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Crystal Structure of a Human K-Ras G12D Mutant in Complex with GDP and the Cyclic Inhibitory Peptide KRpep-2d

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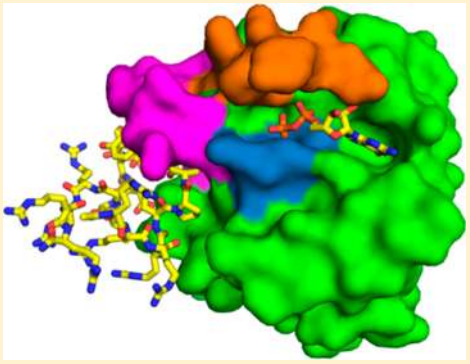
Supporting Information

ABSTRACT: The Ras proteins play roles in cell differentiation, proliferation, and survival. Aberrant signaling through Ras-mediated pathways in tumor cells occurs as a result of several types of mutational damage, which most frequently affects the amino acids G12, G13, and Q61. Recently, KRpep-2d was identified as a K-Ras(G12D) selective inhibitory peptide against the G12D mutant of K-Ras, which is a key member of the Ras protein family and an attractive cancer therapeutic target. In this study, the crystal structure of the human K-Ras(G12D) mutant was determined in complex with GDP and KRpep-2d at 1.25 Å resolution. This structure revealed that the peptide binds near Switch II and allosterically blocks protein−protein interactions with the guanine nucleotide exchange factor. This discovery of a unique binding pocket provides valuable information that will facilitate the design of direct Ras inhibitors.



KEYWORDS: *K-Ras, G12D mutation, inhibitor, peptide, protein−protein interaction, X-ray crystallography*

The Ras (rat sarcoma) proteins are small guanine nucleotide-binding proteins that act as molecular switches in signaling pathways associated with the regulation of cell growth, proliferation, and differentiation. The Ras family includes three main members, H-Ras, N-Ras, and K-Ras, all of which have been found to drive cancer development and progression.[1] Specifically, Ras mutations are found in approximately 30% of human cancers.[2] Of these, K-Ras is the most frequently mutated isoform, and it has been associated with the highest incidence of activating mutations in various carcinomas.[3] K-Ras mutations have also been associated with increased tumorigenicity and poor prognosis.[4] Common mutations in K-Ras include G12D, G12V, and G12C, which abolish GTP hydrolysis through the steric hindrance of GTPase-activating proteins (GAPs).[5] These mutations therefore render Ras constitutively GTP-bound and active, leading to the constant activation of downstream effector pathways. Accordingly, K-Ras is considered an attractive target in several exceptionally important cancers.

In recent decades, reports have described direct small-molecule K-Ras inhibitors with various mechanisms of action.[6−10] One mechanism involves guanine nucleotide-exchange inhibition or the block of protein−protein interactions (PPIs) with guanine nucleotide exchange factors (GEFs), such as the son of sevenless homologue (SOS). GEF catalysis-mediated conversion from an inactive GDP-bound state to an active GTP-bound state activates Ras and initiates downstream signaling cascades. For instance, small-molecule inhibitors that bind directly to the K-Ras(G12D) mutant and interfere with downstream signaling pathways were discovered via fragment-based screening.[11,12] Synthetic peptide inhibitors with micromolar binding affinity, based on an α-helix from SOS, were developed using a peptidomimetic approach,[13,14] and artificial cyclic peptide inhibitors with submicromolar affinity were identified from a random peptide-displayed bead library.[15,16] Nevertheless, K-Ras remains a substantially challenging target, and it has been considered an "undruggable" target because of its smooth surface, which lacks deep binding pockets.

Previously, we successfully discovered a 19-mer cyclic inhibitory peptide formed via disulfide bonding between two Cys residues, KRpep-2d (Ac-RRRRCPLYISYDPVCRRRR-NH_2), using T7 phage display technology and subsequent sequence optimization (Chart 1).[17] This peptide exhibits potent noncovalent K-Ras(G12D) inhibitory activity, with selectivity against wild-type (WT) K-Ras and the K-Ras(G12C) mutant, in both cell-free enzyme (IC_{50} = 1.6 nM) and cell-based assays.[17,18] For further characterization, we investigated the binding affinity of KRpep-2d for K-Ras proteins using the surface plasmon resonance (SPR) technique. Then, we performed structural determination of K-Ras(G12D) in complex with GDP and KRpep-2d, which allowed an investigation of the protein binding site and peptide binding conformation. This structural analysis indicated a unique

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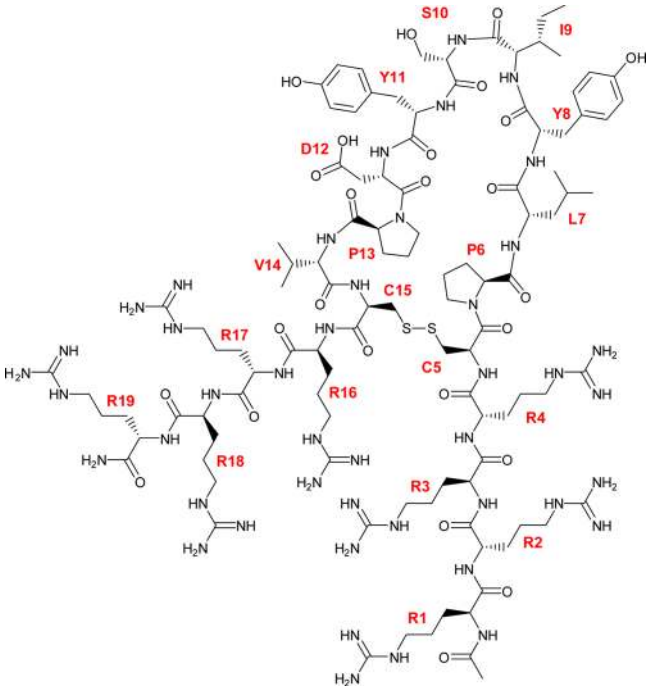
Chart 1. Chemical Structure of KRpep-2d



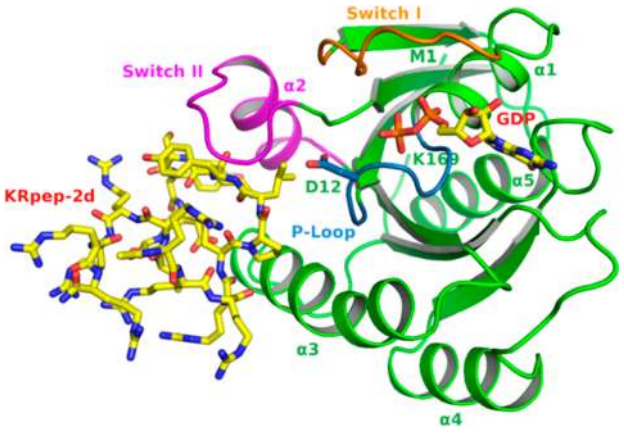


Figure 1. Ribbon diagram of the K-Ras(G12D)−KRpep-2d complex. GDP and KRpep-2d are depicted as yellow stick models, with Switch I, Switch II, and the P-loop indicated in orange, magenta, and blue, respectively.

binding site for small molecules, and it might therefore provide a valuable basis for the future design of K-Ras inhibitors.

SPR measurements revealed that KRpep-2d binds to K-Ras(G12D) with a K_D of 8.9 nM and approximately 6-fold selectivity over WT K-Ras, leading to an extended dissociation half-life (Table 1). The crystal structure of K-Ras in complex with GDP and KRpep-2d was determined at 1.25 Å resolution. The crystallographic processing and refinement statistics are summarized in Table S1. One molecule was included per asymmetric unit. Except for two N-terminal residues derived from the expression tag, the K-Ras(G12D) polypeptide chain is sufficiently well-ordered to allow structural feature interpretation. The crystal structure revealed that KRpep-2d binds near Switch II and the α3 helix in an extended and shallow cleft composed of two α-helices (Figure 1). The main-chain atoms of KRpep-2d were well-ordered in crystal packing, although the electron densities of the side-chain atoms of N- and C-terminal arginine residues (Arg1−Arg4 and Arg16−Arg19) were relatively ambiguous (Figure S1). Four arginine residues at each terminus were exposed to solvent, and therefore, they did not interact specifically with K-Ras, except for crystal-packing interactions (Figure S2). Notably, the inhibitory activity (IC_{50}) and binding affinity (K_D) of KRpep-2d were approximately 5-fold stronger than those of KRpep-2 (Ac-RRCPLYI-SYDPVCRR-NH_2), a lead peptide of KRpep-2d (ref 17 and Table S2). It was suggested that these arginine residues at both termini participate in indirect KRpep-2 interactions. The crystal structure showed that KRpep-2d has an intramolecular hydrogen bond between the acetyl oxygen of the N-terminus and the main-chain nitrogen of Arg19 of the C-terminus (Figure S3). Along with the intramolecular disulfide bond, the hydrogen bond between terminal residues might contribute to stabilizing the overall peptide conformation to adopt favorable direct interactions with K-Ras.

The loop conformation of KRpep-2d results from the formation of the intramolecular disulfide bond between Cys5 and Cys15. Both hydrophilic and hydrophobic interactions were observed at the binding interface between KRpep-2d and K-Ras(G12D) (Figures 2 and S3). The main-chain nitrogen and oxygen of Leu7 form hydrogen bonds with the side-chain oxygen and nitrogen of Gln99 in the α3 helix, respectively. The main-chain nitrogen and oxygen of Tyr8 form hydrogen bonds with the main-chain oxygen of Gln61 and the side-chain nitrogen of Arg68 in the α2 helix, respectively. The main-chain nitrogen and side-chain oxygen of Ser10 interact via hydrogen bonding with the side-chain oxygens of Asp69 in the α2 helix. The side chain of Pro6 interacts hydrophobically with the side chain of Tyr96 in the α3 helix. The side chain of Leu7 occupies a small hydrophobic pocket involving Val9, Thr58, Arg68, Met72, and Tyr96. The side chain of Ile9 interacts hydrophobically with the side chain of Met72 in the α2 helix. The side chain of Tyr11 forms a water-mediated hydrogen bond with the main-chain oxygen of Val103 in the α3 helix. The side chain of Asp12 forms hydrogen bonds with the side-chain nitrogens of Gln99 and Arg102 in the α3 helix. The side chain of Val14 interacts via van der Waals stacking with the side chain

Table 1. Kinetic Parameters of KRpep-2d Binding[a]

peptide	K-Ras	ligand	K_D (nM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	chi^2 (RU^2)	binding $t_{1/2}$ (s)
KRpep-2d	G12D	GDP	8.9	1.3×10^6	1.1×10^{-2}	2.52	61
	G12C		35	1.0×10^6	3.5×10^{-2}	3.21	20
	WT		58	1.6×10^6	9.3×10^{-2}	1.39	7.5
	G12D	GTP	11	2.8×10^6	3.0×10^{-2}	9.95	23
	G12C		250	3.7×10^5	9.2×10^{-2}	7.29	7.5
	WT		200	6.1×10^5	1.2×10^{-1}	1.93	5.8

[a]WT, wild-type; $t_{1/2}$, half-life.

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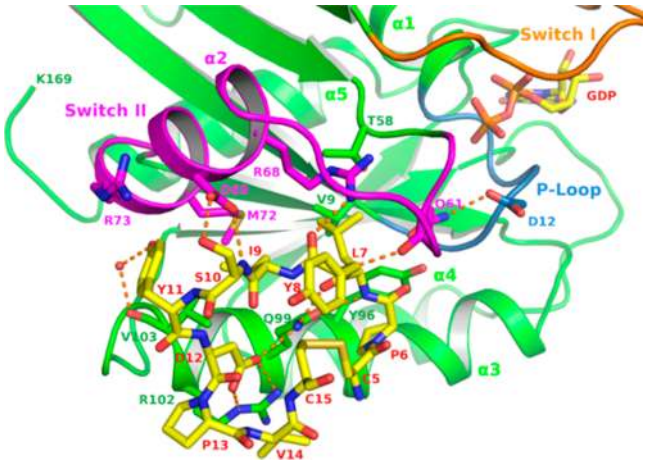

Figure 2. Binding interaction of KRpep-2d with K-Ras(G12D). The color scheme is as described for Figure 1. Orange dashed lines indicate hydrogen bonds. Arginine residues at both termini of the peptide were excluded for clarity.

of Arg102. Together, these results indicate that the conformation of KRpep-2d, when anchored by the intramolecular disulfide bond, is optimal for interaction with the shallow and extended cleft of K-Ras. Alanine scanning of KRpep-2d prior to the structural analysis revealed that Leu7, Ile9, and Asp12 are especially critical for the inhibition of K-Ras(G12D).[18] SPR binding analysis also demonstrated that replacement of Leu7, Ile9, and Asp12 with alanine significantly attenuated the peptide binding affinity for K-Ras (Table S2). The structural information regarding the binding interaction is highly consistent with the structure−activity relationships.

To evaluate structural rearrangement of K-Ras, the K-Ras(G12D)−GDP−KRpep-2d complex structure was compared with the active GTP-bound and inactive GDP-bound K-Ras(G12D) structures, as guanine nucleotide exchange was reported to induce large conformational changes in the Switch I and Switch II regions. The structural comparison revealed that the Switch II region resembles the GDP-bound state (Figure S4A). In the K-Ras(G12D)−GDP and K-Ras(G12D)−GDP−KRpep-2d complexes, the side chain of Asp12 formed a direct or water-mediated hydrogen bond with Gln61 of Switch II, whereas in the K-Ras(G12D)−GMPPCP and K-Ras(G12D)−GMPPNP complexes, the side chain of Gln61 was exposed to solvent, resulting in a loss of interaction with the side chain of Asp12 (Figures S4B and S4C). In the K-Ras(G12D)−GTP analogue complexes, the α2 helix of Switch II overlapped with the KRpep-2d binding site. Although the Switch II conformation significantly differs between the GDP- and GTP-bound states, the binding affinity of KRpep-2d for both states is approximately equivalent (Table 1). The increased structural flexibility of Switch II in the GTP-bound state may be tolerable to KRpep-2d binding.

Intriguingly, KRpep-2d exhibits inhibitory selectivity against K-Ras(G12D) compared with the G12C mutant and WT for both states. To understand the specificity, our ternary K-Ras(G12D) complex was compared with the K-Ras(G12C)−GDP and K-Ras(WT)−GDP complexes. The Switch II conformation in the K-Ras(WT)−GDP complex is similar to that of K-Ras(G12D) in the GDP state (Figure S5A), whereas the Switch II conformation in the K-Ras(G12C)−GDP complex is similar to that of K-Ras(G12D) in the GTP state (Figure S5B). Despite of the structural differences of Switch II between K-Ras(WT) and K-Ras(G12C), the binding affinity of KRpep-2d for both K-Ras proteins is relatively weak in comparison with that for K-Ras(G12D) to the same extent. Structural comparison indicated that a hydrogen-bonding interaction between Asp12 and Gln61 likely stabilizes the Switch II conformation for peptide binding. As a result, the stabilized conformation of Switch II may facilitate the binding capability of KRpep-2d.

To explore the inhibitory mechanism of KRpep-2d, our structure was compared with the Ras−GEF complex. GEFs such as SOS catalyze the release of GDP and permit the binding of GTP. Therefore, Ras−GEF inhibition could feasibly decrease the proportion of GTP-bound Ras. An SPR binding competition assay demonstrated that KRpep-2 competes for binding to SOS and indicated that KRpep-2 and SOS could share binding sites on K-Ras(G12D).[17] The αH helix within the helical hairpin motif of SOS is known to play an important

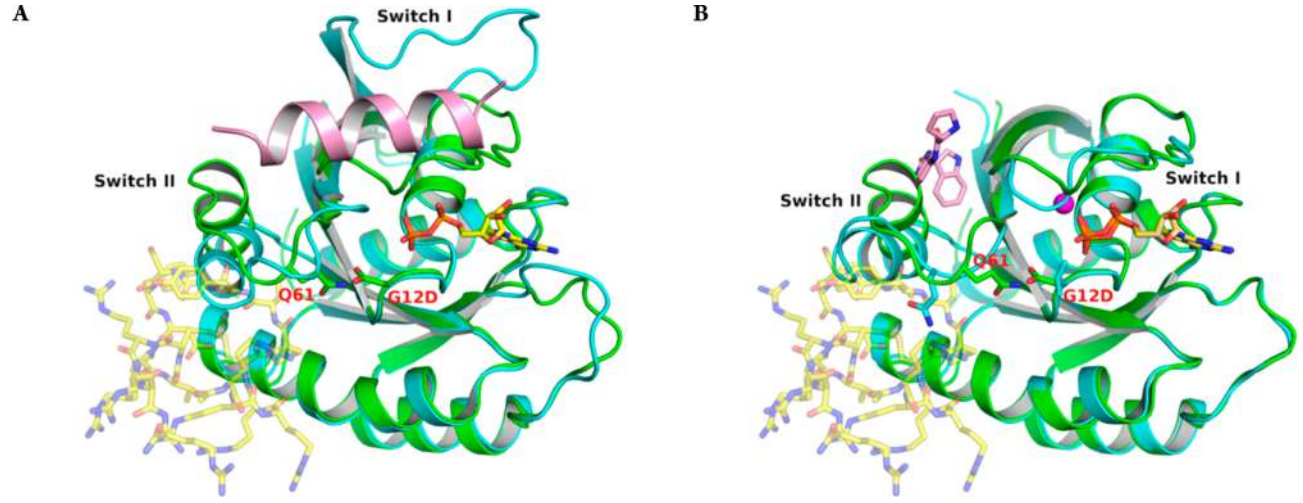

Figure 3. Structural comparison of the K-Ras(G12D)−GDP−KRpep-2d (green) complex with other Ras complexes (cyan). (A) The H-Ras−SOS complex (PDB code 1NVW). (B) The K-Ras(G12D)−GDP−**13** complex (PDB code 4EPY). KRpep-2d is shown transparently for clarity. Only the αH helix of SOS (residues 929−944) is drawn for clarity. The carbon atoms of compound **13** and the α-helix of SOS are colored pink. The magnesium ions are represented as magenta spheres.

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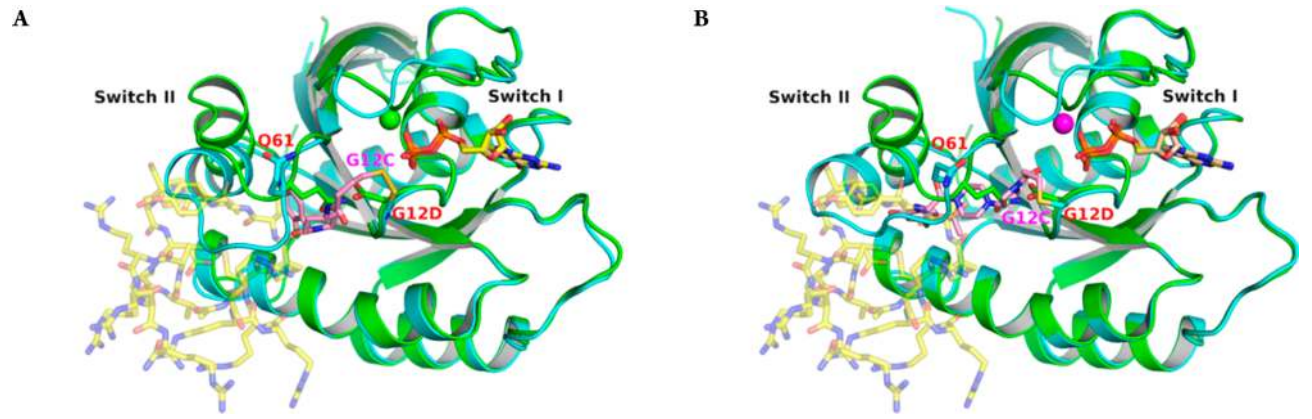

Figure 4. Structural comparison of the K-Ras(G12D)−GDP−KRpep-2d (green) complex with other K-Ras complexes (cyan). (A) The K-Ras(G12C)−GDP−disulfide **6** complex (PDB code 4LUC). (B) The K-Ras(G12C)−GDP−ARS-853 complex (PDB code 5F2E). KRpep-2d is shown transparently for clarity. The carbon atoms of disulfide 6 and ARS-853 are colored pink. The magnesium and calcium ions are represented as magenta and green spheres, respectively.

role in the nucleotide-exchange mechanism.[19] Structural comparison with the H-Ras1−SOS complex showed that the binding site of KRpep-2d is distal to the binding region of the αH helix of SOS, which displaces Switch I and Switch II to mediate GDP release (Figure 3A). KRpep-2d binding stabilized the Switch II conformation and caused steric hindrances with the SOS αH helix, suggesting that KRpep-2d is likely a nonorthosteric inhibitor of the nucleotide-exchange reaction that interferes allosterically with nucleotide binding or release. Although the helical hairpin of SOS is critical for its function, other regions of the catalytic cdc25 domain of SOS also interact with Ras.[20,21] As the binding site of KRpep-2d is adjacent to the αB, αD, and αK helices of the cdc25 domain, the inhibitory mechanism of KRpep-2d remains controversial (Figure S7).

To characterize the KRpep-2d binding site, the structure of our ternary complex was compared with other structures complexed with small-molecule inhibitors. Several small-molecule inhibitors were identified via NMR-based fragment-based ligand discovery.[11,12] One representative fragment, compound **13** (Figure S6), was selected for comparison because all fragments are known to bind to the same hydrophobic cavity via distinct manners. A structural comparison with this fragment revealed that the fragment occupied the same side of Switch II as the SOS helix, which was shifted by fragment binding (Figure 3B), and are considered orthosteric inhibitors. Several K-Ras(G12C) mutant-specific irreversible inhibitors have also been reported (Figure S6).[22,23] Disulfide **6** and ARS-853 are covalently attached to Cys12 and extend into a hydrophobic pocket in the vicinity of Switch II (Figure 4). The pocket was referred to as the Switch II pocket, and it is composed of the central β-sheet and the α2 and α3 helices.[22] Relative to known structures of Switch II−bound irreversible inhibitors, KRpep-2d induces structural rearrangements of the α2 helix to accommodate the ligand in a distinct hydrophobic pocket. This hydrophobic pocket is occupied by the side chain of Leu7 of KRpep-2d. Interestingly, the Switch II conformation of the K-Ras complex with published small-molecule inhibitors was relatively similar to that of the active GTP-bound state. Although the KRpep-2d binding interface adjacent to Switch II overlaps that of irreversible inhibitors, the binding interface contains the unique structural feature of KRpep-2d recognition, suggesting that Switch II is significantly mobile and has wide conformational variability in the inactive GDP-bound state.[6,10] Structural analysis revealed a unique potential binding region for inhibitors of the allosteric Ras−GEF interaction. Taken together, our results provide valuable insights into the molecular basis of the K-Ras protein, and they could provide direct guidance for the design of small-molecule K-Ras inhibitors for cancer therapy.

The PPIs between Ras−GTP and its effectors, which initiate various downstream signaling cascades, are also challenging targets. Structural information may facilitate a detailed understanding of the binding capabilities of inhibitors. KRpep-2d also binds to the GTP-bound state of K-Ras(G12D) with comparable affinity (K_D = 11 nM; Table 1). It remains to be determined whether KRpep-2d inhibits Ras−effector interactions. One orthosteric inhibitor of Ras−effector interactions, Kobe (Figure S6),[24] is known to bind to a similar site adjacent to Switch II as compound **13** (Figure S8A), indicating the importance of the Switch II conformation for Ras−effector binding.[25] According to one plausible hypothesis, KRpep-2d could prevent Switch I and Switch II from adopting the correct conformations for Raf-Ras binding domain (RBD) binding (Figure S8B). However, preliminary analysis of K-Ras(G12D) binding with Raf-RBD is still under investigation. Further investigation may facilitate an understanding of the mechanism of action of KRpep-2d.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00128.

Experimental procedures related to peptide synthesis, recombinant protein production, and X-ray crystallography; figures of electron density maps, a close view of the KRpep-2d binding site, schematic diagram of interactions, chemical structures of K-Ras inhibitors, and structural comparisons with other K-Ras protein complexes; and binding affinities of KRpep-2d derivatives (PDF)

Accession Codes

Atom coordinates and structure factors have been deposited in the Protein Data Bank with the accession code 5XCO, and the data will be released upon publication.

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Author Contributions

‡These authors contributed equally. S.S. performed X-ray crystallography experiments. Y.K. conducted the SPR analysis. M.M. prepared the recombinant protein. A.N. synthesized the peptide. K.S. led the discovery of the peptide. All authors reviewed the results and approved the manuscript.

Notes

The authors declare no competing financial interest.

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