the interaction of RIP1 with RIP3 (Christofferson and Yuan, 2010; Vandenabeele et al., 2010). The kinase domains of RIP1 and RIP3 share 33% sequence identity and 53% sequence similarity.

Necrostatins, isolated as specific and potent small-molecule inhibitors of necroptosis, directly inhibit the kinase activity of RIP1 (Degterev et al., 2005, 2008). Inhibition of RIP1 by necrostatins has been shown to ameliorate tissue damage in animal models of ischemic brain injury (Degterev et al., 2005), retina ischemia-reperfusion (Rosenbaum et al., 2010), myocardial infarction (Lim et al., 2007), and traumatic brain injury (You et al., 2008). The specificity and activity of three necrostatin-1 analogs have been examined thoroughly both in vitro and in vivo, providing important information about necrostatin-1 in disease models (Takahashi et al., 2012).

Despite rigorous effort, there is no atomic-resolution structure for RIP1 or its homolog RIP3. The lack of structural information hinders functional and mechanistic understanding of the RIP kinases and limits the optimization of necrostatins and rational drug design. In this study, we present the crystal structures of the RIP1 kinase domain bound to three different necrostatins. Our results reveal the structural basis of necrostatin-mediated RIP1 inhibition and may facilitate the design and development of RIP1-specific small-molecule inhibitors.

RESULTS AND DISCUSSION

Characterization and Crystallization of the RIP1 Kinase Domain

The human RIP1 kinase domain (residues 1–312) was expressed in insect cells and purified to homogeneity. To characterize the activities of necrostatins, we reconstructed an
autophosphorylation assay as previously described (Degterev et al., 2008). Using this assay, a chemically improved derivative of necrostatin-1, (R)-5-((7-chloro-1H-indol-3-yl)methyl)-3-methylimidazolidine-2,4-dione (Nec-1a) (Figure 1A) (Degterev et al., 2008), exhibited an inhibitory constant (IC\textsubscript{50}) of 0.32 M for RIP1 (Figure 1B; Figure S1A available online). By contrast, necrostatin-4, (S)-N-(1-[2-chloro-6-fluorophenyl]ethyl)-5-cyano-1-methyl-1H-pyrrole-2-carboxamide (Nec-4) (Figure S2A), and a necrostatin-3 analog, 1-((3S,3aS)-3-[3-fluoro-4-[6 trifluoromethoxy]phenyl]-8-methoxy-3,3a,4,5-tetrahydro-2H-benzol[g]indazol-2-yl]-2-hydroxyethanone (Nec-3a) (Figure S2B), exhibited IC\textsubscript{50} values of about 0.37 and 0.44 M, respectively (Figure S1A).

Despite rigorous effort, the human RIP1 kinase domain, both by itself and in complex with necrostatins, defied crystallization. Reasoning that flexible sequences might hinder crystallization, we generated a number of constructs by trimming the hydrophilic sequences at the N and/or C terminus. One such construct (residues 1–294) gave rise to small crystals only in the presence of Nec-1a. However, these crystals diffracted X-rays weakly. Replacement of four cysteine residues by alanine in the RIP1 kinase domain led to marked improvement of crystal size and morphology. The engineered RIP1 kinase domain (residues 1–294, C34A, C127A, C233A, and C240A) was also crystallized in the presence of Nec-4 and Nec-3a. All crystals appear in the space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}, with different cell dimensions (Table 1).

There are two molecules of RIP1 kinase domain in each asymmetric unit. Because these two molecules display identical features relevant for discussion, we only focus on one such molecule.

**Overall Structure of the RIP1 Kinase Domain**

We attempted, and were successful at, structure determination by molecular replacement using the atomic coordinates of B-RAF kinase domain (Protein Data Bank [PDB] code 3C4C) (Tsai et al., 2008). The X-ray structure of the RIP1 kinase domain (referred to as RIP1 hereafter) bound to Nec-1a was refined at 2.25 Å resolution (Figure 1C; Table 1). RIP1 exhibits a canonical kinase fold, with an N-lobe, a C-lobe, and an intervening activation loop (also known as the T-loop). Nec-1a is bound between the N- and C-lobes, in close proximity to the activation loop (Figure 1C). Notably, the Nec-1a-bound RIP1, where Nec-1a binds largely outside of the ATP-binding pocket, does not contain any nucleotide. Similar to B-RAF and other protein kinases, the N-lobe comprises an antiparallel, five-stranded β sheet and an activation helix (commonly known as the alpha C-helix) (Figure 1C; Figure S1B). The C-lobe contains six α helices and a pair of β strands (Figure 1C; Figure S1B). All essential amino acids for ATP binding and hydrolysis in canonical kinases are conserved in RIP1, including the catalytic triad residues Lys45/Glu63/Asp156 and key residues in the P-loop (residues 24–31) and the catalytic loop (residues 136–143) (Figure S1B). Most of these amino acids are visible in the electron density map and are placed in the vicinity of the ATP-binding site in RIP1.

**Recognition of Nec-1a**

Nec-1a is buried in a relatively hydrophobic pocket between the N-lobe and the C-lobe (Figure 1D; Figure S1C). The indole ring of Nec-1a interacts with six amino acids, Met67, Leu70, Val75, Leu129, Val134, and His136, through van der Waals contacts, whereas the five-membered ring is surrounded by hydrophobic amino acids Val76, Leu78, Val90, Met92, Leu157, Leu159, and Phe162 (Figure 1D). These hydrophobic interactions likely provide the major driving force for binding. Consistent with the structural observations, mutation of Phe162 to Glu led to decreased inhibition of RIP1 by Nec-1 (Degterev et al., 2008).

The vast majority of these amino acids are highly conserved among RIP3 and RIP1 orthologs in mouse, frog, and fish (Figure S1B). This binding feature is consistent with the hydrophobic nature of Nec-1a, which, compared to a solubility of at least 500 mM in dimethyl sulfoxide (DMSO), is soluble only up to about 2 mM in aqueous solution.

In addition to the hydrophobic interactions, there are three specific hydrogen bonds (H-bonds) at the interface, which
appear to anchor the orientation of Nec-1a in the greasy pocket of RIP1. The indole ring of Nec-1a contributes one H-bond, between its nitrogen atom and the hydroxyl oxygen of Ser161 on the activation loop (Figure 1D). The other two H-bonds are mediated by the five-membered ring of Nec-1a, involving the carbonyl oxygen of Val76 and the amide nitrogen of Asp156.

Inactive Conformation of RIP1

Binding by Nec-1a presumably locks RIP1 in an inactive conformation. Structural comparison between RIP1 and the catalytic subunit of protein kinase A (PKA; PDB code 2CPK) (Knighton et al., 1991a, 1991b) confirms this notion (Figure 2). Most notably, the activation helix in RIP1 is rotated by approximately 40° relative to that in PKA, and the space vacated by the RIP1 activation helix is partially occupied by the activation loop and inhibitor Nec-1a (Figure 2A). Consequently, one of the catalytic triad residues, Glu63 in RIP1, is about 15 Å away from Lys45. (Figure 1D). Consistent with a dominant role by hydrophobic contacts, mutation of Ser161 to Ala only led to a 4-fold increase of the IC50 value (Figure S1D). The moderate increase is caused by loss of one H-bond; Ala can be nicely accommodated in the Nec-1a binding pocket. By contrast, mutation of Ser161 to a bulkier, negatively charged amino acid Glu resulted in a 10-fold decrease in efficiency of inhibition by Nec-1a (Figure S1D). Nonetheless, the S161E mutation is tolerated by Nec-1a binding, suggesting a remarkable conformational adjustability in the binding pocket of RIP1.

Table 1. Statistics of Data Collection and Refinement

<table>
<thead>
<tr>
<th>Data</th>
<th>Nec-1a-Bound RIP1</th>
<th>Nec-4-Bound RIP1</th>
<th>Nec-3a-Bound RIP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2,2,2,1</td>
<td>P2,2,2,1</td>
<td>P2,2,2,1</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
<td>47.13, 93.60, 129.07</td>
<td>46.84, 97.14, 128.12</td>
<td>82.63, 91.41, 103.33</td>
</tr>
<tr>
<td>Unit cell (%)</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.0000</td>
<td>0.9793</td>
<td>0.9793</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>40~2.25</td>
<td>40~2.18</td>
<td>40~2.90</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>5.9 (20.1)</td>
<td>6.0 (22.1)</td>
<td>7.3 (81.8)</td>
</tr>
<tr>
<td>Rwork/Rfree (%)</td>
<td>21.2 (5.2)</td>
<td>24.8 (6.6)</td>
<td>35.0 (3.8)</td>
</tr>
<tr>
<td>Number of measured reflections</td>
<td>144,351</td>
<td>235,759</td>
<td>171,368</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>51,908</td>
<td>54,270</td>
<td>18,400</td>
</tr>
<tr>
<td>Redundancy</td>
<td>2.8 (2.7)</td>
<td>4.3 (4.4)</td>
<td>9.3 (9.7)</td>
</tr>
<tr>
<td>Wilson B factor (Å²)</td>
<td>40.2</td>
<td>20.1</td>
<td>96.6</td>
</tr>
<tr>
<td>Rmerge/Rfree (%)</td>
<td>21.90/24.80</td>
<td>20.03/21.98</td>
<td>22.70/26.65</td>
</tr>
</tbody>
</table>

No. atoms

- Overall: 4,233, 4,051, 38
- Protein: 4,051, 4,278, 42
- Ligand: 38, 42, 0
- Water: 132, 578, 0
- Other entities: 12, 9, 0

Average B value (Å²)

- Overall: 44.12, 44.30, 31.75
- Protein: 44.30, 28.37, 17.67
- Ligand: 31.75, 17.67, 0
- Water: 41.38, 35.61, 0
- Other entities: 54.09, 30.43, 0

rmsd

- Bonds (Å): 0.010, 0.009, 0.010
- Angle (°): 1.249, 1.261, 1.366

Ramachandran plot statistics (%)

- Most favorable: 89.1, 91.6, 85.8
- Additionally allowed: 9.6, 7.8, 13.0
- Generously allowed: 0.7, 0.6, 1.3
- Disallowed: 0.7, 0.0, 0.0

Values in parentheses are for the highest-resolution shell. $R_{\text{merge}} = \Sigma_i \Sigma_j |I_{hi,j} - I_{h,j}| / \Sigma_i \Sigma_j I_{hi,j}$, where $I_{hi,j}$ is the mean intensity of the $i$ observations of symmetry-related reflections of $h$. $R = \Sigma |F_{\text{calc}} - F_{\text{obs}}| / \Sigma F_{\text{calc}}$, where $F_{\text{calc}}$ is the calculated protein structure factor from the atomic model ($R_{\text{free}}$ was calculated with 5% of the reflections selected).
conformations during catalysis (Kornev et al., 2006). In PKA, the hydrophobic spine comprises Leu106, Leu95, Phe185, and Tyr164, which appear in a spatially linear order and stack against each other through van der Waals interactions (Figure 2D). In RIP1, the corresponding residues Leu78, Met67, Leu157, and His136, no longer form a linear spine. Rather, Met67 and Leu157 move away from the spine and from each other, resulting in an approximate square distribution (Figure 2D).

**RIP1 Bound to Nec-4 and Nec-3a**

The crystal structures of the RIP1 kinase domain bound to Nec-4 and Nec-3a were determined at 1.8 and 2.9 Å, respectively (Figures S2A and S2B; Table 1). These two RIP1 structures are very similar to that of Nec-1a-bound RIP1. Nec-1a-bound RIP1 exhibits root-mean-squared deviation (rmsd) values of ~0.55 Å over 223 aligned Cα atoms with Nec-4-bound RIP1 and 0.63 Å over 222 aligned Cα atoms with Nec-3a-bound RIP1 (Figure 3A). All structural elements important for catalysis and inhibitor binding are invariant among these three structures. Notably, despite their very different chemical formulas, all three necrostatins are bound in the same general location of RIP1 (Figure 3A). The conformation of the P-loop in Nec-4-bound RIP1 adopts a well-defined conformation and is quite different from that in the ATP-bound CDK2 structure (PDB code 1FIN) (Jeffrey et al., 1995) (Figure S2C). The P-loop in Nec-4-bound RIP1 occupies the same general location as that required for ATP binding (Figure S2D), which explains why a nucleotide can no longer bind to Nec-4-bound RIP1. In addition, the Nec-4-binding pocket is far away from the ATP-binding site (Figures S2C and S2D).

In both structures, necrostatin is bound in a hydrophobic pocket, similar to that in Nec-1a-bound RIP1. The same set of hydrophobic amino acids is involved in binding to Nec-1a, Nec-4, and Nec-3a, except that the exact van der Waals interactions are adjusted to account for the different chemical structures of the three necrostatins. The RIP1 amino acids that are involved in the specific H-bonds with necrostatins are also similar in these three structures. Nec-4 forms two H-bonds with the carbonyl oxygen of Ile154 and the amide nitrogen of Asp156 (Figure 3B), and Nec-3a makes three H-bonds with the amide nitrogen and carbonyl oxygen of Asp156 (Figure 3C), and the side chain of Ser161 (Figure 3C). These conserved structural features are likely to serve as an important guide for improvement of these RIP1-specific inhibitors. Nonetheless, the chemical groups in these necrostatins that are involved in the interactions are quite different among the three structures (Figures 3B and 3C).

**Comparison between RIP1 and PLX4032-Bound B-RAF**

The human Ser/Thr protein kinase B-RAF, encoded by the proto-oncogene BRAF, is a key signaling molecule in the RAF-MEK-ERK pathway for the regulation of cell growth, proliferation, and differentiation (Robinson and Cobb, 1997; Wan et al., 2004). A number of activating B-RAF mutations have been found to cause cancers including melanoma, ovarian cancer, and colorectal carcinoma (Röring and Brummer, 2012). A small-molecule inhibitor of B-RAF, named Vemurafenib, has been successfully used in the clinic to treat late-stage melanoma with a V600E mutation in the B-RAF protein. Vemurafenib (also known as
The human RIP1 kinase domain shares 28% sequence identity and 47% similarity with the kinase domain of B-RAF (Figure S1B). Consistent with the degree of sequence conservation, the Nec-1a-bound RIP1 structure is quite similar to that of PLX4032-bound B-RAF, with an rmsd of 1.041 Å over 140 aligned Cα atoms (Figure 4A). Most notably, necrostatins and PLX4032 bind to the same general locations in RIP1 and B-RAF, respectively. A portion of the more extended and larger molecule, PLX4032, overlaps with Nec-1a (Figure 4A, inset). However, the binding interactions for necrostatins are quite different from those for PLX4032. Compared to the hydrophobic environment for necrostatins (Figure 4B), PLX4032 appears to be bound in a relatively hydrophilic pocket that also contains a number of hydrophobic amino acids (Figure 4C). Consequently, PLX4032 forms four H-bonds with the main-chain groups of amino acids Gln530, Cys532, Gly596, and Asp594 (Figure 4A, inset).

Given its important role in diseases, RIP1 has been pursued as a target for potential therapeutic intervention. The advent of the crystal structure may facilitate rational drug design and development. Our study serves as an important framework for mechanistic understanding of RIP1.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**

Nec-1a, Nec-4, and Nec-3a were kindly provided by Stephen M. Condon of the TetraLogic Pharmaceuticals, and all of them are single enantiomers. Necrostatins were dissolved in 100% DMSO with a stock concentration of 200 mM. All other chemicals were purchased from Sigma-Aldrich.

**Protein Preparation and Crystallization**

The RIP1 constructs were subcloned into the Nde I and Xho I sites of pFastBac (Invitrogen) with an N-terminal 10xHis-tag and an engineered cleavage site for the caspase drICE. The cleavage site of drICE is Asp-Glu-Val-Asp-Ala, and cleavage occurs between Asp and Ala. The linker between the drICE cleavage site and RIP1 is Gly-Ser-Gly. Bacmids were generated in DH10Bac cells, and the resulting baculoviruses were generated and amplified in SF-9 insect cells. After infection by baculoviruses for 48 hr, the cells were harvested in a buffer containing 25 mM Tris (pH 8.0) and 150 mM NaCl. The RIP1 kinase domain was purified to homogeneity by nickel affinity chromatography (QIAGEN), anion-exchange chromatography (Source-15Q, GE Healthcare), and gel-filtration chromatography (Superdex-200, GE Healthcare). An additional step of drICE cleavage was performed to remove the 10xHis tag just prior to gel filtration. The purified RIP1 was in a buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, and 5 mM dithiothreitol.

About 20 baculoviruses, each expressing an RIP1 kinase domain with a distinct N- and C-terminal boundary, were generated for screening of the optimal RIP1 construct for crystallization. On the basis of protein solution behavior, the wild-type RIP1 kinase domain (residues 1–294) was chosen for extensive crystallization screening. The purified RIP1 (residues 1–294) was concentrated to ~9 mg/ml, and the necrostatin analogs were added at a final concentration of 2.5–5 mM (Nec-1a, 2.5 mM; Nec-4, 5 mM; Nec-3a, 4 mM). Crystals were grown at 18°C using the hanging-drop vapor-diffusion method. Unfortunately, these crystals diffracted X-rays weakly. To help improve diffraction, we attempted dehydration, seeding, crystal aging, construct reengineering, and cysteine mutation. In the end, introduction of four cysteine mutations...
into the RIP1 kinase domain (C34A, C127A, C233A, and C240A) resulted in significant improvement of diffraction.

All mutations were generated with two-step PCR and verified by plasmid sequencing. Crystals of the Nec-1a-bound RIP1 kinase domain appeared after 2 days in a well buffer containing 0.25 M NaI, 20% polyethylene glycol (PEG) 3350, and 0.03 M glycyglycyglycine. Crystals of the Nec-4-bound RIP1 kinase domain appeared after 1 day in a well buffer containing 0.15 M NaI and 17% PEG 3350. Crystals of the Nec-3a-bound RIP1 kinase domain appeared in a well buffer containing 0.1 M HEPES, pH 7.0, and 15% PEG 20,000.

Data Collection and Structure Determination

Despite different crystallization conditions, all crystals of necrostatin-bound RIP1 kinase domain are in the same space group, P212121, but have different unit-cell dimensions. There are two molecules of RIP1 in each asymmetric unit. All diffraction data sets were collected at Shanghai Synchrotron Radiation Facility beamline BL17U and processed using HKL2000 (Otwinowski and Minor, 1997). Further data processing was carried out using programs from the CCP4 suite (CCP4, 1994). Human B-RAF (PDB code 3C4Q) (Tsai et al., 2008) was selected as the search model for molecular replacement. To make a more accurate model, the program Chainsaw (Stein, 2008) was applied to make a modification of the structure. The sequence alignment between B-RAF and RIP1 was used as input to Chainsaw. The newly modified structure was used as the initial search model for molecular replacement.

In vitro Kinase Activity Assay

For the in vitro kinase activity assay, wild-type or mutants of the RIP1 kinase domain (residues 1–312) were used. RIP1 protein at 3 μM was incubated in 50 μl reaction buffer containing 25 mM HEPES (pH 7.0), 10 mM MgCl2, 50 mM NaCl, and 1 mM dithiothreitol for 15 min at 25°C in the presence of varying concentrations of necrostatins. For these assays, compounds were diluted to appropriate concentrations in DMSO and added to the reactions in varying concentrations of necrostatins. For these assays, compounds were diluted to appropriate concentrations in DMSO and added in the assay systems with a final concentration of 1% DMSO. Kinase reactions were initiated by addition of 10 μM cold ATP and 1 μCi of [γ-32P]ATP, and the reactions were carried out at 25°C for 30 min. Reactions were stopped by adding SDS-PAGE sample buffer and subjected to 16% SDS-PAGE. RIP1 band was visualized by autoradiography.

ACCESSION NUMBERS

The atomic coordinates and structure factor files of RIP1 bound to Nec-1a, Nec-4, and Nec-3a have been deposited in the Protein Data Bank with the accession codes 4ITH, 4ITJ, and 4ITI, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information contains three figures and can be found with this article online at http://dx.doi.org/10.1016/j.str.2013.01.016.

ACKNOWLEDGMENTS

We thank J. He from the Shanghai Synchrotron Radiation Facility beamline BL17U for assistance. This work was supported by funds from the National Natural Science Foundation of China (projects 31021002 and 31130002).

REFERENCES


