Structure of the ABL2/ARG kinase in complex with dasatinib

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ABL2/ARG (ABL-related gene) belongs to the ABL (Abelson tyrosine-protein kinase) family of tyrosine kinases. ARG plays important roles in cell morphogenesis, motility, growth and survival, and many of these biological roles overlap with the cellular functions of the ABL kinase. Chronic myeloid leukemia (CML) is associated with constitutive ABL kinase activation resulting from fusion between parts of the breakpoint cluster region (*BCR*) and *ABL1* genes. Similarly, fusion of the *ETV6* (*Tel*) and *ARG* genes drives some forms of T-cell acute lymphoblastic leukemia (T-ALL) and acute myeloid leukemia (AML). Dasatinib is a tyrosine kinase inhibitor used for the treatment of CML by inhibiting ABL, and while it also inhibits ARG, there is currently no structure of ARG in complex with dasatinib. Here, the co-crystal structure of the mouse ARG catalytic domain with dasatinib at 2.5 Å resolution is reported. Dasatinib-bound ARG is found in the DFG-in conformation although it is nonphosphorylated on the activation-loop tyrosine. In this structure the glycine-rich P-loop is found in a relatively open conformation compared with other known ABL family-inhibitor complex structures.

1. Introduction

ABL1/ABL and ABL2/ARG comprise the ABL family of nonreceptor tyrosine kinases in vertebrates. These kinases (ABL and ARG) are ubiquitously expressed and regulate diverse cellular functions including cell proliferation, survival, invasion, morphogenesis, adhesion and migration (Bradley & Koleske, 2009; Colicelli, 2010; Greuber *et al.*, 2013; Wang, 2014). ABL and ARG have a similar domain structure at their N-termini, comprising Src homology 3 (SH3), Src homology 2 (SH2) and kinase domains. This SH3–SH2–kinase cassette is highly conserved between ABL and ARG, with about 90% sequence identity (Bradley & Koleske, 2009). The C-terminal halves of ABL and ARG are less well conserved and contain distinct domains that interact with diverse binding partners, including scaffolding proteins and the actin and microtubule cytoskeletons (Bradley & Koleske, 2009). ARG knockout mice exhibit defects in neuronal dendrite stability (Gourley *et al.*, 2012; Moresco *et al.*, 2005; Sfakianos *et al.*, 2007), and ABL and ARG double-knockout mice are embryonically lethal, exhibiting defects in neural tube and heart development (Koleske *et al.*, 1998). ABL and ARG activation is associated with cancers of organs such as breast and lung, and importantly is dysregulated in chronic myeloid leukemia (CML) and other leukemias (Ben-Neriah *et al.*, 1986; Cazzaniga *et al.*, 1999; Ganguly *et al.*, 2012; Iijima *et al.*, 2000; Mader *et al.*, 2011; Rikova *et al.*, 2007; Srinivasan & Plattner, 2006).

ABL is regulated by an autoinhibitory snaplock mechanism that is similar to that found in Src (Hantschel & Superti-Furga,

2004; Harrison, 2003; Nagar *et al.*, 2003). In ABL, the SH2 and SH3 domains interact with the distal side of the catalytic domain and lock the kinase into an autoinhibited conformation. This conformation is stabilized by insertion of a myristoyl group into a hydrophobic pocket in the kinase domain that causes a conformational change to create a binding surface for intramolecular interaction with the SH2 domain (Nagar *et al.*, 2003). These autoinhibitory interactions are frequently destabilized in ABL- and ARG-related cancers, shifting the equilibrium of the kinase to the active state. This observation has created opportunities for kinase-targeted small-molecule drug discovery (Greuber *et al.*, 2013). Indeed, the Philadelphia chromosome, which results in a fusion of BCR and ABL, is the archetypal target for tyrosine kinase small-molecule drug discovery, with imatinib (STI-571, Gleevec; Novartis) considered to be the paradigm-changing drug for CML (Druker *et al.*, 1996). Imatinib, however, is also susceptible to acquired resistance mutations, and consequently second-generation drugs were developed, including dasatinib (Sprycel; Bristol-Myers Squibb), which has different conformational specificity for ABL and has been approved for clinical use (Quintás-Cardama *et al.*, 2007). However, the other second-generation inhibitors are also susceptible to resistance. Dasatinib can inhibit ARG (Roberts *et al.*, 2014). Although there are some structures of ARG in complex with other inhibitors such as imatinib and tozasertib (VX-680; Salah *et al.*, 2011), the co-crystal structure of dasatinib and ARG has not been determined.

Here, we present the co-crystal structure of the ARG kinase domain in complex with dasatinib at 2.5 Å resolution. This dasatinib-bound ARG is found in the DFG-in conformation and is nonphosphorylated on the activation-loop tyrosine. The glycine-rich P-loop is found in a relatively open conformation compared with other ABL family-inhibitor complex structures.

2. Materials and methods

2.1. Expression and purification of the ARG kinase domain

The mouse ARG kinase domain, consisting of residues Asp279–Ser546, was cloned into pFastBac-HTA (Invitrogen), and baculovirus was generated in Sf9 insect cells (Life Technologies) as described previously (Tanis *et al.*, 2003). High Five (Hi5) insect cells (Life Technologies) were infected at a density of 2×10^6 with baculovirus for 48 h before spinning and snap-freezing the cell pellets in liquid nitrogen. The cell pellets were stored at -80°C until purification.

Cells expressing the mouse ARG catalytic domain were harvested and suspended in lysis buffer [20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP), cOmplete EDTA-free protease-inhibitor cocktail (Roche)] and lysed by sonication. The supernatant was affinity-purified using a HisTrap chelating column (GE) and the hexahistidine (6×His) tag was removed by the addition of a 1:20 ratio of TEV protease during overnight dialysis against 20 mM Tris–HCl pH 8.0, 100 mM NaCl. Following cleanup on

Table 1

Data-collection and refinement statistics.

Data for the high-resolution shells are shown in parentheses for the 2.59–2.50 Å shell and in square brackets for the 2.69–2.59 Å shell (where available).	
Data collection	
Space group	$P6_5$
X-ray source	24-ID-E, APS
Detector	ADSC Q315
Wavelength (Å)	0.97916
Unit-cell parameters (Å, °)	$a = 109.7$, $b = 109.7$, $c = 121.5$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution range (Å)	50.00–2.5 (2.59–2.50) [2.69–2.59]
No. of unique reflections	29610
Completeness (%)	100 (100) [100]
$R_{\text{p.i.m.}}$ (%)	10.2 (102.0) [85.0]
$CC_{1/2}$ (%)	(37.8) [42.7]
Mean $\langle I \rangle / \langle \sigma(I) \rangle$	8.5 (1.2) [1.56]
Wilson B factor (Å ²)	48.8
Multiplicity	11.1 (11.0) [11.0]
Refinement statistics	
Resolution range (Å)	7.5–2.5 (2.56–2.50)
R factor (%)	15.4 (26.2)
R_{free} (%)	20.5 (37.6)
R_{free} reflections (%)	5.1 (5.2)
No. of R_{free} reflections	1478 (103)
Residues built	
Chain A	279–321, 326–546
Chain B	279–295, 300–546
No. of water molecules	32
Mean B factor (Å ²)	
Protein (chain A/chain B)	58.3/58.7
Dasatinib (chain A/chain B)	53.0/59.3
Zn ²⁺	37.1
Water	45.4
Model statistics	
R.m.s.d., bond lengths (Å)	0.019
R.m.s.d., bond angles (°)	2.015
Ramachandran plot (%)	
Favored	95.2
Allowed	4.6
Disallowed	0.2

a HisTrap chelating column (GE), the protein was resolved by FPLC on a Resource Q (GE) column. ARG was then loaded onto a Superdex 75 10/300 GL (GE) column. ARG eluted as a monodisperse peak in 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM dithiothreitol (DTT).

2.2. Crystallization and data collection

The ARG kinase domain was concentrated to 4 mg ml^{−1} for crystallization trials and mixed with 1 mM dasatinib dissolved in dimethyl sulfoxide (DMSO) prior to setting up crystallization drops. Following overnight incubation of the ARG catalytic domain and dasatinib, the complex was centrifuged and the supernatant was used for crystal screening. Initial crystallization screening was performed using the sitting-drop method with a Matrix Hydra II eDrop crystallization robot. Initial crystals were observed after three months at room temperature against precipitant conditions consisting of 100 mM HEPES pH 7.0, 1.1 M sodium malonate, 5%(v/v) Jeffamine ED-2001. After screening for optimal crystal-growth conditions, the best crystals grew over three months in 100 mM HEPES pH 7.0, 1.3 M sodium malonate, 2.5%(v/v) Jeffamine ED-2001. For data collection, crystals were cryo-protected in reservoir buffer supplemented with 30%(v/v)

glycerol. Data were collected on beamline 24-ID-E at the Advanced Photon Source (APS).

2.3. Structure determination and refinement

Crystallographic data were processed to 2.5 Å resolution using the *HKL-2000* package (Otwinowski & Minor, 1997). Molecular replacement using *Phaser* (McCoy, 2007) gave an initial solution using the previously determined crystal structure of human ARG (PDB entry 2xyn; Salah *et al.*, 2011) as the search model. The structure was refined using *REFMAC5* (Murshudov *et al.*, 2011) with a maximum-likelihood target and TLS (translation, libration, screw) refinement. Model building was conducted in *Coot* (Emsley *et al.*, 2010) and model validation using *MolProbity* (Chen *et al.*, 2010) with

almost (99.8%) all built residues found within favored or allowed Ramachandran regions. Good electron density is observed for both the kinase domains and the inhibitors. The final *R* and *R*_{free} values are 15.4 and 20.5%, respectively (Table 1).

3. Results and discussion

3.1. Structure determination and refinement of the ARG–dasatinib complex

Residues 279–546 of the mouse ARG and human ARG kinase domains (UniProt codes F8VQH0 and P42684, respectively) are 99% identical at the protein level. We expressed the mouse ARG kinase domain (residues Asp279–

Figure 1

Purification, crystallization and overall structure of ARG complexed with dasatinib. (a) Size-exclusion chromatography (SEC) of the ARG kinase domain on a Superdex 75 column. SDS–PAGE shows the eluted ARG kinase. (b) Crystals of ARG kinase in complex with dasatinib. (c) Overall view of the ARG kinase domain with secondary-structure elements labeled. Boxes show the $2F_{\text{obs}} - F_{\text{calc}}$ refined electron-density map of dasatinib in blue and unbiased $F_{\text{obs}} - F_{\text{calc}}$ electron density prior to the addition of dasatinib to the model.

Figure 2

Analysis of ARG in complex with dasatinib. (a) A zinc ion is found between the two copies of the ARG kinase domain in the asymmetric unit. The detailed view shows conserved His536 and Glu398 residues coordinating the ion (top) and a *LIGPLOT* analysis (bottom; Wallace *et al.*, 1995). (b) Superposition of ARG-dasatinib (green), ARG-imatinib (PDB entry 3gvu; coral), ARG-JNJ-7706621 (PDB entry 3hmi; gray) and ARG-VX-680 (PDB entry 2xyn; gold; Salah *et al.*, 2011). These ARG-inhibitor structures share similar overall structure, but conformational differences are observed in the glycine-rich P-loop of ARG-dasatinib. The box shows an alternate view of the P-loop. (c) Superposition of ARG-dasatinib (in green) and ABL-dasatinib (PDB entry 2gqg; pink; Tokarski *et al.*, 2006). Close-up showing hydrogen bonding in ABL-dasatinib. (d) Comparison of ARG and ABL structures with dasatinib, highlighting conformational differences in the dasatinib binding site and P-loop. Dashed red lines indicate hydrogen bonding between kinase (red residues) and bound dasatinib.

Ser546) using a baculovirus system and purified it by affinity chromatography. Following removal of the N-terminal 6×His tag by TEV protease, the ARG kinase domain was further purified by ion-exchange and size-exclusion chromatography. The ARG kinase domain elutes from size-exclusion chromatography as a monomeric, monodisperse protein (Fig. 1*a*). Dasatinib was dissolved in DMSO, mixed with purified ARG and incubated overnight at 4°C. Precipitate was removed by centrifugation at 14 000 rev min⁻¹ for 10 min and the ARG–dasatinib mixture was then used for co-crystallization. Diffraction-quality crystals were found in similar conditions consisting of 100 mM HEPES pH 7.0, 1.3 M sodium malonate, 2.5%(v/v) Jeffamine ED2001 after three months (Fig. 1*b*).

3.2. Structure determination

A 2.5 Å resolution data set was acquired from 180° of data collected from a single crystal on the NE-CAT 24-ID-E beamline at the Advanced Photon Source, Argonne National Laboratory. The ARG crystal belonged to space group *P*6₅ and contained two molecules in the asymmetric unit. This crystal packing has not previously been observed for ARG or ABL structures. We used a human ARG structure (PDB entry 2xyn) as the search model for molecular replacement. The refined model has good electron density, with the exception of the β3–αC loop in molecule *A* (residues 322–325) and part of the P-loop in molecule *B* (residues 296–299). The structure has been deposited in the Protein Data Bank under accession code 4xli.

3.3. Overall structure

The overall structure of the ARG kinase domain in complex with dasatinib shows a typical kinase-domain fold, with a mostly β-stranded N-lobe and a mostly α-helical C-lobe sandwiching the catalytic cleft, which is occupied by the ATP-competitive inhibitor dasatinib. We find that the DFG motif of the ARG activation segment is in the DFG-in conformation and that the activation loop is extended and in an active-like state; however, we do not observe phosphorylation of the activation-loop tyrosine residue Tyr439. The ATP-competitive small-molecule inhibitor dasatinib displays good electron density in both copies of the asymmetric unit (Fig. 1*c*).

3.4. Crystallographic asymmetric dimer mediated by a zinc ion

In the asymmetric unit there are two molecules of the ARG catalytic domain. These two molecules show a highly similar conformation, with a root-mean-square deviation (r.m.s.d.) of 0.34 Å over 260 C^α residues. Interestingly, a metal ion mediates dimerization of the two ARG kinase domains, being coordinated by residues Glu398 and His536 in helices αE and αI, respectively, both of which are strictly evolutionarily conserved. Using the *CheckMyMetal* server (Zheng *et al.*, 2014), we concluded that the bound metal is a zinc ion because it is tetrahedral and the bond lengths are 1.97 Å to Glu398 in molecule *A*, 1.83 Å to Glu398 in molecule *B*, 2.1 Å to His536 in molecule *A* and 2.16 Å to His536 in molecule *B* (Fig. 2*a*).

These zinc-coordinating residues are distal from the previously observed kinase-domain interactions with SH3 and SH2 domains in autoinhibited ABL (Nagar *et al.*, 2003). We did not find a similar metal-mediated interface in previous structures of ARG or ABL, and we believe that this may represent a crystallographic event rather than a physiologically relevant homodimerization interface.

3.5. Analysis of the interaction between ARG and dasatinib

Recently, several crystal structures of human ARG with inhibitors have been determined. These are complexes with imatinib, the Aurora kinase inhibitor VX-680 and the Aurora kinase inhibitor JNJ-7706621 (PDB entries 3gvu, 2xyn and 3hmi, respectively; Structural Genomics Consortium, unpublished work; Salah *et al.*, 2011). The most notable differences between the current structure and previous ARG structures are found in the glycine-rich P-loop, the loop between the β3 strand and the αC helix, and the activation loop (Fig. 2*b*). The previous structures were determined with more closed P-loop conformations (ARG–imatinib, PDB entry 3gvu; ARG–JNJ-7706621, PDB entry 3hmi; ARG–VX-680, PDB entry 2xyn; Fig. 2*b*). In the ARG–dasatinib co-crystal structure, the hydrogen bonding observed in the previous structures between the P-loop and the C-lobe is not observed and the P-loop is conformationally more open. This open conformation does not seem to have resulted from interactions with dasatinib, because in the ABL–dasatinib complex a closed P-loop conformation is also observed that displays hydrogen bonding between the P-loop and the C-lobe (PDB entry 2gqg; Tokarski *et al.*, 2006). We do not believe that this P-loop conformation is the result of crystal packing, but instead believe that it illustrates the conformational flexibility accessible to protein kinases when they are bound to ATP-competitive inhibitors. This correlates with previous NMR studies of the ABL kinase domain that showed altered flexibility on binding to different inhibitors (Vajpai *et al.*, 2008).

3.6. Comparison of the ABL–dasatinib and ARG–dasatinib structures

The overall binding of dasatinib to ARG (*K*_d = 0.17 nM; Karaman *et al.*, 2008) is highly similar to that previously observed for dasatinib to ABL (*K*_d = 0.53 nM; PDB entry 2gqg; Karaman *et al.*, 2008; Tokarski *et al.*, 2006). The small molecule binds in the ATP pocket between the N- and C-lobes, and the aminothiazole moiety resides in the ATP adenine-binding site. The 2-chloro-6-methyl ring of dasatinib is located near the gatekeeper residue Thr361, and hydrogen bonding is observed between Thr361 and the dasatinib amide N2 atom (Fig. 2*d*).

4. Conclusion

We have determined the structure of the kinase domain of ARG complexed with dasatinib. ARG is found in the in DFG-in state and is nonphosphorylated on the activation loop. On comparing the ARG kinase domain–dasatinib structure with

other reported ABL-inhibitor structures, we observed some differences in the P-loop conformation and the orientation of Met336. The current structure therefore helps to further map the conformational space accessible to ABL-family kinases and their interactions with ATP-competitive small-molecule inhibitors.

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