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Split luciferase-based biosensors for characterizing EED binders

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ABSTRACT

The EED (embryonic ectoderm development) subunit of the Polycomb repressive complex 2 (PRC2) plays an important role in the feed forward regulation of the PRC2 enzymatic activity. We recently identified a new class of allosteric PRC2 inhibitors that bind to the H3K27me3 pocket of EED. Multiple assays were developed and used to identify and characterize this type of PRC2 inhibitors. One of them is a genetically encoded EED biosensor based on the EED[G255D] mutant and the split firefly luciferase. This EED biosensor can detect the compound binding in the transfected cells and in the *in vitro* biochemical assays. Compared to other commonly used cellular assays, the EED biosensor assay has the advantage of shorter compound incubation with cells. The *in vitro* EED biosensor is much more sensitive than other label-free biophysical assays (e.g. DSF, ITC). Based on the crystal structure, the DSF data as well as the biosensor assay data, it's most likely that compound-induced increase in the luciferase activity of the EED[G255D] biosensor results from the decreased non-productive interactions between the EED subdomain and other subdomains within the biosensor construct. This new insight of the mechanism might help to broaden the use of the split luciferase based biosensors.

1. Introduction

Polycomb repressive complex 2 (PRC2) is a methyltransferase complex that can specifically methylate lysine 27 of histone H3. It plays a key role in the epigenetic regulation of gene expression. At least three subunits are required for the enzymatic activity of PRC2: EzH2 (Enhancer of zeste homolog 2), EED (embryonic ectoderm development) and Suz12 (suppressor of zeste 12) [1–5]. EzH2 is the catalytic subunit of PRC2 which contains the binding sites for the cofactor SAM. EED contains a binding site for tri-methylated lysine 27 (H3K27me3), the product of the PRC2 reaction. Binding of H3K27me3 to EED stimulates the catalytic activity of the PRC2 complex through an allosteric mechanism [6,7]. Thus EED mediates the feed forward regulation of PRC2. In cells, binding of EED to an H3K27me3-bearing nucleosome could also bring the PRC2 complex to its substrate and facilitate the subsequent methylation of the adjacent H3K27me0/1/2-bearing nucleosome.

Recently, we disclosed a novel class of allosteric PRC2 inhibitors that bind to the H3K27me3 pocket of EED [8,9]. We refer this class of inhibitors in this paper as EEDi. Binding of EEDi competitively inhibited the binding of H3K27me3 peptide, thus blocked the H3K27me3-mediated stimulation of PRC2 activity. Surprisingly, EEDi also inhibit the basal enzymatic activity of PRC2 measured in the absence of any stimulatory peptide. The exact mechanism for this allosteric effect is not fully understood as the complex structure of EEDi with the PRC2 complex is not available. In the co-structure of EEDi with EED, binding of EEDi causes significant change in the positions of the three aromatic residues (F97, Y148 and Y365) while the conformational change outside the aromatic cage is subtle. Yet it might be significant enough to affect the intricate interaction of EED with the stimulation responsive motif

Abbreviations: EED, embryonic ectoderm development; PRC2, polycomb repressive complex 2; DSF, differential scanning fluorimetry; ITC, isothermal titration calorimetry; EZH2, enhancer of zeste homolog 2; SUZ12, suppressor of zeste 12; SAM, S-Adenosyl methionine; EEDi, EED inhibitor; SRM, stimulation responsive motif; *ELISA*, enzyme-linked immunosorbent assay; SAR, structure-activity relationship; wt, wild type; luc, luciferase; N-Luc, N-terminal domain of luciferase; C-Luc, C-terminal domain of luciferase; EBD, EED binding domain (EZH2 residue 40–68); AEBP2, *adipocyte enhancer-binding protein 2*; RbAp48, retinoblastoma binding protein 48; SAH, S-Adenosyl homocysteine; LC, liquid chromatography; MS, mass spectrometry; AlphaScreen, Amplified Luminescence Proximity Homogenous Assay Screen; HADCi, histone deacetylase inhibitor; LMW, low molecular weight; RMSD, root mean-square deviation.

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(SRM) of EzH2 [10–12]; thus prevents PRC2 from adapting the catalytically active conformation.

Several cell-based assays have been developed for characterizing PRC2 inhibitors. The H3K27me3 ELISA assay is the one being used extensively to support the SAR effort. It's a proximal assay that directly read out the consequence of PRC2 inhibition [8]. However, like other histone markers, the apparent half-life for H3K27me3 in cells is very long. It often takes about 48–72 h compound incubation to see any significant change in the H3K27me3 level. The prolonged incubation time makes this assay inadequate for characterizing early stage compounds that are usually not potent enough and often have off-target toxicity issues.

To overcome this drawback associated with the H3K27me3 ELISA assay, we evaluated the feasibility of using the split firefly luciferase based biosensor approach to characterize EEDi. Split-luciferase complementation technologies have been widely used to study protein-protein interactions and protein-ligand interactions [13–17]. The underlying mechanism for most of the published split luciferase biosensors is compound-induced large conformational change. For example, in the “kinase activity sensors” [17], a substrate peptide motif and a phospho-S/T/Y binding motif are inserted in the middle of the luciferase. Once phosphorylated, the substrate peptide will “bite” back to the phospho-S/T/Y binding motif and thus change the overall conformation of the biosensor. In the “Abl conformational sensor” reported by Zhou [18], the authors took advantage of the large conformational difference between the activated Abl and the inactivated Abl, and inserted the entire Abl sequence (including the CAP, SH2, SH3 and the catalytic domain) directly in the middle of the firefly luciferase. This sensor was able to detect the conformational change of Abl in cells after treatment with the ATP-pocket inhibitors as well as the myristate-pocket inhibitors [18].

In this paper, we report the development and validation of a robust EED biosensor (based on the EED[G255D] mutant) that can be used to characterize EEDi both in cells and *in vitro*. We also provide data to suggest that compound-induced increase of the luciferase activity of the EED biosensor is likely from the change in the thermal dynamics of the EED motif rather than from compound-induced large conformational change.

2. Materials and methods

2.1. Protein expression and purification

EED wt (residues 40–441) sensor and EED[G255D] (residues 40–441) mutant sensors were both cloned in a multiple expression host compatible vector of pTriEx-3, with N-terminal domain of the firefly luciferase (N-Luc) and the C-terminal domain of firefly luciferase (C-Luc) flanking at the N- and C- terminal regions of EED protein respectively, and a FLAG tag was inserted between the N-luc and EED protein, and a His8 tag was added just before the stop codon. The EED sensor proteins were expressed in BL21(DE3) and purified first by a His FF(GE) affinity column followed with a Mono S (GE) column, and finally purified with a superdex 200 16/60 (GE) column in 20 mM HEPES (pH 8.0), 50 mM NaCl, 5% Glycerol, 5 mM DTT. EED wt and EED [G255D] proteins used in AlphaScreen, ITC and DSF were expressed and purified as previously described [8].

2.2. Crystallization and structure determination of EED[G255D]-EBD-EED226 complex

Crystals of EED[G255D] in complex with the peptide and compound were obtained by vapor diffusion in sitting drops incubated at 20 °C by mixing 0.1 μL of protein solution (8.4 mg/mL) including 0.5 mM EBD peptide (EZH2 residue 40–68, referred as EED-binding domain), and 0.6 mM EED226 compound and 0.1 μL of reservoir solution containing 10% polyethylene glycol (PEG) 6000, 0.1 M Bicine (pH 9.0). Crystals appeared at the second day. Reservoir solution supplemented with 30% glycerol was used as cryo-solution. Crystals were mounted and flash-frozen in liquid nitrogen. Diffraction data to 2.03 Å resolution were collected at the Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U1 and processed using HKL2000 [19]. The structure was solved by molecular replacement using Molrep in the CCP4 suite [20] with the previous EED wt structure (PDB entry 5GSA) as a model [8]. The model was built using COOT [21] and refined using Buster [22]. The statistics of the structure refinement and the quality of the final model are summarized in Table 1. The atomic coordinates and structure factors for the EED226 compound bound EED [G255D] co-crystal structures (accession code 5WUK) has been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

2.3. PRC2 complex enzymatic activity assay

PRC2 complex (EZH2/EED/SUZ12/AEBP2/RbAP48) enzymatic assays were performed as previously described [8,9,23,24]. The assay employs liquid chromatography mass spectrometry technology using either histone H3(21–44, K27me0) peptide or recombinant mono-nucleosome as substrate. The co-product SAH formation was detected and quantified by LC/MS/MS using SAH-d_4 as an internal standard (IS) control. For enzymatic activity test of PRC2 wt and PRC2 [EED-G255D] mutant complex, the conditions were as following: 30 nM PRC2 wt or PRC2 [EED-G255D], 5 μM SAM, 5 μM H3(21–44, K27me0) peptide, reaction time 2.5 h. For histone H3(21–44, K27me3) stimulation on PRC2 wt or PRC2 [EED-G255D] mutant complex, the assay was performed as following: 40 nM PRC2 wt or PRC2 [EED-G255D], 1 μM SAM, 0.5 μM recombinant mono-nucleosome, various concentration of histone H3(21–44, K27me3) peptide (0–50 μM), reaction time 2 h.

Table 1
Data collection and statistics for the structure of EED[G255D]-EBD-EED226 complex.

	EED[G255D]-EBD-EED226
PDB	entry 5WUK
Data collection	
Space Group	P 21 2121
Unit Cell Parameters [Å]	53.1, 80.3, 108.4
αβγ[°]	90.00 90.00 90.00
Contents of ASU[a]	
Protein Molecules	1 EED[G255D]-EBD
Ligand Molecules	1 EED226
Resolution [Å]	64.52–2.03 (2.14–2.03)[b]
Unique Reflection	30739 (4427)
Completeness [%]	99.9 (99.9)
Redundancy	6.2 (6.4)
Rmerge [%]	10.9 (47.0)
I/σ(I)	5.0 (1.6)
Refinement	
Resolution [Å]	26.00–2.03 (2.10–2.03)
No. of Reflections	30632 (2976)
Completeness [%]	99.8 (99.9)
Rwork[%]	16.2 (16.7)
Rfree[c][%]	19.1 (21.4)
Average B-factor [Å2] Overall	27.7
rmsd bonds[Å]/angles[°]	0.010/1.04
Ramachandranplot (allowed)	100%

[a] Asymmetric Unit.
[b] Numbers in parenthesis are for highest resolution shell.
[c] Test set uses 5.1% data.