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A Quantitative Comparison of Wild-Type and Gatekeeper Mutant Cdk2 for Chemical Genetic Studies with ATP Analogues

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Chemical genetic studies with enlarged ATP binding sites and unnatural ATP analogues have been applied to protein kinases for characterisation and substrate identification. Although this system is becoming widely used, there are limited data available about the kinetic profile of the modified system. Here we describe a detailed comparison of the wild-type cdk2 and the mutant gatekeeper kinase to assess the relative efficiencies of these kinases with ATP and unnatural ATP analogues. Our data demonstrate that mutation of the kinase alters neither the substrate specificity nor the phosphorylation site specificity. We find comparable K_M/V_{max} values for mutant cdk2 and wild-type kinase. Furthermore, F80G cdk2 is efficiently able to compensate for a defective cdk in a biological setting.

Introduction

The study of protein kinases and the elucidation of the pathways involved in cell regulation is key to our understanding of both healthy and diseased states. Small molecule inhibitors have become widely used to infer the requirement of a particular kinase in a specific phosphorylation event or pathway. Most kinase inhibitors bind to the ATP binding site of their targets. However, given that there are over 500 different protein kinases in the human genome that all share the same conserved ATP binding site,[1] it is often difficult to precisely state that a particular phenotype is due to one particular kinase. Knockout/knockdown technologies are useful to circumvent this problem, but can cause secondary problems such as the upregulation of related proteins to compensate for the missing activity (such as cdk1 compensating in cdk2 knockouts[2,3]).

In 1997 the Shokat lab pioneered a chemical genetic approach to analyse protein kinase function.[4] In this study a mutation was used to enlarge the ATP binding site of v-Src; this enabled the kinase to accept a [γ-^{32}P]ATP analogue with a bulky addition to the N^6 position. This approach provided almost complete selectivity for the mutant v-Src over cellular kinases (Figure 1 A). The same group then went on to show that selective inhibitors of the enlarged ATP binding site can be used to the same effect (Figure 1 A) with the advantage that many are cell permeable and hence can be used with living cells and organisms.[5] The specific residue mutated in order to enlarge the ATP binding site was termed the "gatekeeper" residue[6] and is conserved in many protein kinases and makes the approach widely applicable.[7,8] These techniques have subsequently been used to study a wide variety of kinases[9–11] and have led directly to the identification of some novel kinase substrates.[12,13]

Whilst the chemical genetic approach outlined above is attractive (and is indeed being utilised with increasing frequency), there is little quantitative data available for the serine/threonine protein kinases. Studies on mutant c-Src tyrosine kinases show that binding of an N^6-modified ATP analogue does not alter the phospho-acceptor binding site, nor did it affect the site specificity of a range of peptide targets.[14] Mutation of c-Src to the analogue sensitive kinase not only allowed the N^6-modified ATP to be utilised, but the N^6-modified ATP was a much more efficient phosphate donor than native ATP.[15] With respect to serine/threonine kinases, there is limited kinetic data already published with N^6-modified ATP analogues.[16] In addition, recent work has demonstrated that substitution at the gatekeeper residue can have profound effects on kinase catalytic efficiency.[17] In order to clarify these issues

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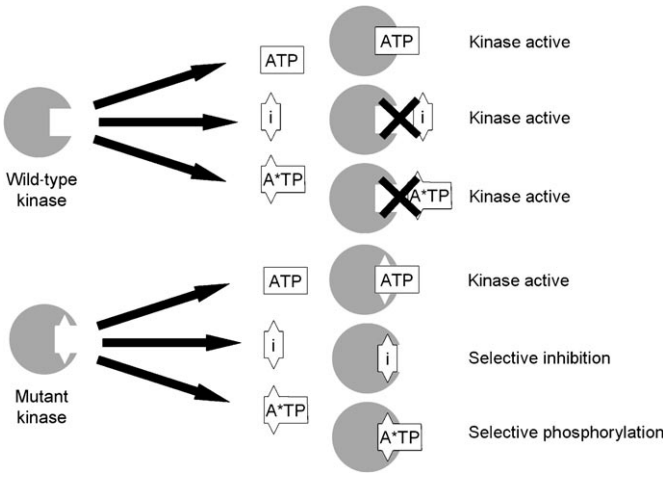

Figure 1. ATP-based chemical genetic approaches to protein kinases. Wild-type protein kinases accept ATP as a phosphate donor but not sterically enlarged inhibitors (shown as i) or ATP molecules (shown as A*TP). By enlarging the ATP binding domain the mutant kinase is still able to utilise ATP as a phosphate donor but is also able to bind sterically enlarged molecules that utilise the enlarged domain such as bulkier inhibitors and ATP analogues.

with respect to serine/threonine kinases, we have performed experiments to quantitatively compare the kinetic parameters of wild-type cdk2 with the analogue sensitive mutant F80G cdk2[16, 18, 19] and have assessed a range of ATP analogues and a variety of known substrates. Although some kinetic evaluation has been performed with wild-type and analogue-sensitive v-Src and Cdk2/cyclin E previously[16] our work here substantially expands on this by examining multiple protein substrates and ATP analogues and quantitatively comparing the kinetics of two cdk2 holoenzymes with distinct cyclin subunits.

Results

Generation of mutant Cdk2 and ATP analogue pairs

Gatekeeper mutations can be used to enlarge the active site of protein kinases to enable them to use analogues of ATP (see Figure 1). Analysis of the crystal structure of cdk2 bound to ATP[20] identified three amino acids which, when mutated, might allow bulky groups on the N^6 position of ATP to access the hydrophobic pocket behind the gatekeeper residue. These three amino acids were V64, F80 and L134 (Figure 2). Mutant kinases were prepared containing V64S and/or F80G and/or L134A mutations. Analysis of the activities of these kinases with ATP variants showed that V64 and L134 substitutions were largely inactive whilst the F80G mutant kinase retained substantial catalytic activity (data not shown and Figure 3). The F80 residue is equivalent to the gatekeeper site identified by Shokat et al.[7] and further studies were carried out using the F80G mutant cdk2.

An initial screen was undertaken to find N^6-modified ATP analogues that could be utilised as a phosphate donor by the F80G cdk2 but not by wild-type cdk2. Initial investigations utilised two known substrates of cdk2/cyclin E1: Rb and $p27^{Kip1}$ (hereafter p27). A phospho-specific antibody was used to observe p27 phosphorylation and a gel shift assay was used to measure retinoblastoma protein (Rb) phosphorylation, as hyperphosphorylated Rb migrates with a retarded mobility in SDS PAGE when compared to un/hypophosphorylated Rb (Figure 3). Of the N^6-modified ATP analogues screened, two were effective phosphate donors and selective for the F80G cdk2 mutant; N^6-(cyclohexyl)ATP (CxATP) and N^6-(cyclopentyl)-ATP. The smallest cyclic adduct tested, N^6-(cyclopropyl)ATP, could be used by both wild-type and F80G cdk2, albeit to a lesser extent by the wild-type kinase (Figure 3). The largest cyclic adduct tested, N^6-(cyclooctyl)ATP, was selective over wild-type kinase/ATP in combination with the substrate p27 but, as no hyperphosphorylation was observed with Rb, it was concluded to be a poorer phosphate donor than the cyclopentyl and cyclohexyl variants. N^6-(pyrrolidinyl)ATP was inactive as a phosphate donor both with the F80G mutant and the wild-type kinase.

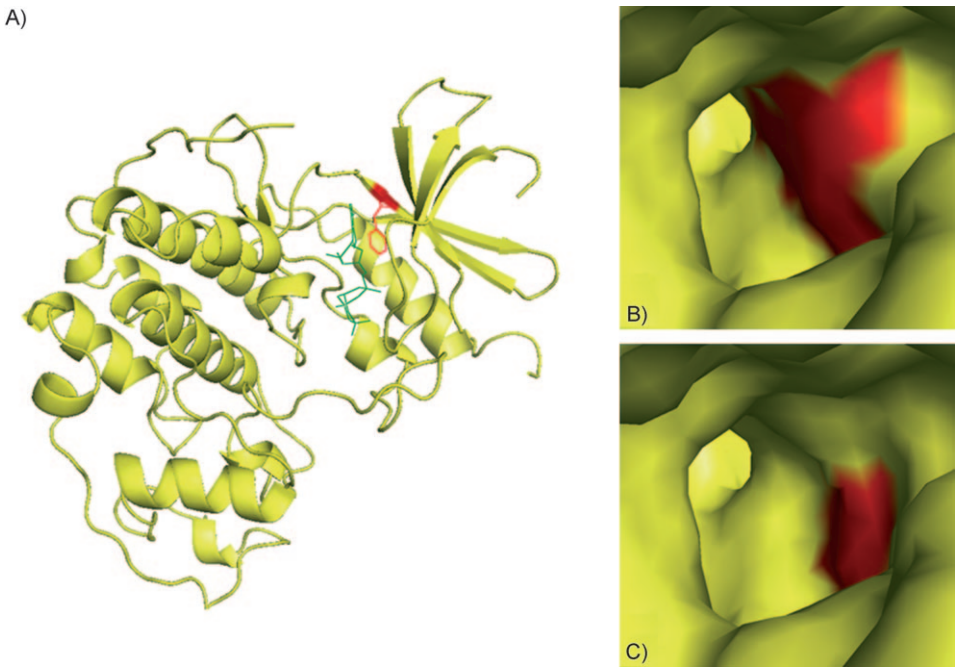

Figure 2. Mutation of the ATP binding domain in cdk2. A) ATP binding site of cdk2 that highlights the residues that interact with the N^6 region of ATP. V64 is highlighted in blue, F80 in red and L134 in green. B) A close up of the ATP binding domain showing the F80 residue in red. C) Mutation of the F80 residue to glycine is shown in red. Enlargement of the hydrophobic pocket is shown. Images were created using PyMOL software, based on coordinates from protein data bank 1HCK.[37]

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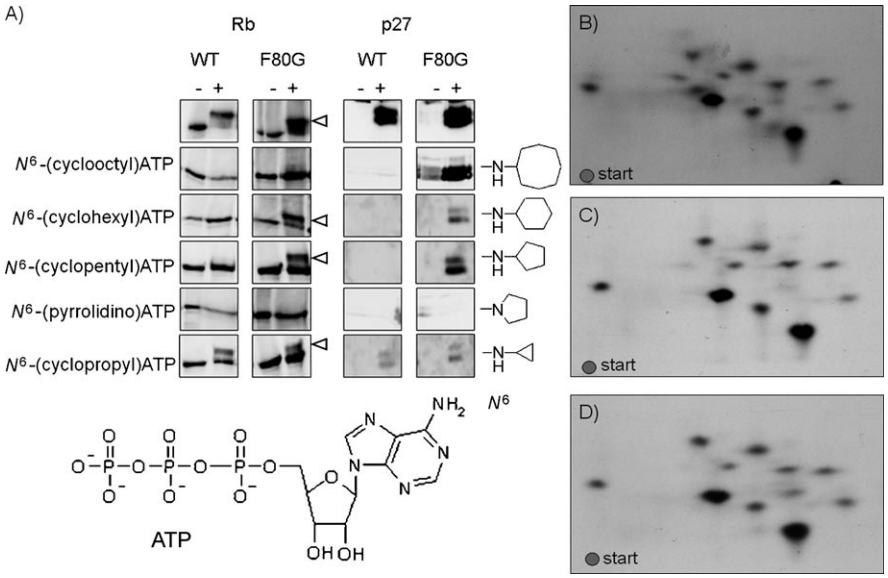

Figure 3. A) Testing the compatibility of N^6-modified ATPs with F80G cdk2. GST purified Rb or p27 was phosphorylated with cyclin E1/wild-type cdk2 or cyclin E1/F80G cdk2 in the presence or absence of 1 mM ATP analogues. Phosphorylation was identified by the presence of a gel shifted hyperphosphorylated band for Rb (open triangles) or using a phospho-specific p27 antibody for p27. B–D) Phosphopeptide mapping of Rb. Phosphopeptide maps of Rb fragments phosphorylated with B) wild-type cdk2 and ATP, C) F80G cdk2 and ATP or D) F80G cdk2 and N^6-(cyclohexyl)ATP. The origin is marked "start".

Analysis of Rb phosphorylation sites

After establishing that F80G cdk2 and some N^6-modified ATP analogues can act as bio-orthogonal pairs to phosphorylate known substrates, we addressed whether the phosphorylation site selection in a protein substrate was identical to that of wild-type cdk2. Retinoblastoma protein contains multiple phosphorylation sites targeted by cyclin E1/cdk2.[21,22] We employed phosphopeptide mapping to generate a fingerprint indicative of the sites of phosphorylation targeted in the substrate. Rb was phosphorylated by [γ-^{32}P]ATP with both wild-type cdk2 and F80G cdk2, and by [γ-^{32}P]N^6-(cyclohexyl)ATP ([γ-^{32}P]CxATP) with F80G cdk2. The hyperphosphorylated Rb was then digested with trypsin, the tryptic fragments separated in two dimensions and visualised using autoradiography (Figure 3). Comparing maps obtained with different ATP/kinase pairs demonstrated that identical patterns of phosphorylated peptides were obtained from all kinase/ATP combinations (Figure 3); this indicated that neither the mutant kinase nor the [γ-^{32}P]CxATP altered the site specificity of Rb phosphorylation. In addition, the intensity of phosphorylation of individual peptides was very similar irrespective of which kinase/ATP pair was employed; this indicated comparable efficiencies of selection of individual target sites. In addition, confirmation of Thr187 phosphorylation on p27 (the only available phosphorylation site within this clone) was demonstrated by mass spectroscopy analysis (see the Supporting Information), and showed unambiguously wild-type phosphorylation site specificity with all ATP/kinase pairs tested.

Comparison of wild-type and mutant Cdk2 activities

The activity of cdk2 is regulated by binding to cyclins. The two main cyclin types that bind to cdk2 in vivo are E-type and A-type cyclins, which are required for progression through the G1/S and S phases, respectively.[23] In order to compare the activity with both cyclins, baculovirus expression was used to coexpress a GST-tagged kinase and the appropriate cyclin. The cdk/cyclins were purified and used to phosphorylate Rb. Rb gel shift was used to quantify the fraction of hyperphosphorylated Rb from total Rb over time (Figure 4). We established that the reaction was within the linear range of phosphorylation (that is, ATP is not limiting and no more than 10% of the available ATP was used, see the Supporting Information), and Lineweaver–Burke plots were produced using different concentrations of ATP or CxATP and the resulting K_M and V_{max} values calculated (Table 1 and Figure 4).[24]

Comparable K_M values were obtained with native ATP for both wild-type and F80G cdk2 in combination with cyclin A2 ($K_{M(WT/ATP)} = 0.03 \pm 0.007$ mM, $K_{M(F80G/ATP)} = 0.03 \pm 0.02$ mM) and cyclin E1 ($K_{M(WT/ATP)} = 0.05 \pm 0.02$ mM, $K_{M(F80G/ATP)} = 0.08 \pm 0.05$ mM). This indicates that enlargement of the ATP binding site with the F80G mutation is likely to have made no significant difference to the kinetics of ATP binding/ADP release or product formation. When mutant F80G cdk2 was used in combination with either cyclin, N^6-(cyclohexyl)ATP resulted in a slight increase in K_M compared to ATP (with cyclin A2 $K_{M(F80G/CxATP)} = 0.04 \pm 0.01$ mM and cyclin E1 $K_{M(F80G/CxATP)} = 0.06 \pm 0.03$ mM), indicating that N^6-(cyclohexyl)ATP has a marginally lower efficiency as a phosphate donor that is, more N^6-(cyclohexyl)ATP is required to give half maximal activity. In all cases no phosphorylation was observed when N^6-(cyclohexyl)ATP was used as a substrate for wild-type cdk2, therefore kinetic parameters could not be established for these holoenzymes.

Maximal activity values (V_{max}) between all kinase/ATP pairs were also established. In all cases a comparable V_{max} were observed with ATP for wild-type and F80G cdk2 for cyclin E ($V_{max(WT/ATP)} = 27.1 \pm 7.7\%$, $V_{max(F80G/ATP)} = 24.6 \pm 1.4\%$) and cyclin A2 ($V_{max(WT/ATP)} = 13.1 \pm 4.3\%$, $V_{max(F80G/ATP)} = 8.7 \pm 3.3\%$). N^6-(cyclohexyl)ATP in combination with F80G cdk2 with cyclin A2, although increased, was not significantly different from wild-type cdk2 and ATP ($V_{max(F80G/CxATP)} = 19.5 \pm 0.6\%$) and similarly F80G cdk2 with cyclin E1 gave consistent V_{max} values for all three combinations of kinase and ATP ($V_{max(F80G/CxATP)} = 25.6 \pm$

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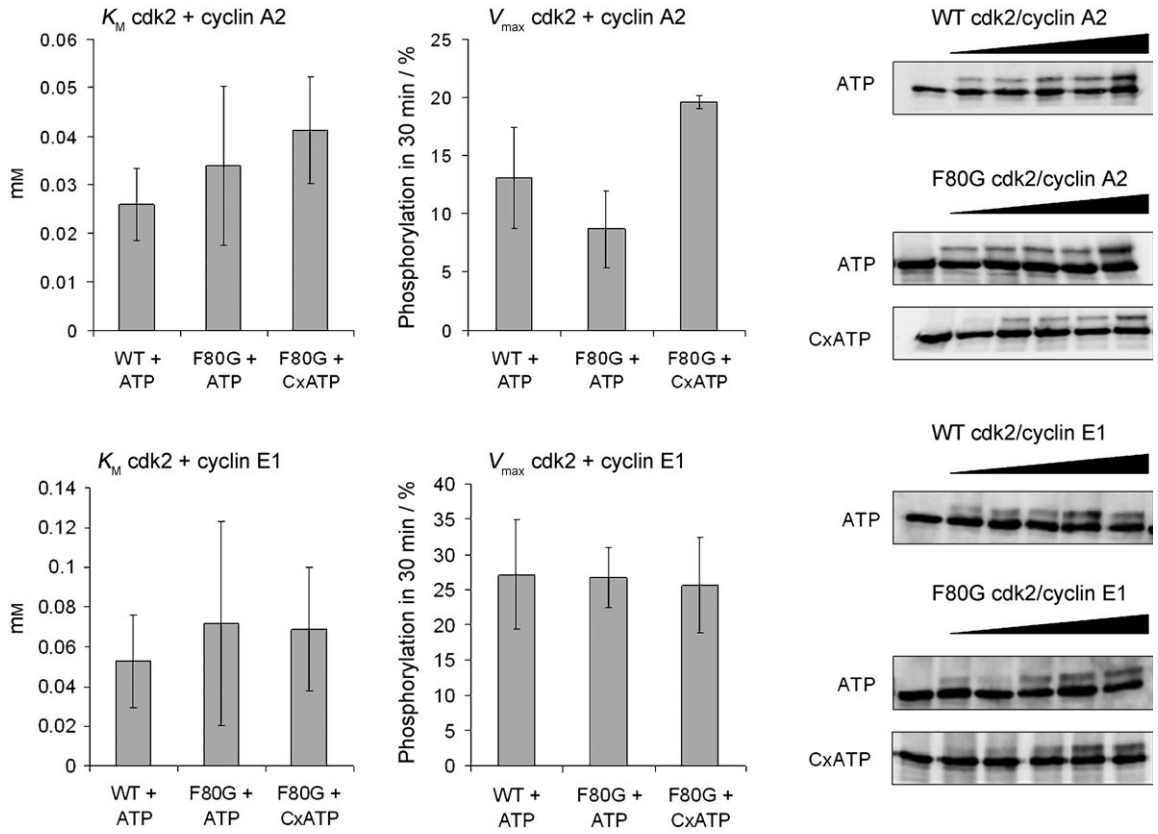

Figure 4. Determination of K_M and V_{max} for wild-type and F80G cdk2 with cyclin E1 or cyclin A2 as binding partners. Rb was phosphorylated with increasing concentrations of ATP/N^6-(cyclohexyl)ATP (CxATP) from 0.01 mM to 1 mM for 30 min. Phosphorylation of Rb was measured by immunoblotting and quantitative analysis of gel shifted hyperphosphorylated Rb (open triangle) and expressed as a percentage of total Rb. From these calculations Lineweaver–Burke plots were obtained and the K_M and V_{max} values determined.

Table 1. K_M and V_{max} values for wild-type and F80G cdk2 kinases.

	ATP		CxATP	
	K_M	V_{max}	K_M	V_{max}
WT cdk2/E	0.05 ± 0.02	27.1 ± 7.7		
F80G cdk2/E	0.08 ± 0.05	24.6 ± 1.4	0.06 ± 0.03	25.6 ± 6.7
WT cdk2A	0.03 ± 0.007	13.1 ± 4.3		
F80G cdk2A	0.03 ± 0.02	8.7 ± 3.3	0.04 ± 0.01	19.5 ± 0.6

6.7%) indicating comparable phosphorylation efficiencies with all kinase/ATP pairs. Although the N^6-(cyclohexyl)ATP and native ATP gave similar K_M values we wondered if a less hydrophobic substitution would result in an analogue that was preferentially used by the mutant kinase by disrupting potential hydrophobic interactions between the cyclohexyl moiety of the ATP analogue and the hydrophobic cavity that is revealed by the gatekeeper mutation (see Figure 1). We therefore synthesised N^6-[(4-amino)-piperidinyl]ATP as a less hydrophobic N^6-modified ATP variant; reduced hydrophobicity was implied by reduced retention times compared to N^6-(cyclohexyl)ATP on reverse phase HPLC. However, analysis of the rate of phosphorylation of Rb showed that N^6-[(4-amino)-piperidinyl]ATP was less efficient than N^6-(cyclohexyl)ATP (Figure 5).

Alternative substrate phosphorylation rates

After determining the kinetic values for the phosphorylation of Rb, we next investigated whether the rates of phosphorylation of other substrates are similarly affected. For this study, as we did not have the option of bandshift quantification, we used [γ-^{32}P]-labelled ATP/N^6-(cyclohexyl)ATP and two other known substrates of cdk2/cyclin E1; cdc25a[25] and histone H1.[26] Following 30 min exposure to the radiolabelled ATP derivative the intensity of phosphorylation reached a maximum and the intermediate time points were then expressed as a percentage of this total phosphorylation (Figure 5). Autophosphorylation of cyclin E1 gave a comparable rate of phosphorylation in all combinations tested. We obtained the percentage phosphorylation values as above (that is, assuming maximum phosphorylation at 30 min). In order to account for minor differences in kinase concentration, these values were then normalized relative to the phosphorylated cyclin E1 in each sample. This represents a relative amount of the total *active* kinase available. Following normalization ATP was shown to be a more efficient phosphate donor than N^6-(cyclohexyl)ATP (Figure 5); for example, on comparison of histone phosphorylation, 50% of the total phosphorylation was observed after 1.3 min for ATP whereas with N^6-(cyclohexyl)ATP this took 2.6 minutes (Figure 5).

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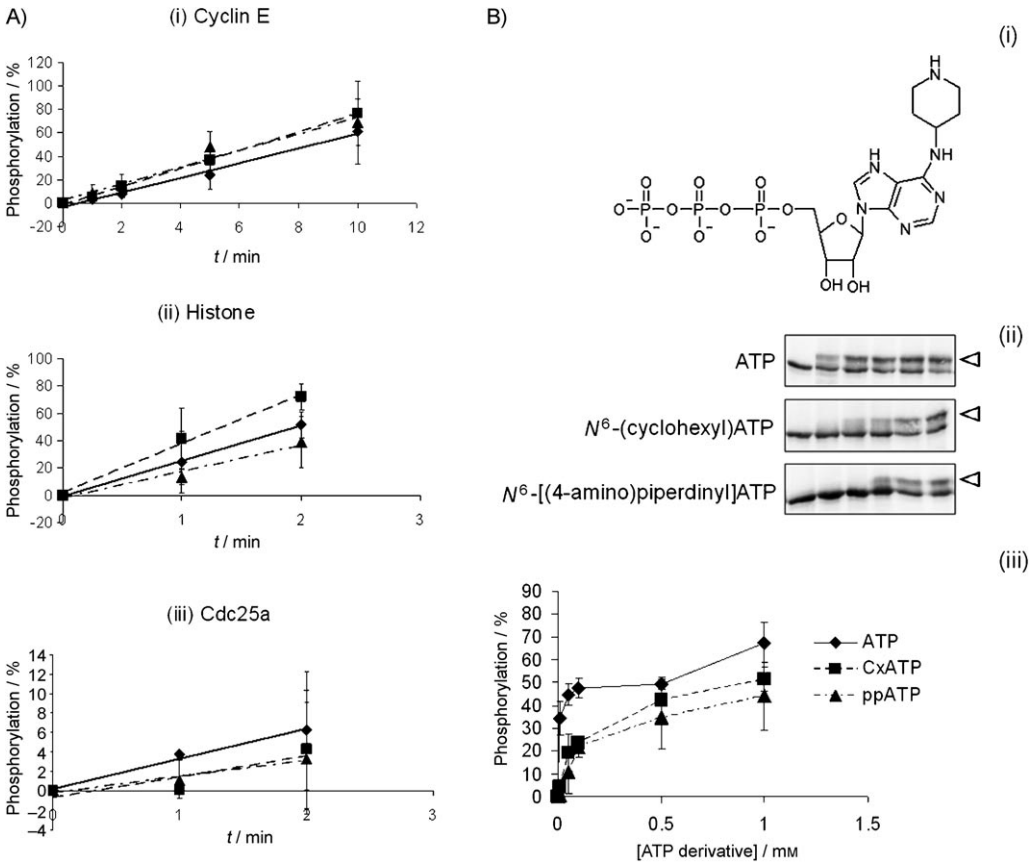

Figure 5. A) Measuring the rate of phosphorylation of cdk2 substrates. Recombinant proteins were phosphorylated with purified cyclin E1/cdk2 and [γ^{32}P]ATP/N^6-(cyclohexyl)ATP for the time indicated. Samples were separated by SDS PAGE and quantified by phosphoimaging. The initial rates of phosphorylation are shown for i) cyclin E1, ii) histone and iii) cdc25a each phosphorylated with wild-type cdk2+ATP (◆, solid line), F80G cdk2+ATP (■, dashed line) or F80G cdk2+CxATP (▲, dotted and dashed). B) Decreasing the hydrophobicity of N^6-(cyclohexyl)ATP does not improve the rate of Rb phosphorylation. Rb was phosphorylated with F80G cdk2 and either ATP, N^6-(cyclohexyl)ATP or N^6-[(4-amino)-piperidinyl)]ATP (shown in (i)). Quantitative analyses of the rates of phosphorylation were measured by taking the density of hyperphosphorylated Rb (open triangles) from the total Rb in each sample (ii). Data is represented by a Michaelis Menten plot (iii) of the % phosphorylation of Rb observed after 60 min with increasing ATP concentrations.

Complementation studies

Our enzymatic analyses indicated that F80G cdk2 was comparable to wild-type cdk2 in terms of its kinetic properties. In order to assess the ability of F80G cdk2 to function in a biological setting we employed the budding yeast *Saccharomyces cerevisiae* as a model system. Human cdks have been shown to complement defects in the yeast cdk *CDC28* and this system provides a simple test of the biological efficacy of a cdk.[27] We performed complementation assays using a strain of the budding yeast *S. cerevisiae* containing a temperature-sensitive mutation in *CDC28*. In this strain, the yeast proliferate normally at 30 °C but are unable to grow at 34.5 °C. We expressed either wild-type or F80G cdk2 into the temperature sensitive strain and tested for growth at the permissive and restrictive temperatures. As shown in Figure 6, both wild-type and F80G cdk2 were indistinguishable in their ability to complement the growth defect at 34.5 °C, whereas yeast transformed with the empty vector control were unable to grow. These data indicate that the F80G cdk2 is functional in vivo and is able to compensate for the loss of wild-type *CDC28* in yeast.

Discussion

The utilisation of a protein kinase with a mutationally enlarged ATP binding site in combination with an ATP analogue has proved to be an innovative and informative chemical genetic technique in the study of protein kinases.[9–11] Whilst the technique is becoming widely used, there are few studies rigorously analysing the kinetics and specificity of the mutant kinase/ATP analogue pairing with serine/threonine kinases. To be fully applicable, the kinase/ATP analogue pairing should reflect the properties of the unaltered components as closely as possible. In this study, we have examined this requirement for the cell cycle regulatory protein serine/threonine kinase cdk2 and a range of nucleotide analogues and known protein substrates.

Three different cdk2 mutants were assessed, each with amino acid side chains positioned in close proximity to the N^6 nitrogen of the adenine base of ATP: F80G, L134A and V64S (Figure 2). All proteins were expressed well through baculovirus infection of Sf9 cells but only the F80G variant had activity comparable to wild-type cdk2 when complexed with a cyclin

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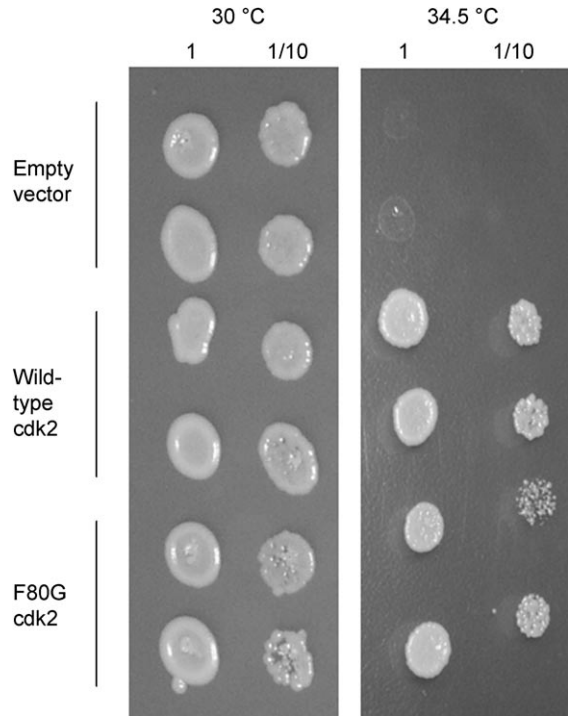

Figure 6. Complementation of defective CDC28 by F80G cdk2. *S. cerevisiae* with a temperature sensitive allele of *CDC28* was transformed with either empty vector or vectors expressing either wild-type or F80G cdk2. Cultures were grown from two independent transformants for each plasmid at the permissive temperature and then spotted onto duplicate plates at two concentrations (marked "1" and "1/10") and incubated at either 30 °C (permissive) or 34.5 °C (restrictive) for three days.

(Figure 3 and data not shown). Recent computational analysis has indicated that L134 of cdk2 is likely to form part of the so-called C spine, a stack of hydrophobic residues that is predicted to be important for optimisation of the catalytic process.[28] Our observation that the L134A mutant is substantially less active than the wild-type cdk2 is in agreement with this computational analysis, as the substitution with an alanine is likely to destabilise this hydrophobic stacking hence reducing kinase catalytic ability.

F80 is equivalent to the gatekeeper residue in cdk2 and as such is the most commonly targeted site in protein kinases for enlargement of the ATP pocket. Recent studies on tyrosine kinases have indicated that this residue can have a profound effect on the activity of the kinase.[17] Many endogenous tyrosine kinases have threonine at this gatekeeper site; in v-Src and some activated versions of c-ABL, this residue is changed to a more bulky hydrophobic amino acid such as isoleucine. These changes result in activation of the tyrosine kinase through stabilisation of a second hydrophobic spine (the R spine) that holds the kinase in an active conformation.[17,28] In contrast, many serine/threonine kinases already have a bulky hydrophobic amino acid at this position (F80 in cdk2). Our enzymatic analysis, in agreement with previous studies,[16] does not indicate any substantial decline in enzymatic activity of cdk2 upon substitution of the gatekeeper residue with an amino acid lacking a side chain. The interaction with a cyclin assists in holding the cdk in an active conformation and this may be a factor in the retention of activity with F80G cdk2. These in vitro observations are substantiated by the demonstration that F80G cdk2 is able to complement the loss of CDC28 activity in *S. cerevisiae* and attests its capability in a biological system (Figure 6).

Mutation of the gatekeeper residue[29] allows bulky additions at the N^6 position of ATP to sit within the ATP binding site and hence modified ATPs to be utilised as phosphate donors. Of the variety of ATPs with bulky N^6 additions tested, we found only N^6-(cyclopentyl), N^6-(cyclohexyl) and N^6-[(4-amino)piperidinyl] ATPs to be productive binding partners resulting in substrate phosphorylation that was comparable to that observed with ATP. None of these analogues were phosphate donors with wild-type cdk2. Further work employed N^6-(cyclohexyl)-ATP.

Ideally, to discover novel protein kinase substrates, F80G cdk2 would need to be expressed in cells or a cellular environment in the presence of ~1 mM ATP.[30] Current gatekeeper mutations such as F80G cdk2 do not preclude the kinase from using standard ATP but allow it to additionally employ to the N^6-substituted ATP analogues as phosphate donors. We therefore set out to compare the activity of the wild-type and mutant kinases with ATP and the F80G mutant with the N^6-(cyclohexyl)ATP analogue. The F80G mutation did not significantly affect the kinetics of phosphorylation of several known cdk2 substrates with native ATP, and gave comparable K_M and V_{max} values; this indicates that the kinase retains the enzymatic properties of its wild-type counterpart. Ideally to compete successfully with intracellular ATP, the F80G cdk2 would have greater catalytic efficiency with N^6-(cyclohexyl)ATP than with ATP which was not observed here as shown in Table 1. Further modifications to the ATP moiety may prove to be more efficient selective substrate donors. For example, construction of dual bump hole system[31] would allow absolute specificity and circumvent competition from endogenous ATP; such a system could be engineered on top of the existing model by, for example, reducing the ribose unit whilst introducing a compensatory bulky amino acid sidechain in the appropriate portion of the kinase. A previous study in chemical genetic mutants F80G cdk2 and T338G c-Src kinases, the K_M values for the analogue N^6-(benzyl)ATP was much lower than that of ATP indicating that in this example the analogue ATP was in fact a better substrate than the native ATP.[16] The differences between this study and that by Kraybill et al. are likely to be due to the specific combination of the kinase and the ATP analogue. Thus, it is clearly important to further evaluate mutant kinases with different ATP analogues to identify optimal combinations, and further detailed study is required.

Protein serine/threonine kinases generally select substrates through interaction of the kinase with a docking site on the substrate (at least partially through the cyclin in the case of cdks). This sterically aligns the target residue close to the catalytic site.[28,32,33] Given that the gatekeeper residue is deep within the adenine-binding pocket of the kinase, far from these surface interactions, it seemed unlikely that its mutation would affect substrate selection. However, recent studies have suggested that substantial interconnectivity exists between

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spatially disparate portions of protein kinases;[28] this makes it important to assess the effects of gatekeeper mutations on substrate selection. Our observations indicate no significant change in substrate phosphorylation, both in terms of the selection of individual proteins for phosphorylation and the relative efficiency of targeting specific sites within a single substrate undergoing multi-site phosphorylation (Figures 4 and 5). These observations greatly validate the use of gatekeeper mutations for studying protein serine/threonine kinase function and suggest that this residue is not contributing substantially to long range interactions within the protein serine/threonine kinase scaffold.

Experimental Section

Materials: Primary antibodies p27 (C-19), p27 T187 phospho-specific (sc16324R) and GST (B-14) were from Santa Cruz Biotechnology (Heidelberg, Germany) NeutrAvidin-HRP from Molecular Probes (Eugene, OR, USA). All secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). All other materials were from Sigma unless stated otherwise.

Synthesis of ATP derivatives: All syntheses carried out according to literature procedures.[4] Further details and full analytical data can be found in the Supporting Information.

Generation of constructs: GST-tagged cdk2 constructs were created by subcloning cdk2[34] into pAcGEX by using BamHI/EcoRI. Mutant cdk2 clones were created by PCR mutagenesis using the following primers (coding strands only shown): for F80G: 5′-CTC TAC CTG GTT GGC GAA TTC CTG CAC CAA G-3′; V64S 5′-CCT AAT ATT TCG AAG CTG CTG-3′; L134 A 5′-AAC CTC AGA ATC TCG CGA TTA ACA CAG AG-3′. All clones were cloned into a T-tail vector and sequenced before being subcloned into pBlueBacHisA using BamHI/HindIII for baculovirus expression. Wild-type and F80G cdk2 were then further subcloned into pAcGEX for expression with a GST tag and into pGBDU-C1 for yeast expression studies. Other constructs have been described.[21,34,35]

Cell culture: NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with newborn calf serum (NCS, 10% *v/v*). Sf9 cells were cultured in Grace's media supplemented with foetal bovine serum (FBS, 10% *v/v*). Recombinant baculoviruses were produced using the BaculoGold system (Pharmingen, Oxford, UK).

Baculovirus expression and purification of wild-type and F80G cdk2: Infections with recombinant viruses producing GST-tagged proteins were performed such that the cyclin expressing virus was present in ~tenfold excess compared to the GST-cdk2 virus to ensure maximal binding of cyclin to each cdk. Cells were lysed in NETN (10 mL of: 20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% NP40) by repeated passage through a 21-gauge needle. Debris was removed by centrifugation at 5000 rpm at 4 °C for 5 min and the lysate was then incubated with glutathione-Sepharose beads (500 μL) for 2 h at 4 °C. The beads were thoroughly washed in NETN (3×10 mL) before use in assays as described below. Preparations were assayed against histone H1 and contained activities of phosphate transfer per mg of kinase from two independent assays in the following ranges: cyclin E1/wild-type cdk2: 53.24–78.21 $nmol^{-1}\,mg^{-1}\,min^{-1}$; cyclin E1/F80G cdk2: 1.40–4.96 $nmol^{-1}\,mg^{-1}\,min^{-1}$; cyclin A2/wild-type cdk2: 7.74–19.55 $nmol^{-1}\,mg^{-1}\,min^{-1}$; cyclin A2/F80G cdk2: 3.48–5.86 $nmol^{-1}\,mg^{-1}\,min^{-1}$.

Kinase assays: Kinase assays were performed in 100 μL volume, which contained GST-purified kinase (1–2 units where 1 unit = 35% Rb shift after 30 minutes incubation), GST-purified substrate (~1 μg of Rb (aa 414–960), p27 (aa 105–198), cdc25a and cyclin E co-purified by using cdk2-GST as above) or histone H1 (Roche), ATP (1 mM) and K buffer (25 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.1% (*v/v*) 2-mercaptoethanol, 0.1 mM EDTA). For K_M/V_{max} experiments, Rb-GST (1 ng) was used as a substrate. The samples were incubated for up to 1 h at 30 °C (for Lineweaver–Burke plots, samples were incubated for 30 min). Reactions were terminated by adding 5× SDS-PAGE loading buffer. The protein samples were separated by SDS-PAGE and visualized western blotting and ECL detection (Millipore). Analysis and quantification of blots was performed using Aida software.

His prep of NDPK: Nucleotide diphosphate kinase (NDPK) cDNA was expressed as a His-tagged protein in *E. coli* strain BL21. Bacterial cultures (200 mL) were centrifuged at 1000 rpm for 5 min at 4 °C and resuspended in buffer A (10 mL of: 20 mM Tris at pH 8, 0.5 M NaCl). Cells were lysed by sonication and the debris removed by further centrifugation at 5000 rpm for 5 min at 4 °C. His-tagged protein was purified by immobilised metal affinity chromatography. Beads were washed in buffer B (3×20 mM Tris pH 8, 0.5 M NaCl, 40 mM imidazole) before being adjusted to 50% glycerol and stored at −20 °C prior to further use.

Enzymatic synthesis of [γ-^{32}P] N^6-(cyclohexyl)ATP: His-tagged NDPK beads were washed in ammonium bicarbonate (0.1 M). [γ-^{32}P]ATP (5 nmol) was added to beads and the reaction incubated at 30 °C for > 1 h. Unreacted [γ-^{32}P]ATP was washed from the beads with ammonium bicarbonate (0.1 M) and discarded, until washes contained < 5 cpm. N^6-(cyclohexyl)ADP (CxADP, 0.1 nmol) was added to the beads followed by incubation at 30 °C for > 1 h. Following brief centrifugation, the supernatant was removed and the remaining labelled CxATP washed from beads. The supernatant was concentrated by centrifugal evaporation and subsequently the [γ-^{32}P]CxATP was resuspended in K buffer.

Phosphopeptide mapping of Rb: For the phosphopeptide analysis, in vitro kinase assays were performed as described above except that [γ-^{32}P]ATP/CxATP (3 nmol) and GST-Rb (amino acids 378–928) (1.5 ng) were used. The phosphorylated GST-Rb products were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and digested with trypsin prior to phosphopeptide analysis as described.[36]

Complementation studies in yeast: *S. cerevisiae* strain Y246a (MATa cdc28–4 trp1 ura3–52 tyr1; a gift from John Diffley) was transformed with either empty vector (pGBDU-C1) or plasmids directing expression of either wild-type or F80G cdk2 using a standard lithium acetate protocol. Transformants were grown in liquid culture at 30 °C and then spotted onto selective agar plates and incubated at either 30 °C or 34.5 °C for 3 days.

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