Characterisation of the Molecular Complexes that Regulate the G_2/M Checkpoint of the Eukaryotic Cell Cycle

Melanie Collings

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Abstract

The cell cycle is one of the fundamental processes in nature, and is primarily concerned with the faithful replication of cellular contents, followed by even division to produce two identical daughter cells. It is made up of five discrete biochemical steps, comprising the interphase (G_1, S and G_2) and the mitotic phase (mitosis and cytokinesis), with two major regulatory checkpoints at G_1 and G_2. The focus of this research is the G_2 checkpoint, which ensures the successful achievement of DNA replication, prior to the initiation of mitosis. Arrest or progression is principally mediated by the CDK1/cyclin B1 complex; phosphorylation of CDK1 by wee1 kinase prevents progression to mitosis, and subsequent dephosphorylation by the CDC25 phosphatases, initially the B isoform, leads to mitotic onset.

The aim of this research was the biophysical and/or biochemical characterisation of the molecular complexes that form at the G_2 checkpoint to regulate entry into mitosis. CDK1 and cyclin B1 were separately expressed and purified from baculovirus-infected Sf9 cells. The wee1/14-3-3β complex was also expressed and purified, incorporating either full length wee1 or a truncated version from which the N-terminal domain of wee1 was deleted. Both exhibited wee1 kinase activity, at equivalent levels, $p<0.001$, with a 2.4 fold increase in kinase activity when wee1 is bound by 14-3-3β, $p>0.001$. Tryptic digestion of the complex indicated that its architecture was likely to be flexible and open, particularly within the N-terminal domain of wee1. CD analyses indicated that the wee1/14-3-3β complex was folded, with 30-40% α-helical content and 10-20% β-sheet content. Dissociation experiments were unsuccessful, however, indicating a high strength of interaction between wee1 and 14-3-3β. The empirical stoichiometry of the complex was determined as 1:1; subsequent native molecular weight determination suggested that the minimal functional unit is likely to be a 2:2 wee1/14-3-3β arrangement. It was proposed that the structural architecture of this complex may be similar to the serotonin N-acetyltransferase/14-3-3ζ complex. Experiments to determine the structure experimentally, using either TEM or x-ray crystallography, were unsuccessful, as the complex appeared to exhibit a high degree of flexibility in solution.

CDC25B was also expressed and purified, and was found to co-purify with a putative Sf9 14-3-3 protein. Consequently, it was re-cloned to co-express with 14-3-3β, and subsequent analysis of the resulting CDC25B/14-3-3β complex indicated that the empirical stoichiometry was 1:1, with the functional organization likely to be a 2:2 arrangement. It was proposed that the structural arrangement of this complex is most likely to be similar to that of the wee1/14-3-3β complex.

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Abbreviation:	Definition:
14-3-3β	human 14-3-3 protein, β polypeptide, NCBI accession number NP_003395
AMP-PNP	5'-adenylyl-β,γ-imidodiphosphate, non-hydrolysable ATP analogue
amt	Amount
ATP	Adenosine triphosphate
AU	absorbance units
β-ME	β-mercaptoethanol
BG	background
C167S	mutation of cysteine residue 167 to serine
C238S	mutation of cysteine residue 238 to serine
C350S	mutation of cysteine residue 350 to serine
CD	circular dichroism
CDC25B	human cell division cycle 25B protein, isoform 1, NCBI accession number NP_068659
CDK1	human cyclin dependent kinase 1, isoform 1, NCBI accession number NP_001777
cDNA	complementary DNA
C-terminal	carboxyl terminal
cyclin B1	human cyclin protein, isoform B1, NCBI accession number NP_114172
°C	degrees celsius
DEPC-treated water	Water treated with diethylpyrocarbonate
dephosphorylation	the enzymatic removal of a phosphate group from a protein
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
E183A	mutation of glutamic acid residue 183 to alanine

E184A	mutation of glutamic acid residue 184 to alanine
EDTA	ethylene diamino tetraacetic acid
FBS	fetal bovine serum
G_0	an opting out gap phase of the eukaryotic cell cycle
G_1	the first gap phase of the eukaryotic cell cycle
G_2	the second gap phase of the eukaryotic cell cycle
GST	glutathione-S-transferase
HeLa cells	Human lymphoma-derived cell line from Helen Lane, ATCC Number CCL-2
His-tagged	polyhistidine purification tag
IEF	isoelectric focussing
IMAC	immobilised metal affinity chromatography
IPG	immobilised pH gradient strip
IPTG	isopropyl-β-D-thiogalatopyranoside
LAU	laser absorbance units, for Sypro Ruby analysis
MPD	2-methyl-1,3-propanediol
MPF	mitosis promotion factor
Mr	molecular weight
MWCO	Molecular weight cut-off point
NCBI	National Centre for Biotechnology Information
NP40	nonionic P40 detergent
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB ID	protein data bank identification number
Pfx	*Pyrococcus furiosus* polymerase enzyme
phosphorylation	the enzymatic addition of a phosphate group to a protein
pI	isoelectric point
RMS	root-mean-square
RNA	ribonucleic acid
RNAse	ribonuclease enzyme
rTEV	recombinant tobacco etech virus protease

S phase	the synthesis phase of the eukaryotic cell cycle
SD	standard deviation, a measure of the variance of a dataset, calculated as the RMS deviation of the individual values from the mean value
SDS	sodium dodecyl sulfate
SE	standard error of the mean, an unbiased estimate of the error of the population mean
Sf9	*Spodoptera frugiperda*-derived cell line, ATCC Number CRL-1711
SOC	superoptimal broth with catabolite repression
TAE buffer	tris-acetate-EDTA buffer
TE buffer	10 mM Tris.HCl pH 8.0, 1 mM EDTA
TEM	Transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
tr. wee1	truncated wee1
tris	tris(hydroxymethyl)aminomethane
truncated wee1	wee1 construct consisting of residues 215 to 646 only
v/v	volume per volume
w/v	weight per volume
wee1	human wee1 kinase, NCBI accession number NP_003381, full length construct
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside