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Extra Views

Selective chemical inhibition as a tool to study Cdk1 and Cdk2 functions in the cell cycle.

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Abstract

Cyclin-dependent kinases are highly conserved among all eukaryotes, and have essential roles in the cell cycle. However, these roles are still only poorly understood at a molecular level, partly due to the functional redundancy of different Cdk complexes. Indeed, mice knockouts have even thrown into some doubt the assumed essential roles for Cdk2-cyclin E in triggering S-phase, but this is almost certainly due to compensation by Cdk1 complexes. By combining both knockout approaches and chemical Cdk inhibition in Xenopus egg extracts, we have shown that one reason for functional redundancy of Cdk control of S-phase is that Cdk activity required to trigger S-phase is very low. Cdk1 contributes to this activity even in the presence of Cdk2, and Cdk activity at this stage does not show "switch-like" regulation, as at the onset of mitosis. It is important to try to confirm and extend these findings to other cell-types, and to explain why different cells might have evolved different requirements for Cdk activity. In this paper, we present data that suggest that selective chemical Cdk inhibition will be a useful tool towards achieving this goal.

The models for Cdk control of the cell cycle have evolved over the years, starting with the initial dramatic discovery that Cdc2 is essential for triggering both mitosis and DNA replication in fission yeast,¹ and that it is a protein kinase². After the discovery that oocyte maturation promoting factor was a homodimer consisting of one molecule of Cdk1 and one cyclin B molecule,³ and that Cdc2 is activated by cyclin-like molecules,^{4,5} the obvious hypothesis became that different roles of Cdk1 might be determined by different cyclin subunits, the prototype "mitotic cyclins" and "G1 cyclins".⁶ Rescue of G1 cyclin mutants in budding yeast by the animal cyclin C, D and E family⁷ tended to agree with this theory, especially given the discovery that cyclin E is rate-limiting for S-phase onset in mammalian cells⁸ and the requirement for cyclin E for S-phase onset in larval cell cycles of drosophila.⁹ However, around the same time, Eg1 (Xenopus Cdk2) was identified as a Cdc2-related protein kinase¹⁰⁻¹² followed shortly after by a plethora of Cdk-like kinases in mammals.¹³ It began to seem that animals had evolved a requirement for Cdks highly homologous to Cdk1 (Cdk2 in all animals, and additionally Cdk3 in mammals) and other cyclins, to orchestrate the G1/S transition and initiation of DNA replication, reserving the presumed ancestral role of Cdk1 and B-type cyclins to the control of cell division.^{10, 14} But once again, this simple model of division of labour between Cdk1-mitotic cyclins and Cdk1/2/3-G1 cyclins was somewhat confounded by genetic studies in yeast. Although G1 cyclins are present in all eukaryotes and the classes are well conserved among metazoans, in fission yeast, the G1-S promoting roles of cyclins of previously unknown function could only be demonstrated in the absence of the mitotic cyclin B, since a single mitotic Cdk-cyclin complex can trigger both DNA replication and mitosis.¹⁵ Similarly, in budding yeast, the mitotic CLB1-4 cyclins can compensate for an absence of the S-phase promoting CLB5/CLB6 duo.¹⁶ The "quantitative" hypothesis was thus born, in which phosphorylation of different substrates at S-phase onset and mitosis could result from quantitative properties of Cdk-cyclin complexes (different affinities for substrates and different kinetic parameters) or, even more simply, different expression patterns of cyclins, rather than absolute specificities between kinase and substrate.^{15, 17} The differential temporal expression patterns of cyclins in both yeast and mammals were indeed demonstrated soon after their discovery.¹⁸ As for

kinetic parameters, Loog and Morgan¹⁹ recently investigated this hypothesis using a yeast system and found that the S-phase Clb5-Cdk1 complex has higher affinity (lower Km) for putative S-phase substrates than the mitotic complex Clb2-Cdk1, and vice-versa, although both kinases could indeed phosphorylate most substrates in vitro.

Results of disrupting cyclin function in different animals have yielded conceptually similar results to G1 cyclin knockouts in yeast.²⁰ Cells can cycle in cyclin D mutants in drosophila, after cyclin D or cyclin E RNAi or mutation in nematodes, and in mice mutant for both E cyclins, or all three D-type cyclins. The majority of phenotypes from Cdk and cyclin knockout animals suggest that Cdk roles evolved to allow fine control over the cell cycle, and possibly other essential cellular processes, during growth and development of complex organisms. However, it is starting to become clear that the whole story cannot emerge from knockout approaches. For example, mice lacking Cdk2 are healthy^{21, 22} even though Cdk2 clearly plays an important role in regulating DNA replication, probably in all cells. It is now evident that this is because (as expected from the quantitative model) Cdk1 can compensate for Cdk2 loss in most cell types.^{23, 24} Indeed, in mice only Cdk1 is essential for the cell cycle: *ie* it has roles that cannot be fulfilled by other Cdks.²⁵ However, there are specific requirements for Cdk2 in some cell types. For example, Cdk1 cannot compensate for loss of Cdk2 in the gonadal lineages in mice, and whereas Cdk2 is apparently non-essential in most cancer cell lines, it is required for proliferation of melanoma cells.²⁶ Cdk2 also has important roles in mitosis,²⁷ S-phase histone gene transcription,²⁸ centrosome duplication,^{29, 30} and other processes. However, to what extent these roles are conserved in different cell types, and which kinase takes over in the absence of Cdk2, is not known.

To investigate functional redundancy of Cdk promotion of S-phase onset in a simple vertebrate system (in which, for example, there is no requirement for transcription) we have recently described a comparison between chemical inhibition and knockdown approaches to discern the roles of Cdks in DNA replication.³¹ In this study we were able to use differential affinities of Cdk1 and Cdk2 for a chemical inhibitor to selectively inhibit Cdk2 but not Cdk1 during a physiological process, demonstrating the complementary nature of "knockout" and chemical inhibition strategies for determining protein function. This allowed us to demonstrate a role for Cdk1 in embryonic DNA replication, even in the presence of Cdk2. We also showed that Cdk activity requirements for initiating at replication origins are vanishingly low. At low activity levels, there seemed to be a direct relationship between Cdk activity, the proportion of pre-replication complexes (pre-RCs) which are converted to pre-initiation complexes (pre-ICs), and the number of replication origins which fire. If the same holds for somatic cell cycles, this might partly explain why siRNA approaches to eliminating Cdk1 and Cdk2 generally have little effect on S-phase onset (for example,^{23, 32}), *ie* the small amount of remaining kinase might be sufficient. Additionally, we found one reason why Cdk2 may be more important for regulating DNA replication in an embryonic system than Cdk1: it seems to be a better promoter of activation of clusters of replication origins. One caveat is that because Cdk1 protein levels are about ten times higher than Cdk2 levels, Cdk1 is much harder to quantitatively deplete than Cdk2. Given the apparent extremely low kinase activity which is required to fire replication origins, trace amounts of Cdk1 might still be physiologically important. Thus, apparent differences between ability of Cdk1 and Cdk2 to regulate replication might still be due to their different relative abundance. Selective chemical inhibition of Cdk1 should throw some light on this question, and we have been attempting this approach (see below). However, there will undoubtedly turn out to be functional differences between Cdk1 and Cdk2 in the control of initiation of DNA replication, the understanding of which will require a detailed description of the kinetics of phosphorylation of respective substrates of different Cdk complexes throughout the cell cycle. At present such a "holistic" understanding of Cdk function is completely lacking.

Nevertheless, chemical Cdk inhibitors are useful tools for understanding roles of Cdk complexes within cells, since the inhibition is essentially instantaneous, and the genes or proteins targeted are still present. As such they would be expected to maintain their interactions with protein substrates and regulators, or put more simply, occupy the same binding sites as when active. This might lead to a very different picture from that where genes or proteins are "knocked down" or "out". The simple substitution of one protein's functions by another might no longer be possible, and the "real picture" should thus emerge. Indeed, due to intense pharmaceutical interest (with a view to cancer chemotherapy) several hundred small-molecules have been found to be active against Cdks, some of them with inhibitor constants (Ki) in the nanomolar (nM) range, and

over 100 crystal structures of Cdk2 complexed with such inhibitors are present in the National Center for Biotechnology Information protein database (PDB). All of these structures show the inhibitor to bind at the ATP-binding site of Cdk2, thereby competing with ATP. However, ATP binding sites are highly conserved among the 518 kinases encoded in the human genome, and the specificity of many of these inhibitors is unknown. Recently, Cohen and coworkers have analysed in vitro specificity of 65 kinase inhibitors (including Cdk inhibitors) and concluded that most were rather aspecific.³³ Furthermore, apparent K_i may be misleading when one considers specificity, a fact usually not addressed in scientific literature. For example, staurosporine has K_i of <10 nM for Cdk1-cyclin B and Cdk2-cyclin A, compared with around 10 μ M for Cdk4-cyclin D1. One might suggest therefore that staurosporine is specific for Cdk1 and Cdk2 when compared to Cdk4. Yet the affinity of the latter for ATP is at least 100-fold lower than that of Cdk1 or Cdk2 (Michaelis constant, or K_m , for ATP is in the millimolar range for Cdk4-cyclin D, compared to micromolar for Cdk1 and Cdk2), and it is therefore highly likely that staurosporine will also be a good inhibitor of Cdk4. Finally, the millimolar in vivo concentrations of ATP often mean that high doses of inhibitors must be used to effectively inhibit the kinase in cells or cell extracts. As such, experiments involving chemical inhibition of protein kinases must be interpreted with a great deal of caution. Thus, in our study, we originally intended to use Nu6102 as a potent inhibitor of both Cdk1 and Cdk2, as described,³⁴ to reinforce our results using depletion approaches in which both Cdk1 and Cdk2 appeared to be involved in DNA replication. However, Nu6102 treatment of the extracts at doses which completely inhibit Cdk2 gave quantitatively very similar results to depleting Cdk2: in the presence of the inhibitor, DNA could still replicate, albeit considerably more slowly, but certainly much more efficiently than if both Cdk1 and Cdk2 were depleted. By investigating different ATP/inhibitor ratios, we found that Nu6102 could in fact be used as a "specific" inhibitor of Cdk2, not significantly inhibiting Cdk1 in the conditions of the extract.

We have now performed similar experiments with another Cdk inhibitor, RO3306,^{35, 36} which is selective for Cdk1 (K_i 35nM for Cdk1-cyclin B) compared with Cdk2 (K_i 340nM for Cdk2-cyclin A). We compared and combined RO3306 inhibition with Nu6102 inhibition in *Xenopus* egg extracts. We confirmed that RO3306, while it can inhibit both kinases at certain concentrations, is selective for *Xenopus* Cdk1 rather than Cdk2 at an inhibitor/ATP ratio of 1:50 (figure 1a), which in egg extracts (which contain an ATP regenerating system and 2mM ATP) would translate to an inhibitor concentration of 40 μ M. At this dose Cdk1 should be completely inhibited whereas Cdk2 should retain at least 50% activity. Since we found in our recent study that immunodepletion of all detectable Cdk1 caused only a modest (but reproducible) delay of DNA replication, as did depletion of at least 80% of both Cdk1 and Cdk2, we expected that 40 μ M RO3306 should have less effect than completely inhibiting Cdk2 with 100 μ M Nu6102. Interestingly, however, RO3306 alone at 40 μ M allowed only 20-30% of DNA to be replicated in the time course (figure 1b, c), at least comparable to the effect of specifically inhibiting Cdk2 with Nu6102. RO3306 treatment showed either only slight synergy with Cdk1 depletion (figure 1b), or no synergy at all (figure 1c), whereas Nu6102 treatment combined with Cdk1 depletion was strongly synergistic (figure 1c). This raises the possibility that Cdk1 is actually much more important for DNA replication, even in the presence of Cdk2, than we previously thought, and that the high concentrations of Cdk1 and the low Cdk activities required for DNA replication might have prevented demonstration of such a role for Cdk1 by depletion approaches. However, it is possible that Cdk2 activity might become much more limiting if Cdk1 is efficiently inhibited, and in this case the expected 50% decrease in Cdk2 activity might be much more significant than we would have predicted from our depletion experiments. As expected, however, RO3306 was also synergistic with Cdk2 depletion or inhibition (figure 1b), but even when Cdk2 depletion is combined with RO3306 inhibition, or when extracts are treated with both Nu6102 and RO3306, up to 10% of DNA can still be replicated during the time course (figures 1b, 1c). This not only confirms that only relatively very low Cdk activities are required for DNA replication, it also suggests that it is extremely difficult to prevent initiation of DNA replication by chemical inhibition strategies. This might have important consequences for Cdk inhibitor-based therapies.

One obvious further question is whether the cell-type influences Cdk requirements for DNA replication. *Xenopus* egg extracts represent a model embryonic system, in which the S-phase checkpoint is inactive, there is no transcription, DNA replicates without specific replication origins and much smaller replicons than somatic cells (5-15kb compared with 50-300kb). However, somatic cell nuclei replicate differently from sperm nuclei in *Xenopus* egg extracts, and we

therefore considered that it might still be possible to test this question using such extracts. In *Xenopus*, erythrocytes are terminally differentiated but nucleated, and their replication in egg extracts is much slower than sperm nuclei due to far greater spacing of replication origins.³⁷ Since origin firing efficiency seems proportional to Cdk activity at low levels, we thought that perhaps the relatively much lower origin usage of erythrocyte nuclei compared to sperm nuclei might mean even greater indifference to Cdk2 inhibition. Surprisingly, we found the opposite. In the same extracts, whereas for sperm nuclei Cdk2 inhibition by Nu6102 reduces DNA replication efficiency from 100% to around 40%, it almost completely abolishes the replication of erythrocyte nuclei (figure 2), suggesting that Cdk1 cannot compensate in this situation. Since both the biochemical environment and the genome which is replicated are the same with both sperm and erythrocyte nuclei, one possibility might be that the chromatin state of erythrocyte nuclei determines their requirement for Cdk2. Indeed, chromatin is probably remodelled prior to S-phase, as histone acetylation³⁸ is strongly upregulated at the G1/S phase transition, at least in somatic cells. Furthermore, Cdk2 can promote histone acetyltransferase (HAT) activity of p300/CBP.³⁸ We therefore propose that Cdk2 might have roles in remodelling chromatin to render it replication competent, which are less rate-limiting for sperm nuclei than erythrocyte nuclei. Inhibition of HATs by anacardic acid (AA) in *Xenopus* extracts strongly inhibits replication of sperm nuclei³⁷ although it does not affect replication rate of erythrocyte nuclei, probably since in the latter, other chromatin features are rate-limiting and HATs have ample time to execute their functions. One might imagine, then, that both HATs should act prior to initiation of S-phase, and that HAT inhibition might sensitize sperm nuclei replication to Cdk2 inhibition. This indeed seems to be the case: effects of both AA and Nu6102 are remarkable only prior to addition of sperm nuclei, at which point the two inhibitors act synergistically to inhibit replication (figure 3A). However, if nuclei are incubated 20 minutes in the extract, the addition of either inhibitor alone now has little or no effect on replication efficiency. Interestingly, though, simultaneous inhibition of Cdk2 and HATs still has a strong synergistic effect (figure 3B). Nevertheless, HATs can act even if Cdk2 is inhibited. If chromatin purified from extracts containing Nu6102 is transferred to recipient extracts without Nu6102 but containing AA, DNA replication is intermediate between AA->AA extract transfers (which do not replicate) and AA-> control extract transfers (which replicate normally). The converse is also true: Cdk2 can act to promote DNA replication in the presence of AA, since transfer of chromatin from an AA extract to an Nu6102 extract shows replication efficiency intermediate between Nu6102->Nu6102 and Nu6102-> control transfers (figure 3C). Therefore, we suggest that both HATs and Cdk2 may act together to remodel chromatin prior to initiation of DNA replication. However, many more studies will clearly be necessary to unequivocally demonstrate that Cdk2 action to promote DNA replication involves chromatin remodelling, whether or not Cdk1 can compensate for an absence of Cdk2 activity, and what, exactly, Cdks phosphorylate to accomplish this. Another question requiring attention is to what extent cell-type specific requirements for Cdks might depend on their chromatin states.

These results led us to wonder whether we could use similar chemical inhibition approaches to selectively inhibit one Cdk in other cell models, to address similar questions. Firstly, might Cdk1 also be involved in DNA replication in somatic cells, even in the presence of Cdk2? Secondly, given that differentiated nuclei seem to show an absolute requirement for Cdk2 for replication in *Xenopus* egg extracts, we speculated that chemical inhibition might reveal a requirement for Cdk2 for DNA replication in somatic cells, which, due to results of knockdown and knockout experiments, has lately become somewhat controversial. Since we found that, like *Xenopus* Cdks, human Cdk1 is much less sensitive to Nu6102 than human Cdk2,³¹ it might be possible to find conditions which inhibit Cdk2 but allow Cdk1 function. Furthermore, in somatic cells Cdk2 has been implicated in centrosome duplication, histone gene transcription, and maintenance of the mitotic state. Selective chemical inhibition might reveal for which of these processes Cdk2 is essential, and for which other Cdks might compensate. We decided to use primary human fibroblasts (HDF) as a physiological model cell line. Treatment of HDF with different concentrations of Nu6102 suggests that there is a dose-dependent delay in passage through mitosis as well as through S-phase (figure 4a). Delay of mitosis might not be a direct effect due to Nu6102 inhibition of Cdk1, but could be a checkpoint-dependent result of delaying S-phase. Time-lapse video-microscopy indeed suggests that this is the case: at 20 μ M Nu6102, cells are delayed on entry into mitosis compared to control cells, but whereas some cells complete mitosis and divide, most cells appear to complete mitotic division but cannot accomplish

cytokinesis, and many become binucleated (figure 4b, c). We also observed many cells with abnormal centrosome number and problems of chromosome congression at the metaphase plate, resulting in formation of multi-lobed nuclei and micronuclei (figure 4d). If Cdk1 were inhibited one would expect an arrest at the G2/M transition, as with the selective Cdk1 inhibitor, RO3306.^{35, 36} The primary phenotype of Nu6102 therefore seems to be a delay in passage through S-phase, cells can then enter into and progress through mitosis, but intriguingly, often cannot correctly undergo cytokinesis. One is tempted to interpret this as evidence that, in primary cells, Cdk2 inhibition primarily delays DNA replication, but also that Cdk2 activity might be required for normal progression through mitosis. The cytokinesis defect could be direct or indirect, as defects in function or structure of centrosomes (whose duplication is regulated by Cdk2) prevent successful cytokinesis.³⁹ However, one clearly must investigate how Nu6102 affects Cdk1 activity in this situation, and whether or not restoration of Cdk2 function will restore a normal cell phenotype. Interestingly, chemical inhibition of Cdk4 leads to a similar (though milder) phenotype in cancer cell lines.⁴⁰ A careful comparison of selective Cdk1, Cdk2 and Cdk4 inhibition in different cell types would certainly be a worthwhile endeavour. We are therefore currently investing energy into developing conditions with different inhibitors which discriminate as much as possible between these Cdks, and the effects of such conditions on the cell cycle of different cell-types.

Materials and methods

Xenopus

Extract preparation, immunodepletion, replication assays and chemical inhibition procedures were all as recently described.³¹ RO3306 was purchased from Axxora biochemicals. For chromatin transfer experiments, nuclei were purified from 25 μ l extract diluted 20 fold in CPB,³¹ centrifuging through a 1ml 0.7M sucrose cushion in CPB for 5 minutes at 6000g, 4°C, and resuspending the pellet directly into the recipient extract.

Fibroblasts

Early passage neonatal human foreskin diploid fibroblasts, their general culture conditions and method of synchronisation have all been described.⁴¹ For immunofluorescence, cells were fixed with 100% cold ethanol for 5 minutes, followed by washes in PBS and block in PBS / 2% BSA / 0.05% Triton X-100 for one hour. Primary antibodies, anti-gamma tubulin (SIGMA, clone GTU-88) and anti-beta tubulin, were incubated for one hour, followed by five washes in PBS / 2% BSA / 0.05% Triton X-100. All secondary antibodies were AlexaFluor conjugates and used according to manufacturer's instructions (Molecular Probes).

All images were taken on an upright microscope (Leica DMRA) using Metamorph 6.2.6. (Molecular Devices) software.

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Figure legends

1. Cdk1 Inhibition by RO3306 inhibits DNA replication in *Xenopus* extracts and is synergistic with Cdk2 inhibition or depletion.

A. Cdk1 and Cdk2 were immunoprecipitated from 10 μ l interphase egg extract (IEE) and used in a histone H1 kinase assay, with different concentrations of RO3306, at a constant concentration of 50 μ M ATP. B. Mock, Cdk1 or Cdk2 depleted(Δ) extracts were treated with DMSO control or 40 μ M RO3306, and percentage of input sperm nuclei DNA replicated at the indicated times was determined by ³³P-dCTP incorporation, as described.³¹ C. As B., but a comparison of the effects of 40 μ M RO3306 or 100 μ M Nu6102 on a time course of DNA replication in mock or Cdk1 depleted extracts.

2. Nuclei of differentiated cells have different Cdk2 requirements from sperm nuclei for DNA replication in *Xenopus* extracts.

Xenopus sperm nuclei or erythrocyte nuclei were incubated in IEE at a concentration of 1400/ μ l extract including either 100 μ M Nu6102 or equivalent volume of DMSO, and replication assays undertaken as above.

3. In Xenopus extracts Cdks and HATs both act early in S-phase, their chemical inhibition is synergistic with respect to DNA replication, but they can act independently.

A. Time course of DNA replication of sperm nuclei in IEE treated either with 100 μ M Nu6102, 300 μ M anacardic acid, or both. B. As A., but inhibitors were added 20 minutes after nuclei. B. Licensed chromatin from control, Nu6102 or anacardic acid-treated extracts was purified at 30 minutes through a sucrose cushion and resuspended in a control, Nu6102 or anacardic acid-treated recipient extract. Standard DNA replication time-courses were then conducted.

4. Nu6102 treated primary fibroblasts show delay in S-phase and mitosis onset, coupled with abnormal centrosome number, defective cytokinesis and nuclear morphology

A. Asynchronous HDF were treated with DMSO control or different concentrations of Nu6102 for 24h. Cells were fixed at 8h and 24h, stained with propidium iodide and analysed by flow cytometry. B. and C. HDF were synchronised by serum starvation and release, and followed by time-lapse video-microscopy, in the absence or presence of 20 μ M Nu6102. A time-lapse sequence of mitosis of Nu6102 treated cells is shown in B., and mitotic indices are represented graphically in C. Fusion % represents percent of cells which undergo mitosis but in which cytokinesis is defective, resulting in subsequent cell fusion. D. Asynchronous control or Nu6102 (20 μ M) treated cells were fixed at 24h for immunofluorescence with beta-tubulin (red), gamma-tubulin (green) to visualise centrosomes, or Hoechst (blue) to visualise DNA; typical overlays are shown. Right two panels show enlargements of the centrosomes and nucleus of an Nu6102 treated cell (boxed area in lower left panel).

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