Control of Cyclin C Levels during Development of Dictyostelium

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Abstract

**Background:** Cdk8 and its partner cyclin C form part of the mediator complex which links the basal transcription machinery to regulatory proteins. The pair are required for correct regulation of a subset of genes and have been implicated in control of development in a number of organisms including the social amoeba Dictyostelium discoideum. When feeding, Dictyostelium amoebae are unicellular but upon starvation they aggregate to form a multicellular structure which develops into a fruiting body containing spores. Cells in which the gene encoding Cdk8 has been deleted fail to enter aggregates due to a failure of early gene expression.

**Principal Findings:** We have monitored the expression levels of cyclin C protein during development and find levels decrease after the multicellular mound is formed. This decrease is triggered by extracellular cAMP that, in turn, is working in part through an increase in intracellular CAMP. The loss of cyclin C is coincident with a reduction in the association of Cdk8 with a high molecular weight complex in the nucleus. Overexpression of cyclin C and Cdk8 lead to an increased rate of early development, consistent with the levels being rate limiting.

**Conclusions:** Overall these results show that both cyclin C and Cdk8 are regulated during development in response to extracellular signals and the levels of these proteins are important in controlling the timing of developmental processes. These findings have important implications for the role of these proteins in controlling development, suggesting that they are targets for developmental signals to regulate gene expression.

Introduction

Cdk8 and its cyclin partner, cyclin C, are regulators of transcription through association with the mediator complex, a high molecular weight complex which couples transcriptional regulators to the basal transcription machinery [1]. Cdk8 has been postulated to have both a positive and negative role on transcription and to function either by direct phosphorylation of the C terminal domain (CTD) of RNA polymerase II or by phosphorylation of regulatory transcription factors binding to upstream promoter elements. It forms part of a sub-module of four proteins able to associate with the core mediator complex to modulate its activity. Mutation of Srb10, the *S. cerevisiae* equivalent of Cdk8, leads to altered expression of around 30% of genes suggesting this sub-module does not function at all genes but is selectively used to modulate transcription [2].

The mechanism of regulation of Cdk8 activity is not well defined, especially the role of regulation of the levels of the cyclin C subunit which is required for kinase activity. In *S. cerevisiae* proteolysis of Srb11, the orthologue of cyclin C, has been reported in response to elevated temperatures, ethanol, oxidative stress and carbon starvation [3]. The signalling pathways that result in this degradation are complex and operate upon three separate elements within the protein whose importance varies with the stimulus. These results imply that a number of independent signalling pathways act upon the Srb11 protein in response to a variety of stimuli. Alternatively, the levels of Cdk8 itself may be rate-limiting as overexpression of Cdk8 has been found to regulate β-catenin levels in colorectal cancers [4].

Cdk8 has been implicated in regulating transcription during development. In mammalian cells, cyclin C and Cdk8 are recruited to the Hairy/Enhancer of Split (HES1) developmental gene where Cdk8 hyperphosphorylates the Notch ICD (intracellular domain) – an activator of HES1 transcription. This phosphorylation results in degradation of the ICD with a resultant reduction in HES1 transcription [5]. This Cdk8-dependent proteolysis of the ICD at the promoter is analogous to the mechanism of GCN4 and Ste12 regulation by Srb10 in *S. cerevisiae* [6,7,8]. The concept of Cdk8 acting as a developmental gene regulator has been reinforced by a number of whole organism studies. Cdk8 is expressed in the head and trunk regions of the developing zebrafish embryo implying that it may act as transcriptional regulator in a tissue-specific manner. Similarly, the Med12 and Med13 components of the Cdk8 module have been found to be essential for correct vulva and male tail formation in *Caenorhabditis elegans* [9] and for wing and eye formation in *Drosophila melanogaster* [10].
morpogenesis in *Drosophila* [10,11]. More recently, a genetic analysis in this organism indicated that, while the subunits of the Cdk8 module are not essential for cell viability, they are all necessary for the correct development of the multicellular organism [12]. The role of Cdk8 as a developmental regulator has been found to be conserved in the plant kingdom. In *Arabidopsis thaliana*, the HUA ENHANCERS3 (HEN3), which encodes CdkE, a homologue of Cdk8, is involved in the proper formation of stem cells in the floral meristem and in the specification of stamen and carpel identities [13].

Cdk8 is essential to the development of *Dicyostelium discoideum* [14,15]. This haploid organism feeds on bacteria as single cells, but upon starvation enters a multicellular life cycle [16]. Starving cells start to secrete low (nM) pulses of extracellular cAMP and surrounding cells respond to this by relaying the signal and chemotaxing up the gradient of cAMP to form a multicellular mound. This mound behaves as a multicellular organism and undergoes a number of morphogenetic events culminating in the production of a fruiting body containing a head of spores supported by a stalk of dead, vacuolated cells. *Dicyostelium* cells in which the gene encoding Cdk8 has been disrupted fail to aggregate to form mounds correlating with a failure of expression of early developmental genes [14,15]. The requirement for Cdk8 activity early in development suggested that the activity of this protein may be developmentally regulated. Here we report that cyclin C levels are decreased during development in response to high levels of extracellular cAMP and time of development. The signalling pathway triggering the decrease in levels works through intracellular cAMP. The behaviour of Cdk8 also alters in response to high levels of extracellular cAMP, as a decreased proportion is found associated with a high molecular weight complex. However, we see no evidence that loss of cyclin C causes the dissociation of Cdk8 but rather that cyclin C levels increase with availability of Cdk8 partner. Overexpression of cyclin C and Cdk8 was found to increase the rate of the early stages of development, consistent with the level of protein being rate-limiting.

### Materials and Methods

#### Construct and strain generation

A construct to express *Dicyostelium* cyclin C with an N-terminal FLAG tag under the actin 15 promoter (pDXA[act15:FLAG-cycC]) was generated by amplifying the coding region of CycC from the ATG to the stop codon by PCR from genomic DNA, with the FLAG tag incorporated into the N-terminal primer. The resulting fragment was inserted into the BamHI and XhoI sites of pDXA-3C [17] to drive protein expression from the actin 15 promoter present on this extrachromosomal vector. To generate a vector to express cyclin C with an additional C-terminal TAP tag under the cyclin C promoter (pDV[ycycC:ycycC-CTAP]), this fragment was first inserted into the BglII and XbaI sites of pDV-CTAP [18]. The actin 15 promoter was then excised from this vector using SalI and BamHI and replaces with a fragment containing the 641 nucleotides upstream of the start codon of cyclin C. This fragment extends to the gene upstream of cyclin C and so is likely to contain all of the relevant control sequences for correct expression of the cyclin C gene. The construct to drive expression of *Dicyostelium* Cdk8 with an N-terminal myc tag has already been described [14].

The constructs were introduced into *Dicyostelium* Ax2 cells by electroporation and transformants selected by growth in the presence of G418 (10 μg/ml) as the expression plasmids contain the neomycin resistance gene (neoR). *GskA* [ycycC:ycycC-CTAP] and *carC* [ycycC:ycycC-CTAP] cell lines were created by introducing the pDV[ycycC:ycycC-CTAP] plasmid into gskA and carC cells made in a Ax2 background, as previously described [19]. All strains were generated with the approval of the Biochemistry Department Genetic Modification Safety Committee, University of Oxford.

### Growth and development of *Dicyostelium*

*Dicyostelium* cells were grown axenically in HL5 medium at 22°C in shaking suspension. For development in shaking suspension, exponentially growing cells were resuspended in KK2 (16.5 mM KH₂PO₄, 3.8 mM K₂HPO₄) at 2 × 10⁵ cells/ml and shaken at 120 rpm and 22°C for 5 hours, pulsed with 5 μM cAMP every 5 minutes. For filter development exponential axenically growing cells were washed in KK, 3.6 mM K₂HPO₄ and re-suspended in LPS (40 mM KH₂PO₄, 20 mM KCl, 680 μM dihydrostreptomycin sulphate [pH 7.2]) at 3.5 × 10⁶/cm² on Millipore filters on an LPS soaked pad. The filters were incubated at 22°C in the dark.

### Northern and Western blot analysis

Total RNA was extracted from approximately 1 × 10⁷ cells using TRIZOL RNA extraction kit (Sigma) according to the manufacturer’s protocol. Samples (10 μg) of total RNA were separated on a 1% formaldehyde-containing gel, blotted and probed by standard methods. Unless otherwise stated, all the northern blots are representative of at least three independent experiments. Western blot analysis was carried out as previously described [14] and blots shown are representative of at least three independent experiments.

### Size fractionation

1 × 10⁷ cells were harvested washed twice with KK₂ and lysed in 4 ml of nuclear isolation (NI) buffer (50 mM Tris-HCl [pH 7.5], 5 mM magnesium acetate, 10% Sucrose, 2% NP-40 and protease inhibitor cocktail [Roche]) and centrifuged at 2,300 xg in order to collect the nuclei. The nuclei were resuspended in 250 μl of NI buffer before addition of an equal volume of (10 mM HEPES [pH 7.9], 600 mM NaCl, 10% sucrose, 5 mM MgCl₂, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT and protease inhibitor cocktail). After spinning to clear debris, the supernatant was subjected to gel filtration on a 24 ml Superose 6 column (Amersham Pharmacia Biotech) that had been equilibrated in gel filtration buffer (25 mM HEPES [pH 7.4], 300 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol). Fractions of 1 ml were collected and analysed by western blot. The size of protein complexes in each fraction was calculated by using the High Molecular weight gel filtration calibration kit (GE Healthcare).

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*Figure 1. Comparison of cyclin C coding sequences. A. The sequence of the predicted *Dictyostelium* protein (DOB_G0274139 at dictybase.org) was aligned against *Homo sapiens* (H.s.) Cyclin C and Srb11 from *S. pombe* (S.p.) and *S. cerevisiae* (S.c.). Alignment against a generic cyclin box from the NCBI conserved domain database (cd00043) is also shown. B. Cyclin protein sequences were obtained from NCBI (www.ncbi.nlm.nih.gov) nucleotide databases and aligned using Clustal W in Bioedit [36]. A generic cyclin box sequence from the NCBI conserved domain database (cd00043) was used to define the cyclin box domain within each protein. These domains were used to construct a phylogenetic tree in Bioedit using the neighbour joining method. The tree was rooted using Cyc18 from *Arabidopsis thaliana* as an outgroup.*

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Cyclin C in Development

A

Western

| 0 | 5 | 10 | 15 | 20 | 25 | hrs |
|---|---|----|----|----|----|-----|
| α-mouse IgG |
| α-actin |
| cycC |
| IG7 |

Northern

B

| 0 | 3 | 6 | 9 | 12 | 18 | 24 | 30 | hrs |
|---|---|---|---|----|----|----|----|-----|
| cycC |
| 26S |
| 17S |

C

Western

| 7 | 8 | 9 | hrs |
|---|---|---|-----|
| cAMP | - | + | - | + | - | + |

Northern

D

| 7 | 8 | 9 | hrs |
|---|---|---|-----|
| cAMP | low | + | - | + | - | + |
| high | - | - | + | - | - | + |
Results

Identification of Dictyostelium Cyclin C

A putative cyclin C gene (gyC) was identified by a BLAST search of the Dictyostelium genomic database (dictybase.org). The protein contains the characteristic α-helical cyclin box fold domain and exhibits 45% amino acid identity and 62% similarity to the human cyclin C protein (Figure 1A). These levels of similarity were higher than when compared with the S. pombe protein (33% identity, 51% similarity) or the S. cerevisiae protein (32% identity, 46% similarity). In order to identify the evolutionary history of the putative Dictyostelium CycC protein, the cyclin box domains present in a number of cyclin proteins was used to plot a phylogenetic tree (Figure 1B). The Arabidopsis thaliana cyclin CycC1B was used as an out group to root the tree. Cyclin C homologues that had been identified in other organisms were placed in the same clade as the Dictyostelium CycC protein indicating that they shared a common ancestor.

Expression of Dictyostelium cyclin C

The pDV[gyC::gyC-CTAP] plasmid was constructed to express the Dictyostelium cyclin C protein with a C-terminal tandem affinity purification tag (CycC-CTAP) from its endogenous gyc promoter. This promoter fragment stretches 641 nucleotides upstream of the gyc coding sequence to the terminus of the neighbouring gene, and is therefore likely to contain all the elements required for correctly regulated gyc expression. This pDV[gyC::gyC-CTAP] plasmid was transformed into Dictyostelium Ax2′ cells and transformants selected by neomycin resistance to generate Ax2[gyC::gyC-CTAP] cells. Western blot of a whole cell extract from this strain was able to detect a protein of the predicted size of CycC-CTAP. The Ax2[gyC::gyC-CTAP] cells were used to assess the levels of cyclin C expression throughout the developmental life cycle by western blot using antiserum directed against the tag (Figure 2A). The protein was expressed in growing cells and the level of tagged cyclin C protein declined during later development starting at around the stage of tipped aggregate formation. This loss of protein was matched by a loss in mRNA levels consistent with the regulation of protein levels being due, at least in part, to loss of mRNA (Figure 2A). This analysis will detect the transcripts from both the endogenous cyclin C gene and that expressed from the plasmid. However, at the exposure times used to detect expression of the transgene, no endogenous cyclin C mRNA expression was detectable (data not shown). Longer exposure of a developmental time course northern of parental Ax2 cells revealed that the endogenous cyclin C mRNA showed a similar pattern of expression to the transgene, with reduced levels later in development, consistent with the transgene having all the regulatory sequences required for correct expression (Figure 2B). All further northern blots shown are short exposures and are likely, therefore, only to be detecting transgene expression, although it is possible the endogenous transcript contributes to the signal.

Developmental regulation of Dictyostelium cyclin C levels by intracellular cAMP

Two signalling pathways have been implicated downstream of extracellular cAMP at the stage of development at which cyclin C levels decline, both working through seven transmembrane domain cAMP receptors: activation of the serine/threonine kinase GskA (the Dictyostelium orthologue of GSK3) and, through increase in intracellular cAMP, activation of a cAMP-dependent protein kinase [20,21]. We set out to determine if either of these pathways was responsible for the loss in cyclin C observed. GskA is activated by tyrosine phosphorylation by zak1 downstream of the extracellular cAMP receptor cAR3, one of a number of cAMP receptors expressed at this stage of development. Therefore we expressed tagged cyclin C, driven by its endogenous promoter, in gskA−/− or carC−/− cells to see if levels of cyclin C could still be reduced by treatment with high extracellular cAMP. In cells lacking the genes encoding GskA or cAR3, the loss of cyclin C in response to high extracellular cAMP was seen as in parental Ax2 cells, suggesting that this pathway is not essential for the reduction in cAMP levels (Figure 3A), although we cannot rule out that it may alter the efficiency. In order to investigate the role for intracellular cAMP, Ax2[gyC::gyC-CTAP] cells developed in shaking suspension were exposed to 8Br-cAMP. This membrane permeable cAMP analogue does not activate the extracellular cAMP receptors but enters the cells and directly activates cAMP-dependent protein kinase [22]. Addition of 8Br-cAMP to cells at the relevant stage of development is sufficient to bring about a decrease in cyclin C levels, and, as previously, there is a simultaneous decrease in the levels of both cyclin C mRNA and protein (Figure 3B). This implicates intracellular cAMP levels in the pathway leading to down-
Figure 3. Signals regulating expression of cyclin C during development. A. I. Exponentially growing carC[cycC::cycC-CTAP] and gskA[cycC::cycC-CTAP] cells were harvested and developed on filters at a density of 3 x 10^6 cells/cm^2. Samples were collected at the times indicated and analysed by western blot as in Figure 1A. II. Exponentially growing carC[cycC::cycC-CTAP] and gskA[cycC::cycC-CTAP] cells were harvested and shaken in KK2 buffer at 5 x 10^6 cells/ml and pulsed with 50 nM cAMP every 5 mins. After 4 hrs cells were split and shaken in the presence (+) or absence (−) of 500 μM cAMP, added every hour for the duration of the experiment. Samples were taken at the time points indicated and analysed by western blot as above. B. Exponentially growing Ax2[cycC::cycC-CTAP] cells were harvested and shaken in KK2 buffer at 5 x 10^6 cells/ml, pulsed with 50 nM cAMP every 5 mins. After 4 hrs the cells were split and incubated in the presence (+) or absence (−) of 5 mM 8Br-cAMP. Samples were collected at the times indicated and analysed by western blot as in Figure 1A.

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regulation of cyclin C expression in mid-development, although this may not be the only important factor and the rate and extent of loss may be dependent on other pathways.

**Dictyostelium cyclin C levels do not alter in response to oxidative stress**

In *S. cerevisiae*, cyclin C levels have been reported to respond to a number of cellular stresses such as oxidative stress although this has not been reported in other systems. In order to determine if this is a universal phenomenon, we investigated the levels of epitope-tagged cyclin C in cells following treatment with hydrogen peroxide at a concentration known to induce approximately 25% cell death [23]. When expressed from its endogenous promoter the levels of cyclin C protein and mRNA were unaltered following H$_2$O$_2$ treatment (Figure 4 and data not shown). When the cyclin C was expressed from the actin 15 promoter, however, the level of protein did decrease following H$_2$O$_2$ treatment. This was mirrored by a decrease in mRNA levels (data not shown), suggesting that the oxidative shock was leading to a decrease in mRNA levels which led to a subsequent decrease in protein levels. The down-regulation of the actin 15 promoter confirms that the cells were responding to oxidative stress but the fact that cyclin C mRNA and protein levels remained unchanged when expressed from the endogenous cyclin C promoter indicates that the pathway inducing loss of cyclin C in response to these levels of oxidative stress is not present in *Dictyostelium*.

**Association of Dictyostelium cyclin C and Cdk8 with high molecular weight complexes**

Cyclin C and its kinase partner, Cdk8, form part of the high molecular weight mediator complex in mammalian cells and yeast.

In order to determine if the *Dictyostelium* proteins are also found in a complex, a construct to drive expression of *Dictyostelium* Cdk8, tagged with an epitope from c-myc at its N terminus, was transfected into Ax2 cells either on its own or co-transfected with a construct to drive expression of tagged cyclin C. Therefore cells were overexpressing cyclin C, Cdk8 or both. Gel filtration analysis of nuclear extracts from cells expressing both epitope-tagged Cdk8 and cyclin C revealed a high proportion of these proteins as part of a high molecular weight complex (Figure 5A). Protein also elutes in fractions consistent with a lower molecular weight which might represent free subunit or protein only complexed to a smaller number of interacting proteins. In mammalian cells, only a very small proportion of Cdk8 is found outside the high molecular weight complex [24], so this lower molecular weight fraction could be a consequence of overexpressing the protein, saturating the availability of interacting partners.

In order to determine if the reduction in cyclin C levels during development was associated with a reduction in the proportion of Cdk8 found in the high molecular weight complex, the fraction of Cdk8 eluting at high molecular weight was compared in growing cells with cells from two conditions where a reduction in cyclin C levels is seen; firstly following development in shaking suspension and treatment with high extracellular cAMP and secondly following multicellular development to the first finger stage (Figure 5B). The first finger stage is around 15–18 hours of development at a time when the levels of cyclin C are seen to be reduced compared to growing cells (Figure 2A). It is not feasible to carry out the experiment in the mature fruiting body when cyclin C levels were further reduced, as it is difficult to lyse the differentiated stalk and spore cells in conditions that would leave protein complexes intact. In both conditions where reduced levels

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**Figure 4. CycC-CTAP protein levels in response H$_2$O$_2$ treatment.** Ax2[cycC::cycC-CTAP] and Ax2[act15::FLAG-cycC] cells were harvested, washed and shaken in KK$_2$ buffer at 1 x 10$^7$ cells/ml. After 1 hr the cells were split and incubated in the presence (+) or absence (−) of 0.25 mM H$_2$O$_2$. Western samples were taken after 1, 2 and 3 hrs of treatment and analysed on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with an α-mouse IgG secondary antibody and an α-actin antibody to control for loading.

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Figure 5. Size fractionation of complexes containing FLAG-CycC and myc-Cdk8. A. \(1 \times 10^9\) vegetatively growing Ax2[myc-cdk8, FLAG-cycC] cells were harvested and the extracted nuclei lysed and subjected to gel filtration on a Superose 6 column. Fractions (7–18) were resolved on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with α-FLAG and α-myc antibodies. Positions of molecular weight standards are specified above. B. \(1 \times 10^9\) Ax2[myc-cdk8] cells were harvested after either vegetative growth (V), development in shaking suspension for 8 hours with high (500 μM) cAMP added after 4 hours, as in Figure 2, (S) or development on filters up to the first finger stage (F). A nuclear extract from each of these cells was fractionated by gel filtration on a Superose 6 column. Samples of fractions 7 to 19, as indicated, were resolved on a 12% SDS-PAGE gel,
of cyclin C are apparent, a lower proportion of Cdk8 is found in the high molecular weight complex, demonstrating an alteration in Cdk8 behaviour as cyclin C levels decrease. Comparison of cells in shaken suspension in the presence and absence of high extracellular cAMP demonstrates that this signal, at the relevant stage of development, is sufficient to reduce the association of Cdk8 (Figure 5C).

One explanation for this data is that the level of cyclin C is the determining factor for Cdk8 association with the complex, so as cyclin C levels decline following transcriptional down-regulation, an increasing proportion of Cdk8 dissociates. To test this we compared the proportion of tagged Cdk8 eluting in the high molecular weight fraction in cells with normal levels of endogenous cyclin C and cells overexpressing epitope-tagged cyclin C from the strong, constitutive actin 15 promoter (Figure 6A). There was no difference in the level of Cdk8 found in the high molecular weight fraction suggesting that the association of Cdk8 with the complex is not purely determined by the level of cyclin C present in the cell. Conversely, co-expression of tagged cyclin C and Cdk8 in the same cells led to a higher level of stable cyclin C protein. When the levels of cyclin C mRNA expressed from the transgene were compared in cells overexpressing cyclin C alone with that in cells overexpressing both cyclin C and Cdk8 the mRNA levels were equivalent in the presence or absence of Cdk8 overexpression. However, western analysis revealed a higher level of cyclin C protein in cells which also overexpressed Cdk8 indicative of a post-transcriptional effect (Figure 6B). This is consistent with Cdk8 leading to stabilisation of the cyclin C protein. This increase in levels has consequences for development as the rate of early development is considerably accelerated in overexpressing cells (Figure 7A,B). Overexpression of either cyclin C or Cdk8 individually leads to an acceleration of early development but overexpression of both decreases the time at which aggregates form still further such that the majority (94%) of aggregates had developed a tip by 13 hours of development in the double overexpressing strain compared to 0.5% in the control strain. This accelerated development is confirmed by earlier expression of a gene up-regulated during early development, pkaC (Figure 7C).

Discussion

Since its identification as a component of the RNA polymerase II holoenzyme, Cdk8 has been implicated as both a positive and negative regulator of transcription. Much of this analysis has been conducted in yeast and has demonstrated that Cdk8 is not a global regulator of gene expression but is instead involved in regulating gene subsets involved in meiosis and response to environmental stresses [23,26]. Interestingly, Cdk8 has been implicated in the regulation of metazoan development. Studies have detected localised expression of Cdk8 in zebrafish embryos [27], involvement of Cdk8 in the Notch cell fate signalling pathway [5] and a requirement for Cdk8 during Drosophila leg and eye development [12]. These studies are consistent with analysis in Dicyostelium which demonstrated that Cdk8 deficient strains exhibit defects in the early aggregation stages of development [14,15].

In a number of studies, across a range of organisms, the regulatory partner of Cdk8 has been shown to be Cyclin C [28,29,30]. A bioinformatics approach was used to identify a Dicyostelium orthologue of this protein which contained the cyclin box fold domain and exhibited high levels of identity and similarity to both human and yeast cyclin C proteins. Upon phylogenetic analysis, the putative Dicyostelium cyclin C protein fell into the same clade as cyclin Cs from other organisms suggesting that it shares a more recent common ancestor with these proteins than with other cyclins.

As cyclins are typically regulated by controlling their abundance within the cell, a study was undertaken to determine whether cyclin C was regulated by this mechanism. To achieve this, it was necessary to develop a system to detect the protein when expressed from its endogenous cycC promoter. Detection of the endogenous cyclin C protein was attempted by generating a strain in which an epitope tag was inserted into the endogenous gene. However, although Southern analysis of this strain and RT-PCR analysis were consistent with successful insertion and expression of the resulting mRNA, it was not possible to detect protein by western blot using antisera against the epitope tag, even after attempts to enrich by immunoprecipitation (unpublished observations). This is consistent with the reports of low abundance of the mediator complex [31]. In order to obtain detectable levels of protein it was necessary to overexpress tagged protein from its endogenous promoter, including all the sequence upstream of the cyclin C coding sequence as far as the next gene. Such regions have been shown to contain all the necessary sequences for correct developmental regulation of expression of a number of developmentally regulated genes in Dicyostelium and there have been no reports of other regions being required. Overexpression was achieved by the presence of multiple copies of the plasmid in transformed cells, as a result of selection for neomycin resistance by G418 in Dicyostelium [32]. Overexpression of mRNA was confirmed by Northern analysis as the endogenous transcript was not detected at exposures at which the transgene gave a robust signal. However, on long exposure of developmental time course of Ax2 cells, the endogenous cyclin C mRNA levels showed a similar pattern of decreased expression during development.

As Cdk8 has been implicated in Dicyostelium development, it was hypothesised that cyclin C levels may be altered during this process to control Cdk8 activity. In support of this, CycC-CTAP protein levels were observed to decrease throughout development and further analysis suggested that the decrease was promoted by high levels of extracellular cAMP which mimics conditions found at the mound stage of the life cycle. As Dicyostelium development is a consequence of starvation it could be argued that the reduction in CycC-CTAP levels was a reaction to nutrient depletion similar to that observed in yeast [3]. However, the reduction in CycC-CTAP abundance occurred in response to the developmental signalling molecule cAMP suggesting that it is a genuine developmental regulatory event. Northern analysis revealed that expression of the cycC-CTAP transcript decreased throughout development and in response to high extracellular cAMP. This suggested that CycC-CTAP levels were regulated at least in part by changes in mRNA expression rather than protein stability although additional regulation at this level cannot be excluded. However, when expressed from either its endogenous promoter or that of the actin 15 gene, we always observed cyclin C protein levels to mimic the abundance of the mRNA during both development and in response to stress (Figures 2,3,4 and
Figure 6. Analysis of myc-Cdk8 complexes in the presence and absence of FLAG-CycC. A. Nuclear extracts from 1x10^9 Ax2[myc-cdk8, FLAG-cycC] or Ax2[myc-cdk8] cells (annotated as FLAG-CycC + and - respectively) were fractionated by gel filtration on a Superose 6 column and analysed as described in the legend to Figure 5. B and C. Samples were harvested from vegetatively growing cells and analysed by western (B and C) and northern (C) blot. Western blots samples were analysed and probed with the α-FLAG antibody to recognise FLAG-CycC and against the myc epitope to recognise myc-Cdk8. RNA samples were resolved on a 1% formaldehyde gel, transferred to a nylon membrane and probed with a 32P labelled fragment of the cycC gene. The blot was reprobed with a 32P labelled fragment of the IG7 gene to control for loading. doi:10.1371/journal.pone.0010543.g006
unpublished observations). This would be consistent with reports in human cells that cyclin C protein has a short half life and cyclin C levels may be regulated by changes in gene expression rather than protein stability [33,34]. Unlike in *S. cerevisiae*, we could not find any change in cyclin C levels in response to oxidative stress in *Dictyostelium* under the experimental conditions used.
Size fractionation of myc-Cdk8 and FLAG-CycC complexes indicated that the two proteins were both present in a complex of between 669 KDa and 2 MDa in size, which is similar to the reported size of the many Cdk8 and cyclin C containing mediator complexes that have been isolated from other organisms. During late development, cells responded to high extracellular CAMP, the proportion of Cdk8 associated with the high molecular weight complex decreased. Although these events correlated with the decrease in cyclic-CTAP levels, additional analysis hinted that cyclin C abundance may not be the only factor which controls association of Cdk8 with the large protein complex: When a cyclin C protein was overexpressed in the same cells as overexpressed myc-Cdk8 it was found that there was no increase in the level of myc-Cdk8 it was found that there was no increase in the level of cyclin C abundance may not be the only factor which controls association of Cdk8 with the complex is regulated by additional signalling pathways and that the level of cyclin C is not rate limiting.

In human cells it has been demonstrated that, unlike other cyclins, cyclin C is stabilised by an interaction with the Cdk8 protein [35]. Consequently, it was predicted that if the Dictyostelium Cdk8 and CycC proteins are functional partners then the levels of cyclin C would be higher in cells which also expressed the myc-Cdk8 protein. Western blotting indicated that this was the case, while northern analysis confirmed that this was not due to transcriptional regulation of the act15 promoter from which both proteins were expressed. Increasing the levels of either cyclin C or Cdk8 individually was sufficient to increase the rate of early development, though overexpression of both led to a greater acceleration. This is consistent with the levels of the cyclin C and Cdk8 being rate limiting for early development, although the proportion of Cdk8 found in the high molecular weight complex did not change on co-expression of cyclin C.

In conclusion we report a decrease in cyclin C levels in response to extracellular developmental signal working through a cell surface receptor. These experiments demonstrate the importance of Cdk8 and cyclin C in controlling developmental processes, and that the levels of Cdk8 are rate-limiting for early development. Understanding the signalling events controlling the levels of these proteins and their association with other complex members is an important aspect of regulation of transcriptional events during development and differentiation.

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Author Contributions

Conceived and designed the experiments: DMG GP. Performed the experiments: DMG DWH. Analyzed the data: DMG GP. Wrote the paper: CP.

References

1. Conaway RC, Sato S, Tomomori-Sato G, Yao T, Conaway JW (2005) The mammalian Mediator complex and its role in transcriptional regulation. Trends Biochem Sci 30: 230–250.
2. van de Peppel J, Kettelarij N, van Bakel H, Kockelkorn TT, van Leenen D, et al. (2005) Mediator expression profiling epitope reveals a signal transduction pathway with antagonistic submodules and highly specific downstream targets. Mol Cell 19: 511–522.
3. Cooper KC, Mallory MJ, Strick R (1999) Oxidative stress-induced destruction of the yeast C-type cyclin Ume3p requires phosphorylationin specific post-translational modification. Nature 357: 547–551.
4. Friesen R, Bass AJ, Kim SY, Dunn IF, Silver SJ, et al. (2008) CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. Nature 454: 2499-456.
5. Flier CJ, White JR, Jones KA (2004) Mastermind recruits CycC/Cdk8 to Phosphorylate the Notch ICD and Coordinate Activation with Turnover. Mol Cell 16: 509-520.
6. Hirn M, Kobor MS, Kuriakose N, Greenblatt J, Sadowski I (1999) GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase dbrl/Cdk8. Mol Cell 3: 673–678.
7. Chi Y, Huddleston MJ, Zhang X, Young RA, Anam RN, et al. (2001) Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. Genes Dev 15: 1078–1092.
8. Nelson C, Gosto S, Lund K, Huang W, Sadowski I (2003) Srb10/Cdk8 regulates yeast filamentous growth by phosphorylating the transcription factor Sc12. Nature 421: 167–190.
9. Yarda A, Kostue H, Okano H, Sasah H (2003) Components of the transcriptional Mediator complex are required for asymmetric cell division in D. melanogaster. Development 132: 1885–1893.
10. Treiman J (2001) Drosophila homologues of the transcriptional coactivator complex subunits TRAP240 and TRAP230 are required for identical processes in eye-antennal disc development. Development 128: 603-615.
11. Janody F, Martinez-Rey Z, Benali A, Treisman JE (2003) Two subunits of the Drosophila mediator complex act together to control cell affinity. Development 130: 3691–3701.
12. Leuch S, Rehms M, Joulia L, Boscohier C, Werner M, et al. (2007) Distinct roles for Mediator Cdk8 module subunits in Drosophila development. Embry Dev 28: 1045-1054.
13. Wang W, Chen H (2009) HUA ENHANCER3 reveals a role for a cyclin-dependent protein kinase in the specification of floral organ identity in Arabidopsis. Development 133: 3147–3156.
14. Lin HH, Khosla M, Huang HJ, Hsu DW, Michaelis C, et al. (2004) Cyclin-dependent kinase-3 (GSK-3) is regulated during Dictyostelium development via the serpentine receptor cAR3. Development 128: 925–933.
15. Hopper NA, Wang J, Byrnes L, Veron M, Williams JG (1995) Activation of the prespore and spore cell pathway of Dictyostelium differentiation by cAMP: negative selective screening for genic activity. Nature 374: 193–196.
16. Knetsch ML, Tsiavaliaris G, Zimmermann S, Ruhl U, Maglione DJ (2002) Dissecting the regulatory circuitry of a eukaryotic genome. Cell 94: 721–728.
17. Kay RR (1989) Evidence that elevated intracellular cyclic AMP triggers spore maturation in Dictyostelium. Development 105: 753–759.
18. Kay RR, Veron M, Williams JG (1994) Glycogen synthase kinase-3 (GSK-3) is regulated during Dictyostelium development via the serpentine receptor cAR3. Development 126: 301–308.
19. Knetsch ML, Tsiavaliaris G, Zimmermann S, Ruhl U, Maglione DJ (1995) Identification of the prespore and spore cell pathway of Dictyostelium differentiation by cAMP: negative selective screening for genic activity. Nature 374: 193–196.
20. Katoch B, Begum R (2003) Biochemical basis of the high resistance to oxidative stress in Dictyostelium. J Biosci 28: 501–508.
21. Kinsey MT, Meyer KD, Berney C, Taatjes DJ (2009) The human CDK8 subcomplex is a molecular switch that controls Mediator coactivator function. Genes Dev 23: 439–451.
22. Cooper KC, Serich R (2002) Saccharomyces cerevisiae C-type cyclin Ume3p/ Srb10p is required for efficient initiation and execution of mitotic development. Eukaryot Cell 1: 66–74.
23. Holstege FC, Jennings EG, Wyrick JJ,.el al. (1998) Dissecting the regulatory circuitry of a eukaryotic genome. Cell 95: 717–729.
24. Hiraoka H, Harte RB, Carton MW, Grewal IY, Bums L (2002) Cyclin-dependent kinase-3 (GSK-3) is regulated during Dictyostelium development via the serpentine receptor cAR3. Development 128: 925–933.
25. Hoppner NA, Wang J, Byrnes L, Veron M, Williams JG (1995) Activation of the prespore and spore cell pathway of Dictyostelium differentiation by cAMP: negative selective screening for genic activity. Nature 374: 193–196.
33. Oh C, Choi YJ, Kim HG, Lee DH (2006) Osmosensitive gene expression of taurine transporter and cyclin C in embryonic fibroblast cells. Adv Exp Med Biol 583: 49–57.

34. Katona RL, Loyer P, Roby SK, Lahti JM (2007) Two isoforms of the human cyclin C gene are expressed differentially suggesting that they may have distinct functions. Acta Biol Hung 58: 133–137.

35. Barette C, Jariel-Encontre I, Piechaczyk M, Piette J (2001) Human cyclin C protein is stabilized by its associated kinase cdkl8, independently of its catalytic activity. Oncogene 20: 551–562.

36. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, et al. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31: 3497–3500.