The effects of Skp2 N terminal post- translational modification on its interactions with CDK2/cyclin A

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Aims

i) Mutate Skp2 WT-N terminal fragment, to create the phospho-mimetic (Skp2-S64/72/75toE) to test the interaction between the mutant and CDH1.

ii) Test Skp2 acetylation mimetics (Skp2-K68/71toL and Skp2-K68/71toQ) using ITC for their ability to modulate (i) Skp1/Skp2 dimerisation and (ii) the interaction between Skp1/Skp2 and CDK2/cyclin A

iii) Purify CDK2/A, Skp1/Skp2 WT and mutant proteins by established protocol.

iv) Conduct a phosphorylation assay to compare the ability of CDK2/A to phosphorylate Skp2-K68/71toL and Skp2 WT

v) CDK2/A kinase activity was successfully tested by phosphorylation assay, however due to a time restriction we deviated slightly from the original plan in the phosphorylation of Skp2 WT and the Skp2 KL mutant by Pim1 and AKT.

Introduction

Skp2 (S-phase kinase associated protein 2) was first characterized as part of a pentameric CDK2/cyclin A/Skp1/Skp2/Cks1 complex.
Skp2 plays an important role in cell cycle regulation as it drives the cell from G1 to S phase by degrading $p27^{kip1}$ (oncogenic tumour suppressor). In normal cells Skp2 is targeted to the APC (E3 ubiquitin ligase) thus decreasing levels of Skp2 and stabilising levels of p27.
In addition, the acetylation of Lys68 and 71 by p300 also effects Skp2 association with CDH1. This ultimately leads to increased levels of Skp2 and decreased levels of p27, which is essential for cell proliferation and tumorigenesis.
The protein Skp2 is found to be elevated in fibroblast cell lines. It undergoes many post- translational modifications. For example, phosphorylation by Akt at Ser72 promotes further phosphorylation by CK1 at Ser75; causing Skp2 stabilisation and cytoplasmic accumulation.

Methods

1. **Site- directed mutagenesis** of Skp2 WT N terminus amino acid sequence:

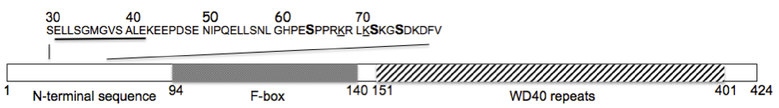

Figure 1. **Skp2 domain organisation-** The protein is divided into three sections and labelled as N-terminal sequence, F-box and WD40 repeats. The N terminal sequence show residues 30-80; the residues underlined illustrate the binding site for cyclin A.

Phosphomimetics –Serine to Glutamine Skp2- S68/72/75toE)

Acetylation mimetics- Lysine to leucine (Skp2-K68/71toL) (seen in figure 1)

2. **Protein Purification-**Skp2 WT, KL mutant and CDK2/A were placed into an expression vector. The proteins were purified via Glutathione purification and GST-tag purification. Once washed with modified HBS buffer, the GST- tag was cleaved with protease 3C. Subsequently the proteins were also ran on a GST 'clean up' column to eliminate the GST before Gel filtration, where the proteins are separated by size to eliminate any contaminants or aggregates.

3. **Phosphorylation Assay**- 10mM of stocks were made up; (ATP, $MgCl_z$, Tris-HCl pH 7.5, CDK2/A and pRb-GST. 6µl of stock, 9µl pRb-GST stock, 6µl 10x kinase buffer and 33µl ddH_20 was pipetted. 6µl of CDK2/A was added to start the reaction, which was stopped after 10/20/40 minutes.

4.**ITC** - Titrations consisted of a preliminary 0.5 µl injection of Skp1/Skp2 K68LK71L followed by 17 injections of 2 µl into a solution of CDK2/cyclin A. Experiments were performed at 25 °C. The protein concentrations for this experiment were as follows: 200 µM Skp1/Skp2 K68LK71L titrated into 20 µM CDK2/cyclin A. Thermodynamic parameters were obtained using Origin software (OriginLab) according to the manufacturer's instructions.

Results

The results obtained include an Agarose Gel electrophoresis DNA gel showing the successful Lysine to Leucine mutation (acetylation mimetic).

A Phosphorylation assay demonstrating the phosphorylation of the Skp1/Skp2 KL mutant and Skp1/Skp2 Wild Type substrates by CDK2/Cyclin A (by SDS-PAGE gel shifts).

An ITC result to illustrate the binding properties of CDK2/cylin A vs. Skp2 KL.

Figure 1.

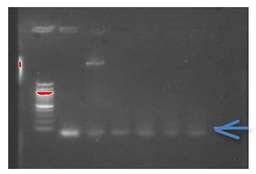

Figure 1. **PCR based-site directed mutagenesis to generate Skp2 mutants.** Agarose Gel Electrophoresis demonstrating successful generation of the Skp2-K68/71toL mutant. Lane 1-MWT markers; Lane 2-(Skp2-S64toE) 2X at 60 degrees; Lane 3-(Skp2-K68/71toL) 5X at 60 degrees-successful; Lane 4-(Skp2-S64toE) 2X at 68 degrees; Lane 5-(Skp2-K68/71toL) 5X at 68 degrees.

The '2X and '10X' denote the dilution of the primers as recommended in the 'Phusion' protocol. The bands that appear at a low MWT on the gel (marked with an arrow) show the excess primers.

Figure 2. (A) (B)

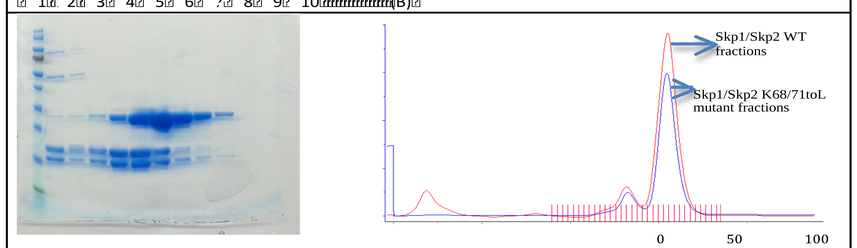

Figure 2. Purification of Skp1/Skp2 by Size Exclusion Chromatography. (A) Chromatogram to show the elution of Skp1/Skp2 and GST dimers from a Superdex 75 26/60 column. The GST dimer and Skp1/Skp2 complex elute at very similar volumes to generate a single broad peak. Red trace, Skp1/Skp2 WT, blue trace Skp1/Skp2 K68/K71toL mutant.

(B) SDS-PAGE analysis of fractions from the Skp1/Skp2 chromatogram in (A). High molecular weight contaminants are seen in lanes 2 and 3. Lane 1-MWT marker; Lane 2-fraction 15; Lane 3-17; Lane 4-18; Lane 5-19; Lane 6-20; Lane 7-21; Lane 8-23; Lane 9-25; Lane 10-26.

Figure 3. (A) (B)

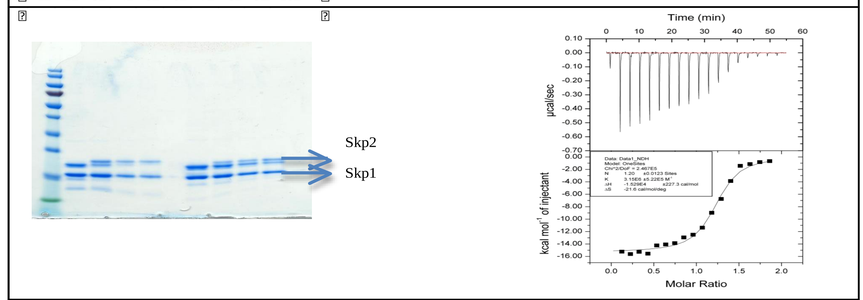

Figure 3. CDK2/A interacts with Skp1/Skp2. (A) Phosphorylation of Skp1/Skp2 WT and Skp1/Skp2 K68/71toL by CDK2/cyclin A. SDS-PAGE to monitor the kinase activity of CDK2/A vs. Skp1/Skp2 WT and Skp1/Skp2 K68/71toL. Samples were taken from the phosphorylation assay at 0, 10, 20 and 40 minutes. Lane 1- MWT marker; Lane 2-(Skp2) WT at 0 mins; Lane 3- WT at 10 mins; Lane 4- WT at 20 mins; Lane 5- WT at 40 mins; Lane 6- Space; Lane 7-(Skp2) KL at 0 mins; Lane 8- KL at 10 mins; Lane 9- KL at 20 mins; Lane 10- KL at 40 mins. As seen on the gel, the Skp2 WT protein appears to be 50% phosphorylated at 10 minutes, whereas Skp2 K68/71toL at 20-40 minutes. (B) ITC to measure the interaction between CDK2/cyclin A and Skp2-K68/71toL. CDK2/cyclin A was placed in the cell and Skp2-K68/71toL was titrated in as the ligand.

Conclusion

When CDK2/ cyclin A kinase activity was assessed with the Skp2 KL mutant substrate, in comparison to the wild type, a difference in kinase activity was observed (figure 3). This shows that the acetylation of Skp2 makes it a weaker substrate for CDK2/cyclin A, which could be due to dimerization which is reported for acetylation of Skp2 in cellulo (Inuzuka *et al*. 2012 Cell). Perhaps this dimerization occludes the Skp2 CDK2 phosphorylation site at S64. Alternatively, the mutations may have also lowered the affinity of CDK2 for the substrate (increasing the Km). Further kinetic characterization would allow these models to be tested. However, ITC analysis yields similar N values suggesting that the stoichiometry between CDK2/ cyclin A and the two Skp1/2 complexes is the same. Therefore, we cannot conclusively state the impact of the KL acetylation mimetic on the interaction of Skp1/Skp2 with CDK2/ cyclin A. However, this work has provided preliminary insights into the regulation of Skp2 via post-translational modification of its N-terminal regulatory domain.

Future Work

Testing the binding and activity of CDK2/cyclin A against other mutants; K-Q, and phosphomimetics S-E, would provide further insight into the Skp2 N terminal regulatory domain.

References

1) Inuzuka *et al*. 2012 Cell

2) Ecker, K. and Hengst, L. (2009). Skp2: caught in the Akt. *Nature cell biology*, 11(4), pp.377--379.
3) Zhang, H. et al., (1995) Cell 82:915-25.
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