The biochemical & structural characterisation of CDK1/2-RingoA complexes and its possible role in cancer

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Introduction

- CDK enzymes tightly regulate mammalian cell cycle progression once activated by cyclin partner binding[1]
- RingoA (Speedy1) are cyclin-like proteins that activate CDK1 and CDK2, but the mechanisms of regulation by RingoA remain understudied[1]
- The published structure of the CDK2-RingoA complex confirms the previously reported findings that CDK2 does not require phosphorylation for its activation once bound to RingoA[1]
- Several studies have also reported elevated RingoA protein expression in various types of cancer[1]

Aims

- To compare kinase (enzyme) activities of CDK1 and CDK2 once activated by Cyclin A and RingoA
- To determine the extent that cyclin identity and phosphorylation dictate substrate preference
- To produce crystals of CDK1/2-RingoA complexes

Methods

1) Production of RingoA and CDK2 proteins through recombinant expression in *E. coli* (Figure 1)
2) Protein purification via GST and amylose resin columns then size exclusion chromatography (SEC) (Figures 2 and 3)
3) Measurement of CDK activities using the ADP-Glo™ kinase assay (Figure 4)
4) Protein crystallisation trials and data analysis[2]

Results

SDS-PAGE Gel of SEC Purified Proteins

GST-CDK2, MBP-RingoA expressed from *E.coli* and GST-CDK1 from insect cells were mixed on a whole cell stage to create a complex. Once purified by affinity tags and by SEC, peaks containing CDK and RingoA were collected and further purified by one round of amylose resin cleaning before being run on a SDS-PAGE gel. Proteins were blotted against RingoA polyclonal antibody to confirm its identity, presence and cleavage.

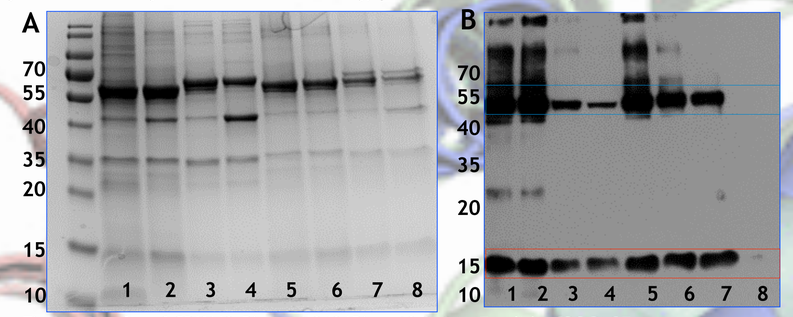

Fig 2A: Results of CDK1/2-Ringo61-213/Ringo68-213 in E.coli by SDS PAGE.
Fig 2B: Results of test expression of CDK1/2-Ringo61-213/Ringo68-213 in E.coli by Western Blot.
= Uncleaved MBP-Ringo bands = Cleaved Ringo with 3C Protease bands

SEC and SDS-PAGE Analysis

GST-CDK2 RingoA complex from 2.5 litres of insect cell culture was bound to glutathione beads under gravity flow and washed with buffer before being incubated overnight with 300 µl of 3C protease. Collected sample has been split into two: one sample for further purification by SEC and another for CDK2 phosphorylation by GST-CAK1 (CDK activating enzyme) in presence of ATP, which was followed by size exclusion chromatography (SEC).

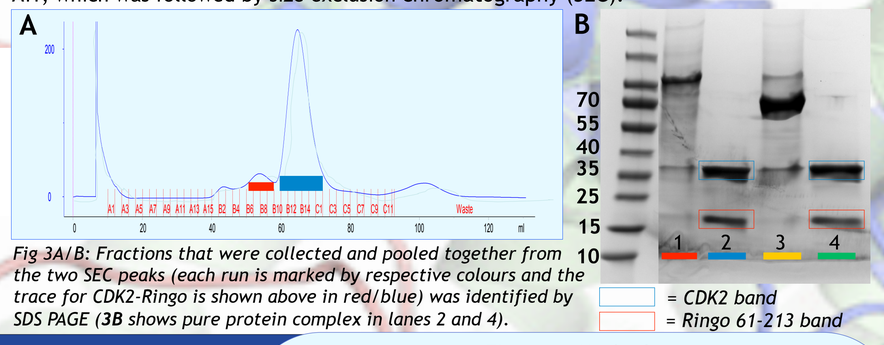

*Fig 3A/B: Fractions that were collected and pooled together from the two SEC peaks (each run is marked by respective colours and the trace for CDK2-Ringo is shown above in red/blue) was identified by SDS PAGE (**3B** shows pure protein complex in lanes 2 and 4).*

= CDK2 band
= Ringo 61-213 band

ADP-Glo Kinase Assay

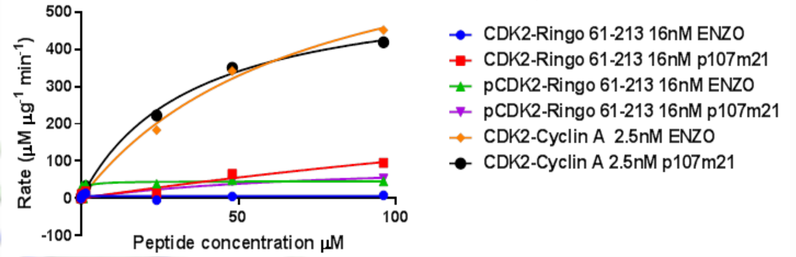

Fig 4: The ADP-Glo™ assay format was used to measure CDK activity towards a p107 or ENZO peptide demonstrating the level of kinase activity. The table below shows the respective Vmax and standard error values for the ADP-Glo kinase assay.

	CDK2-Ringo 61-213 16nM ENZO	CDK2-Ringo 61-213 16nM p107m21	pCDK2-Ringo 61-213 16nM ENZO	pCDK2-Ringo 61-213 16nM p107m21	CDK2-Cyclin A 2.5nM ENZO	CDK2-Cyclin A 2.5nM p107m21
Vmax Best-fit values	6.33	496.30	45.79	100.10	797.50	579.50
Vmax Standard Error values	2.74	953.20	7.97	37.24	71.13	30.03

Discussion & Conclusion

- First, expression trials in *E. coli* failed to generate pure protein (Figure 2), as the MBP tag was still present and the Ringo appeared GroEL associated
- To overcome these difficulties, CDK2-Ringo was co-expressed in insect cells (Figure 3B, the construct was provided by Dr R. Heath)
- Figure 4 suggests that the activity of CDK2 in complex with Ringo is much lower in comparison to CDK2-Cyclin A
- The phosphorylation status of the CDK2 isolated from insect cells as part of a CDK2-Ringo complex could not be determined
- Therefore determining such in future work would help to explain the activity observed
- The results do conclusively suggest that the enzyme activity is far less for CDK2-Ringo complexes and differs with phosphorylation state for substrates
- Protein crystallisation trials were set-up for further analysis

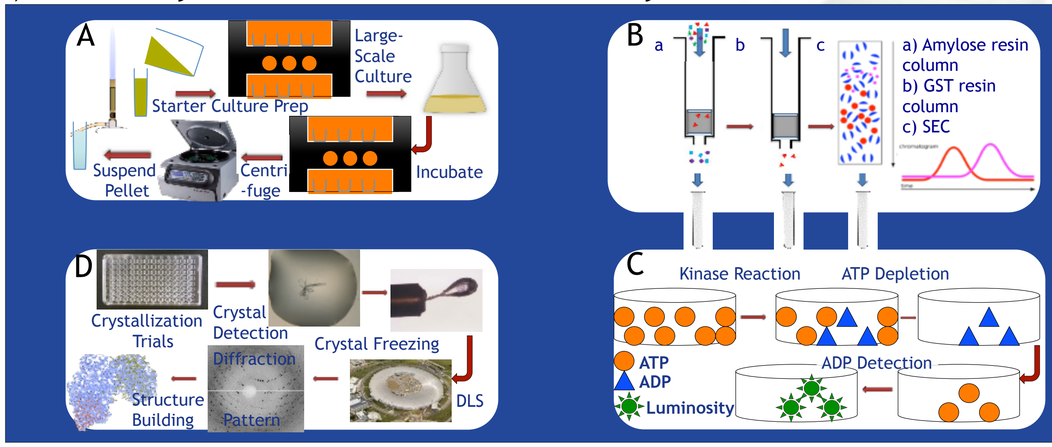

Figure 1 (Methods)

- *E.coli* (strain Rosetta (DE3) pLysS) were transformed with plasmids containing genes of interest (CDK2 and RingoA) followed by expression of proteins in large scale bacterial culture (**figure 1A**) and in insect cell culture (for CDK1 and cloned GSTCDK2-RingoA expression)
- These proteins were made from starter culture, incubation and centrifugation steps (**figure 1A**)
- Next, purification of produced protein complexes took place via affinity chromatography and SEC (**figure 1B**)
- Enzymatic activity was measured using the ADP-Glo™ kinase assay (**figure 1C**)
- Purified protein complexes were subjected to crystallisation trials. The Diamond Light Source would enable data collection for structure determination if crystals of suitable quality were grown (**figure 1D**)

References

1. McGrath DA, Fifield BA, Marceau AH, Tripathi S, Porter LA and Rubin SM. Structural basis of divergent cyclin-dependent kinase activation by Spy1/RINGO proteins. *The EMBO Journal*. 2017; 36: 2251-2262. (background image)
2. Brown NR, Korolchuk S, Martin MP, Stanley WA, Moukhametzianov R, Noble MEM and Endicott JA. CDK1 structures reveal conserved and unique features of the essential cell cycle CDK. *Nature Communications*. 2015; 6: 6769.