Does the phosphorylation status of the Skp2 N- terminal sequence determine its interactions with CDK2/cyclin A and with CDH1?

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Aims

1. Express and purify full length CDH1 and Cdk2/CyclinA
2. Generate WT Skp2 N-terminal fragment, and a corresponding phosphorylation site mutant by site-directed mutagenesis. Test the interactions between the 2 Skp2 constructs with CDH1 and Cdk2/CyclinA.
3. To elaborate the interactions of Skp2 with two of its key regulators that control cell cycle progression .

Introduction

- In order to divide and generate 2 daughter cells, each cell must undergo a complete round of the cell cycle.
- This process is controlled by proteins known as 'Cyclin-dependant Kinases' (CDKs)
- CDK2 activity is inhibited by p27, which in turn is regulated by Skp2. Skp2 is the substrate receptor for the SCF complex which targets proteins for degradation.
- Levels of Skp2 are regulated by the E3 ubiquitin ligase , APC/C, which uses CDH1 to bind to Skp2 to target it for degradation (1).
- If these proteins and their interaction malfunction, then the cell cycle becomes deregulated, which could lead to cancer.
- A short peptide sequence, called the destruction box at the N-terminus of Skp2 is required forSkp2 binding to Cdh1. (2)
- Ubiquitination of Skp2 can be prevented by phosphorylation of residues S64 and S72 within the destruction box. SDM can be used to mutate these residues to alanine or glutamate (a phosphoserine mimetic) to probe the role of phosphorylation in controlling Skp2-CDH1 interaction.

Methods

- Site-Directed Mutagenesis to prepare two Skp2-N constructs.
- Protein expression and purification of full length CDH1, the two Skp2 N-terminal constructs, CDK2 and cyclin A.
- Kinase assay: phosphorylation of Skp2 by CDK2/cyclin A

References

1. Bashir, T., Dorrello, N. V., Amador, V., Guardavaccaro, D. & Pagano, M. Control of the SCFSkp2-Cks1 ubiquitin ligase by the APC/C-Cdh1 ubiquitin ligase. *Nature* **428**, 190-193, doi:10.1038/nature02330 (2004).
2. Liu, W., Wu, G., Li, W., Lobur, D. & Wan, Y. Cdh1-anaphase-promoting complex targets Skp2 for destruction in transforming growth factor beta-induced growth inhibition. *Mol Cell Biol* **27**, 2967-2979, doi:10.1128/mcb.01830-06 (2007).

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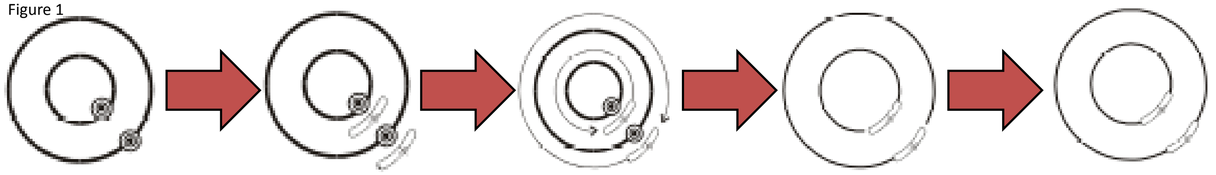
Site-Directed Mutagenesis:

Carried out on Skp2-N construct available (A64,S72,S75) to obtain 2 new constructs; Phospho Null (A64,A72,A75) and a 'Wild Type' Construct (S64,S72,S75). The method is described in fig. 1, results in fig. 4.

Figure 1



Gene within plasmid with target mutation site.

Plasmid denatured and mutagenic primers annealed

DNA polymerase extends and incorporates mutagenic primers

Non-mutated strands digested by 'Dpn1' enzyme

Transformed into competent cells and sent for sequencing, to confirm mutation had occurred

Protein Expression and Purification:

- Each protein was placed in an expression vector and transformed into *E.coli*.
- Full length CDH1 had not been expressed before and so was transformed into 3 different *E.coli* strains; 'Arctic Express', 'BL21DE3' and 'Rosetta' , in order to see which would provide greatest yield.
- Cells grown under varying conditions to determine optimum conditions for expression.
- Cells were then sonicated and spun in a centrifuge.
- The supernatant was then filtered and protein purified as described in figure 2.

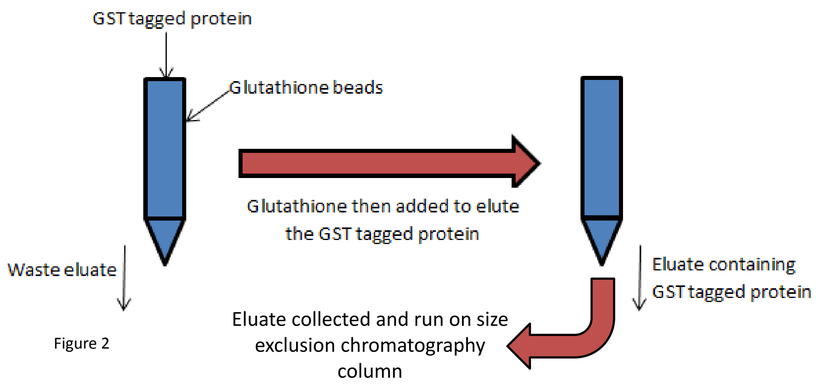


Figure 2

Kinase Assay:

CDK2/Cyclin A added to a reaction mixture, containing the Skp2-N wild type construct. A time course experiment was done, samples were taken at 0, 2, 10 and 20 minutes, which were immediately denatured at 100˚C . Each sample was then run on a 12% SDS-PAGE to determine the extent of phosphorylation by band-shift. Results in fig 6.

Discussion and Future Work

- Full length CDH1 had not been expressed before, and so a variety of *E.coli* strains and conditions were used to optimise expression. Arctic Express shows improved folding, BL21 DE3 has tight regulation of the T7 promoter whilst Rosetta has codon bias correction.
- Expression may not have worked for a number of reasons.
- CDH1 is a multidomain protein, of which the WD40 domain binds Skp2. Future work will focus on expressing this domain alone in both *E. coli* and insect cells.
- Other future work will involve testing the binding of the Skp2 mutants to CDK2/A and CDH1 to see if phosphorylation at S64 and S72 affects their association.

Kinase Assay:

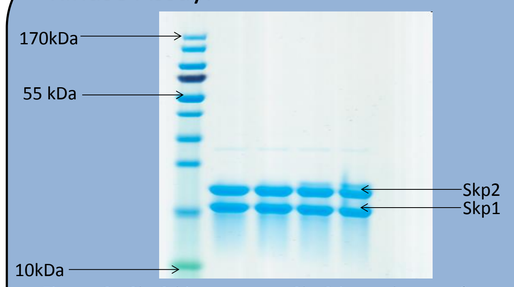


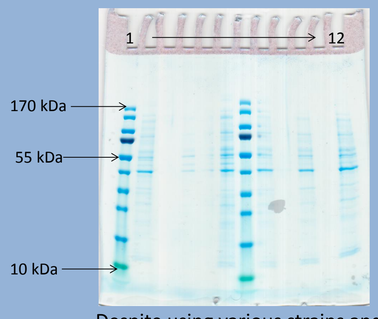
Figure 6 - Skp2 kinase assay. Skp2 is a substrate for CDK2/cyclin A and the reaction can be monitored by gel-shift. This is the first observation in the group that Skp2 phosphorylation can be followed using this method.

Results

CDH1 Expression:

Lane Number	*E.Coli* Strain	Vector
2	Arctic Express	M
4	BL21DE3	M
6	Rosetta	M
8	Arctic Express	J
10	BL21 DE3	J
12	Rosetta	J

Figure 3 CDH1 Test Expression. GST fusion protein from each induction was purified by affinity chromatography and subsequently cleaved with 3C



Despite using various strains and vectors at preferred growing conditions, no soluble full length CDH1 was expressed.

Skp2 Mutagenesis:

Figure 4 – DNA gel of SKP2 mutants.

WT sequence confirmed by 'Sanger Sequencing'. PN sequencing returned false

Figure Legend:

- Lane 1=Marker
- Lane 2=PN
- Lane 3=PN (2)
- Lane 4=WT
- Lane 5=WT (2)

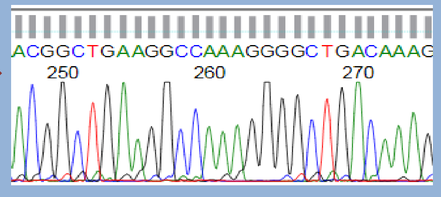


Figure 5 – Chromatogram returned for 'Wild Type' construct sequencing. Good signal to noise ratio makes us confident mutagenesis was a success