Title

Allosteric inhibition of PPM1D serine/threonine phosphatase via an altered conformational state

Keywords

Protein phosphatases; allosteric inhibition; computational modeling; deep mutational scanning; hydrogen-deuterium exchange mass spectrometry; chemotherapeutic resistance

Description (abstract)

PPM1D encodes a serine/threonine phosphatase that regulates numerous pathways including the DNA damage response and p53. Activating mutations and amplification of PPM1D are found across numerous cancer types. GSK2830371 is a potent and selective allosteric inhibitor of PPM1D, but its mechanism of binding and inhibition of catalytic activity are unknown. Here we use computational, biochemical and functional genetic studies to elucidate the molecular basis of GSK2830371 activity. These data confirm that GSK2830371 binds an allosteric site of PPM1D with high affinity. By further incorporating data from hydrogen deuterium exchange mass spectrometry (HDX-MS) and sedimentation velocity analytical ultracentrifugation (SV-AUC), we demonstrate that PPM1D exists in an equilibrium between two conformations that are defined by the movement of the flap domain, which is required for substrate recognition. A hinge region was identified that is critical for switching between the two conformations and was directly implicated in the high-affinity binding of GSK2830371 to PPM1D. We propose that the two conformations represent active and inactive forms of the protein mediated by the position of the flap, and that binding of GSK2830371 shifts the equilibrium to the inactive form. Finally, we found that C-terminal truncating mutations proximal to residue 400 result in destabilization of the protein via loss of a stabilizing N- and C-terminal interaction, consistent with the observation from human genetic data that nearly all PPM1D mutations in cancer are truncating and occur distal to residue 400. Taken together, our findings elucidate the mechanism by which binding of a small molecule to an allosteric site of PPM1D inhibits its activity and provides new insights into the biology of PPM1D.

Sample processing protocol

Deuterium Labeling: Hydrogen-deuterium exchange in PPM1D proteins was monitored by incubating 27 pmoles of protein in storage buffer (50 mM Tris HCl pH 7.4, 500 mM NaCl, 10% glycerol, 10 mM MgCl2, 1 mM TCEP, H_2O) with a 10-fold molar excess of GSK2830371 (270 pmoles) in DMSO, or 3.85% DMSO as a control. Due to the low solubility of the compound, the exact molar excess was unknown. However, the peptide spectra in the presence of the compound presented as single isotopic distributions (except where EX1 is indicated within the text), which suggests a large proportion of the protein was bound to the compound. The slow off-rate of the compound likely plays a role in this. The PPM1D:GSK/DMSO mix equilibrated for 15 minutes at room temperature, followed by addition of a 15-fold excess of labeling buffer (20 mM HEPES, pD 7.5, 25 mM NaCl, 5 mM MgCl2, 0.1 mM TCEP, D_2O) at 25 °C. Analysis of C-terminal truncations of PPM1D were performed without the DMSO and preincubation step. Labeling of

PPM1D$_{\Delta loop}$, was performed with buffer composed of 20 mM HEPES, pD 7.5, 150 mM NaCl, 5 mM MgCl2, 0.1 mM TCEP in D_2O due to solubility issues at low ionic strength (<150mM NaCl). No difference in deuterium uptake was observed in PPM1D$_{1-420}$ in the two different salt concentrations (data not shown). Labeling reactions were quenched with an equal volume of ice-cold quench buffer (0.8M guanidine hydrochloride, 0.8% formic acid, H_2O) at time points ranging from 10s to 4 hrs. Undeuterated control samples were prepared for each of the proteins alone and in complex with GSK, where applicable, using the same procedures as outlined above and using labeling buffer made with H_2O instead of D_2O.

LC/MS: All samples were digested immediately after quench by injecting directly into an M-class nanoAcquity UPLC with HDX technology (Waters) for online digestion with an Enzymate BEH pepsin column [2.1 x 30 mm, 5 µm (Waters)] and UPLC peptide separation. The digestion chamber of the UPLC system was held at 15.0 ± 0.1 °C during analysis and the flow rate over the digestion column was 100 µL /min. The cooling chamber, which contained the chromatography separation, was held at 0.0 ± 0.1 °C for the entire analysis. Peptides were trapped and desalted on a VanGuard Pre-Column trap [2.1 mm × 5 mm, ACQUITY UPLC BEH C18, 1.7 µm (Waters)] for 3 minutes at 40 µL/min, eluted from the trap using a 5%–35% gradient of acetonitrile over 6 minutes at a flow rate of 40 µL/min, and separated using an ACQUITY UPLC BEH C18, 1.8 µm, 1.0 mm × 100 mm column (Waters). The back pressure averaged 8500 psi at 0 °C and 5% acetonitrile:95% water. The error of determining the deuterium levels was ± 0.2 Da in this experimental setup. Therefore, differences ± 0.5 Da were considered meaningful. A blankwash injection was performed between every two samples in which 100 µL wash solution (1.5M GdnHcl, 4% acetonitrile, 0.8% formic acid) was injected over the digestion column and 5%-85% acetonitrile gradients repeated over the trap and analytical columns. Mass spectra were acquired using a Waters Xevo G2-XS QToF mass spectrometer equipped with a standard ESI source set to the following parameters: polarity ES+, capillary voltage, 3.0 kV, cone voltage, 50 V; collision energy, 4 V; cone gas flow, 90 L/h; source temperature, 80 °C; desolvation temperature, 175 °C; and desolvation gas flow, 600 L/h. StepWave parameters were adjusted as previously described (ref). The instrument scanned every 0.4 s from 100 to 2000 m/z in MS^E mode, with collision energy ramp enabled from 20 V to 40 V. All comparison experiments were done under identical experimental conditions such that deuterium levels are reported as relative.

Data Processing: Peptides were identified using PLGS 3.0.2 (Waters) using multiple replicates of undeuterated control samples. Raw MS data were imported into DynamX 3.0 (Waters). Peptides meeting the filtering criteria were further processed automatically by DynamX followed by manual inspection of all processing. Additional experimental details and processing parameters are outlined in Supplementary Table 4. The relative amount of deuterium in each peptide was determined by subtracting the centroid mass of the undeuterated form of each peptide from the deuterated form, at each time point, for each condition. Deuterium uptake values were used to calculate fractional percent uptake as follows:

$$Fractional\ uptake\ \% = \frac{\#\ Da\ incorporated}{peptide\ length - (\#prolines) - 1_{N-term\ amide\ H}}$$

Deuterium uptake values were also used to generate uptake difference maps, where differences are expressed in Daltons and differences greater than 0.5 Da were considered significant.

File name descriptions

Raw file names contain coded descriptions of the constructs used in this study:

D155-166: hinge deletion
F7: flap deletion
t39-95: loop deletion