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Direct Substrate Identification with an Analog Sensitive (AS) Viral Cyclin-Dependent Kinase (v-Cdk)

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Abstract

Viral cyclin-dependent kinases (v-Cdks) functionally emulate their cellular Cdk counterparts. Such viral mimicry is an established phenomenon that we extend here through chemical genetics. Kinases contain gatekeeper residues that limit the size of molecules that can be accommodated within the enzyme active site. Mutating gatekeeper residues to smaller amino acids allows larger molecules access to the active site. Such mutants can utilize bio-orthogonal ATPs for phosphate transfer and are inhibited by compounds ineffective against the wild type protein, and thus are referred to as analog-sensitive (AS) kinases. We identified the gatekeeper residues of the v-Cdks encoded by Epstein-Barr Virus (EBV) and Human Cytomegalovirus (HCMV) and mutated them to generate AS kinases. The AS-v-Cdks are functional and utilize different ATP derivatives with a specificity closely matching their cellular ortholog, AS-Cdk2. The AS derivative of the EBV v-Cdk was used to transfer a thiolated phosphate group to targeted proteins which were then purified through covalent capture and identified by mass spectrometry. Pathway analysis of these newly identified direct substrates of the EBV v-Cdk extends the potential influence of this kinase into all stages of gene expression (transcription, splicing, mRNA export, and translation). Our work demonstrates the biochemical similarity of the cellular and viral Cdks, as well as the utility of AS v-Cdks for substrate identification to increase our understanding of both viral infections and Cdk biology.

Graphical Abstract

Human herpesvirus (HHV) analog sensitive (AS) v-Cdks have larger ATP binding pockets than their wild type (WT) counterparts, depicted here using a model EBV-PK structure (I-TASSER: <http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). The AS-v-Cdks, but not the WT kinases, are inhibited by the AS inhibitor 3-MB-PP1 and can utilize bio-orthogonal ATPs for phosphate transfer and subsequent mass spectrometry analysis to identify direct kinase substrates.

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Author Contributions: AU and RFK designed the experiments. AU performed the experiments. SI performed the luciferase reporter experiments. AU and RFK wrote the paper with comments from all authors.

Supporting Information Available: The Supporting Information is available free of charge on the ACS Publications website at doi: Supporting Information – Table S1: List of primers used in this study
Data File S2: List of proteins identified (XLSX)

Protein kinases catalyze the phosphorylation of specific amino acids in targeted substrates ¹. Analysis of proteomic data estimates that over 50% of proteins are phosphorylated ². This well studied post-translational modification can regulate almost any aspect of protein function, from localization and interactions to stability and activity. Numerous cellular processes are regulated by kinases, such as signal transduction pathways, metabolism, transcription, proliferation, differentiation, and migration. Kinases play critical roles in human health, with at least 244 kinase genes mapped to disease loci ³. There are over 25 small molecule kinase inhibitors that are approved by the US Food and Drug Administration to treat cancer and diabetes, as well as inflammatory and neurological diseases ^{4, 5}.

Understanding the roles that protein kinases play in cell biology and disease, and predicting or managing the consequences of inhibiting a kinase with chemotherapy, requires an appreciation of the array of substrates phosphorylated by a kinase. However, identifying direct kinase targets is challenging. Many substrate identification technologies exist, including prediction based algorithms, phospho-proteomic profiling, array technology, and chemical genetics ⁶. Each methodology has strengths and weaknesses. The chemical genetics approach is particularly attractive because it allows for identification of direct substrates of a kinase in complex, physiologically relevant formats such as permeabilized cells or lysates ⁷. This approach takes advantage of mutant kinases called analog-sensitive (AS) kinases that can utilize bio-orthogonal ATP molecules to directly label substrates. The gamma-phosphate transferred to the substrate can contain a thiol group that may be used to chemically or immunologically enrich phosphorylated proteins to aid in their identification ^{8–10}.

The ATP-binding pockets of kinases are of similar sequence and structure. Kinase co-crystal structures demonstrated that a larger amino acid lying in close proximity to the N6 amino group of a bound ATP molecule controls access to the ATP binding pocket ¹¹. This amino acid was termed the gatekeeper residue. Mutants in which an amino acid with a small side chain (glycine or alanine) substitutes for the gatekeeper residue have a larger ATP binding pocket that can accommodate bio-orthogonal ATP molecules with modifications at the N6 position, and use them to transfer gamma-phosphates to substrate proteins ⁷. The ATP binding pockets of wild type kinases are too small to permit docking of bio-orthogonal ATPs, and thus cannot use them as phosphate donors. Furthermore, purine analogs such as 3-methylbenzyl pyrazolopyrimidine (3-MB-PP1) can enter the ATP-binding pockets of the AS, but not wild type proteins, and thus serve as specific inhibitors of AS kinases ¹². Gatekeeper mutants are functionally silent, can still use normal ATP, have catalytic

parameters similar to their wild type parents, show no changes in substrate specificity, and can biologically complement for the absence of the wild type protein ¹³.

Our interest in the use of chemical genetics to identify direct kinase substrates stems from our work exploring the roles of two viral kinases with functional similarity to cellular cyclin-dependent kinases (Cdks) in viral replication and pathogenesis. Cdks generally require direct physical interaction with a cyclin protein for activity. Humans encode 21 Cdks and 29 cyclins ¹⁴. In broad terms, Cdks control the cell cycle by phosphorylating (and thus inactivating) the Rb family of tumor suppressors to allow for progression through G1 and into the S (DNA synthesis) phase, and phosphorylate Lamin A/C to allow for the disruption of the nuclear envelope required for completion of mitosis ¹⁵. Over 20 small molecule Cdk inhibitors have been developed for the treatment of various diseases, particularly cancer ¹⁶. Recent approval of palbociclib for the treatment of breast cancer has reignited interest in identifying selective kinase inhibitors ^{16, 17}. Lists of substrates and sites for prominent Cdks have been compiled in part through the use of AS derivatives ^{7, 18, 19}. Although cell cycle proteins and transcriptional machinery are well represented ¹⁴, many Cdk substrates have functions that are either unknown or outside of these two major categories, indicating that the influence of Cdks on cellular activities extends beyond proliferation and transcription.

Beta- and gamma-herpesviruses encode kinases with Cdk-like activity referred to as v-Cdks ^{20, 21}. These kinases complement yeast deficient in Cdk activity and phosphorylate Rb and Lamin A/C ^{22–25}. Regulatory mechanisms that control cellular Cdk activity including cyclin association, inhibition by proteins such as p21^{Cip1/Waf1}, and activating or inhibitory phosphorylations do not appear to affect the v-Cdks. Furthermore, individual v-Cdks phosphorylate sites in Rb and Lamin A/C that normally require the activity of more than one Cdk ^{21, 23, 26}. Thus v-Cdks are hyperactive kinases with activities that mimic multiple cellular Cdks ²⁷.

The v-Cdks regulate viral DNA replication, viral gene expression, and capsid egress from the nucleus ²⁸. They greatly enhance but are not absolutely required for productive viral infections *in vitro* ^{29, 30}. However, the v-Cdks are intimately involved in the pathogenesis associated with viral infections *in vivo* ²⁸. Therefore, the v-Cdks represent attractive targets for novel antiviral therapeutics. It is likely that new aspects of v-Cdk biology relevant to viral replication, pathogenesis and treatment will be revealed by an encyclopedic identification of their substrates.

To that end, we have developed AS kinases for the v-Cdks EBV-PK (Epstein Barr Virus-Protein Kinase), and HCMV-UL97 (the 97th gene in the Unique Long segment of the Human Cytomegalovirus genome). EBV is a gamma herpesvirus that causes Burkitt's lymphoma, nasopharyngeal carcinoma, and gastric cancers ³¹. HCMV is the leading viral cause of birth defects, causes severe disease in transplant patients undergoing immunosuppressive therapy, and is associated with glioblastoma multiforme (GBM) brain tumors ^{31–33}. Each of these kinases phosphorylate and thereby activate the antiviral pro-drug ganciclovir ^{34–36}, and UL97 is inhibited by the experimental therapeutic Maribavir ³⁷. We show that the AS-v-Cdks display similar preferences for bio-orthogonal nucleotide utilization as does the AS derivative of cellular Cdk2, whose function they mimic. Furthermore, we use an AS

derivative of EBV-PK to identify novel kinase substrates with important functions in nucleosome remodeling and mRNA splicing.

RESULTS AND DISCUSSION

Generating AS-vCdk

The v-Cdks EBV-PK and HCMV UL97 share low sequence identity with cellular Cdks. For example, EBV-PK is 9.3% identical to Cdk2, and HCMV-UL97 is 5.4% identical to Cdk2³⁸. Despite this modest sequence conservation, v-Cdks retain the conserved residues required for ATP binding and phosphate transfer. By fixing conserved residues within the kinase domain such as the defined catalytic lysine and the GxGxxG motif³⁹, five kinase sub-domains were clearly identifiable²¹. While sequence alignments alone can be used to identify unknown gatekeeper residues, the low similarity of subdomain V sequences (where the gatekeeper is found) prompted us to generate structural models of the v-Cdks. The amino acid sequences for EBV-PK (1–429; full length) and HCMV-UL97 (338–707) were threaded onto the structure of Cdk2 (Figure 1A, 1B, 1C). Potential v-Cdk gatekeeper residues were identified by locating amino acids within the models whose side chains extend into the active site near the predicted position of the N6 amino group of the phosphate-transferring ATP. We used these models, our sequence alignments (Fig. 1D), and the catalog of known gatekeeper amino acids in cellular kinases⁴⁰ to predict putative v-Cdk gatekeeper residues homologous to the Cdk-1 and -2 gatekeeper phenylalanine 80 residue. Our predictions were histidine 146, phenylalanine 149, methionine 150, or phenylalanine 153 for EBV-PK and histidine 411, phenylalanine 414, threonine 416, or methionine 418 for HCMV-UL97 (Figure 1D).

We replaced each of these putative gatekeeper residues with glycine in the hope of making AS1-v-Cdks. We tested substitution mutants for their ability to phosphorylate Rb, a common v-Cdk substrate. Rb phosphorylation is observed as an upward shift in electrophoretic mobility and by detection with an antibody specific for Rb phosphorylated at residues 807 and 811 (Figure 1E and 1F). For EBV-PK, substitution mutants at phenylalanine residues 149 or 153 failed to phosphorylate Rb. Likewise, HCMV-UL97 substitution mutants at residues threonine 416 or methionine 418 also failed to phosphorylate Rb. Substitutions at EBV-PK residue histidine 146 and HCMV-UL97 residue phenylalanine 414 were able to phosphorylate Rb, but like the wild type proteins, were insensitive to the AS-specific inhibitor 3-MB-PP1. Only residue mutants EBV-PK methionine 150 and HCMV-UL97 histidine 411 remained able to phosphorylate Rb and were sensitive to 3-MB-PP1 (Figure 1E, 1F). We further examined these alleles as potential AS1 v-Cdks.

Starting with the potential AS1 v-Cdks, we also generated AS3 alleles. AS3 kinases have an additional mutation to a smaller residue, such as alanine, in the amino acid immediately amino terminal to the conserved DFG motif in kinase subdomain VII. This AS3 mutation is known in some instances to increase AS1 kinase sensitivity to AS-specific inhibitors^{41,42}. We made AS3 v-Cdks by combining the AS1 mutations with one substituting alanine for threonine 218 in EBV-PK or cysteine 480 in HCMV-UL97 (Figure 1D). AS2 kinases have alanine substitutions for the gatekeeper residue but were not made here.

Testing AS-vCdk

EBV-PK-AS1, EBV-PK-AS3, HCMV-UL97-AS1, and HCMV-UL97-AS3, like their wild type counterparts, phosphorylated the Rb protein (Figure 2A) and disrupted the nuclear lamina (Figure 2B) in transient transfection assays^{21, 23}. Rb phosphorylation (Figure 2A) was monitored as described above. Lamina disruption is observed by converting the homogenous nuclear appearance of co-transfected GFP-Lamin A/C into aggregated puncta (Figure 2B). We conclude the AS1 and AS3 v-Cdks retain the ability to phosphorylate their known substrates Rb and Lamin A/C.

Purified AS1 and AS3 v-Cdks displayed *in vitro* kinase activity against an Rb peptide that was attenuated in a dose-dependent manner by the AS kinase selective inhibitor 3-MB-PP1 (Figure 3A). The drug showed statistically significant inhibition of the AS1 and AS3 v-Cdks, but not the wild type proteins (Figure 3B). 3-MB-PP1 also prevented *in vivo* Rb phosphorylation (Figure 2A) and lamina disruption (Figure 2B) by AS1 and AS3 but not wild type v-Cdks. Finally, 3-MB-PP1 had no effect on the demonstrated ability of wild type HCMV-UL97 to inactivate the ability of Rb to repress an E2F-responsive promoter reporter²⁶, but did inhibit the ability of HCMV-UL97-AS1 and HCMV-UL97-AS3 to do so (Figure 3C). We conclude that 3-MB-PP1 inhibits the AS1 and AS3 v-Cdks.

We assembled a panel of ten commercially available bio-orthogonal ATP molecules with modifications at the N6 position (Figure 4A) and tested the ability of wild type, AS1 and AS3 v-Cdks to utilize them *in vitro*. These derivitized ATPs were utilized poorly by the wild type v-Cdks but a subset permitted the AS1 and AS3 v-Cdks to phosphorylate the Rb peptide (Figure 4B). We also tested this ATP panel against Cdk2-AS1 (F80G) and Src-AS1 (T341G). These AS cellular kinases, but not their wild type counterparts, were inhibited in *in vitro* kinase reactions by AS kinase specific inhibitors (Figure 4C). Wild type Cdk2 utilized the derivitized ATPs poorly, but they supported Cdk2-AS1 *in vitro* kinase activity (Figure 4D). Src-AS1 also utilized the derivitized ATPs well, but the wild type protein also showed substantial activity in their presence (Figure 4D).

We calculated usage specificity by comparing the activity of the AS kinase to the wild type kinase for each bio-orthogonal ATP molecule tested (Figure 5). Our results indicate that EBV-PK-AS1 showed a strong similarity in derivitized ATP usage to Cdk2-AS1. HCMV-UL97-AS1 also showed a similar profile to Cdk2-AS1 with an increase in range of ATP analog acceptance, and the AS3 v-Cdks were similar to their AS1 counterparts. In our assays, Cdk2-AS1 did not preferentially use N6-Benzyl-ATP to phosphorylate Rb, whereas previous studies detected preferential use during phosphorylation of cell extracts¹⁹. We conclude that AS v-Cdks show the highest specificity for N6-Phenyl-ATPs similar to Cdk2-AS1, whose function they mimic.

Substrate identification with EBV-PK-AS3

We next utilized EBV-PK-AS3 to directly label substrates in lysates from primary normal human dermal fibroblast (NHDF) cells. Lysates were spiked with either purified wild type or AS3 EBV-PK, and N6-Phenyl-ATP- γ S was added to a subset of the reactions to allow for direct thiophosphorylation by the AS kinase. After the kinase reaction was complete,

thiophosphorylations within the reactions were alkylated with p-nitrobenzylmesylate (PNBM), lysates were separated by SDS-PAGE, and proteins with alkylated thiophosphate esters (TPE) were detected by Western blot. Only reactions that included EBV-PK-AS3 and N6-Phenyl-ATP- γ S and were subsequently treated with PNBM contained labeled proteins detected by the TPE antibody (Figure 6A). We conclude that EBV-PK-AS3 can be used to specifically thiophosphorylate its substrates.

Thiophosphorylated substrates were subsequently identified by tandem mass spectrometry after covalent capture by conjugation to iodoacetyl beads and elution with oxone. Two biological replicates were performed with EBV-PK-AS3 paired with a kinase dead⁴³ derivative of EBV-PK (K102I) as a negative control. A Venn diagram (Figure 6B) displays the overlap of the individual substrates detected between the four reactions. We identified twenty-one proteins with phosphopeptides common to both EBV-PK-AS3 reactions and absent in both negative controls (Table 1) as high confidence EBV-PK substrates. Reassuringly, the list includes the known EBV-PK substrates retinoblastoma tumor suppressor (RB1) and Lamin A/C (LMNA). Of the twenty-four phosphorylation sites mapped in these proteins (Table 1), eighteen (75%) are followed by a proline (Figure 6C), similar to the canonical cellular Cdk consensus phosphorylation site.

Overall, 339 phosphopeptides present in EBV-PK-AS3 samples were identified. From these, unique phosphorylations present in EBV-PK-AS3 samples and not in the kinase dead derivative represented 77 unique proteins (Supplementary file S2: Table 2). Pathway analysis (Figure 6D) on the full list of proteins predicts a novel and under-appreciated role for EBV-PK in essentially all steps of gene expression, including transcription (CHD4, CHD8, HMG1A1, INO80E, KLF3, LMNA, LMNB2, MTA1, NFATC4, NCL, PML, RB1, ZNF318, ZNF687), mRNA splicing (CDC5L, HNRNPA2B1, HNRNPUL2, SF3B1, SRRM2, THRAP3, RALY, UPF3B, ZRANB2), mRNA export (FYTTD1, NUP107, NUP133, UPF3B), and translation (EEF1A1, EEF2, EIF2A, EIF3A, EIF4G3, RPS14). The underlined proteins appeared in both EBV-PK-AS3 biological replicates. Regulation of the cytoskeleton (AHNAK, CALD1, EPB41L3, HSPB1, MYLK, PALLD, PHIP, SVIL), microtubules (MAP1B, MAP4, MAP7D1, MARK3, PSRC1, SYBU) and cellular phosphorylation cycles (AKAP13, PPP1R10, PPP1R12C, PRKAA1, PRKAR2A) by EBV-PK also appears likely based on the identified proteins.

An examination of potential direct EBV-PK substrates by interrogation of a 4,191-member human protein array detected 273 phosphorylated proteins⁴⁴. The high concentration of protein spotted on the arrays may account for the increased number of potential substrates identified in that study compared to our results shown here. In addition, 1,328 proteins phosphorylated in EBV-PK-expressing cells but not in non-expressing cells have been identified, although these proteins are not necessarily direct substrates of EBV-PK. In total, we identified 3 known EBV-PK substrates, 14 proteins implicated as potential substrates by previous high-throughput analyses⁴⁵ and 4 novel substrates. These previous studies implicated EBV-PK in the DNA damage response and mitosis, and we certainly identified proteins that participate in those processes such as CHD4, CHD8, and NUMA1. Likewise, the gene expression pathways we identified downstream of EBV-PK were also implicated in the high-throughput studies cited above. Finally, low-throughput studies have implicated

EBV-PK in regulating some of the processes identified by the more global studies presented here and previously^{44, 45}, including transcription^{46–48}, translation⁴⁹, nuclear pore function^{50, 51}, microtubules⁵², and cellular kinase activity⁵³.

Conclusion

Our work demonstrates the analog sensitive kinase approach for substrate identification is readily applicable to the v-Cdks, thus adding chemical genetics to the arsenal of methods available to understand the function of virally-encoded kinases. The AS-v-Cdks we generated showed activity profiles identical to their wild type derivatives but were inhibited by the AS-specific inhibitor 3-MB-PP1. We identified a panel of ATP analogs the v-Cdks can utilize to phosphorylate a known target, and quantitated their usage efficiency. While the AS analogs could use a subset of the ATPs tested, N6-Phenyl-ATP displayed the highest specificity for all four of the AS kinases we tested. Therefore, this bio-orthogonal ATP might be the molecule of choice when AS derivatives are used to reveal kinase targets. These AS derivatives will be useful to us and others for identifying novel substrates of the v-Cdks to aid in our understanding not only of herpesviral infections, but also general Cdk biology. Indeed, our initial substrate identification revealed the previously under-appreciated and widespread potential for EBV-PK-mediated regulation of multiple steps in the pathways of gene expression.

METHODS

Modeling

The crystal structure of Cdk2 (PDB:1HCK)⁵⁴ was used as a template to generate model structures using I-TASSER⁵⁵. Amino acid predominance analysis for phosphopeptides was generated using WebLogo⁵⁶. Amino acid alignments of the v-Cdk kinase domains with cellular kinases were generated with ClustalW/MEGA 4 with the Blosum matrix and these parameters: Pairwise alignment – gap opening penalty 5; gap extension penalty 1²¹.

Cell lines and plasmids

Saos-2, U-2 OS, and HEK293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen and Sigma) supplemented with 10%(vol/vol) fetal bovine serum (FBS) (Sigma), 100U/ml penicillin, 100µg/ml streptomycin, and 0.292mg/ml glutamine (PSG) (Invitrogen). To generate tagged kinases for purification, HA-UL97, HA-UL97 KD, Cdk1, Cdk2, or c-Src kinases were amplified by PCR (see Supplementary Table S1 for primers) and cloned using an In-Fusion cloning kit (Clontech) into AsiSI and XhoI restriction sites in a pFC14a vector (Promega) adding a HaloTag at the C-terminus of the kinases. PCR templates for the constructs were as follows: pCGN-HA-UL97²¹, pUHD-Cdk1-WT-HA (addgene #27652), pCMV-Cdk2-HA (addgene #1884), and pcDNA3-c-SRC (addgene #42202). The pFN21a-EBV-PK and pFN21a-EBV-PK-KD constructs (a kind gift from Dr. Shannon Kenney) add a HaloTag at the N-terminus of the EBV-PK kinases. The HaloTag containing constructs with the wild type kinases were then used as templates to create site directed mutants of each kinase by standard cloning methods (see Supplementary Table S1 for primers) and confirmed by sequencing.

Transfections and *in vivo* assays

For Rb phosphorylation assays, Saos-2 cells were seeded at 5×10^5 cells per 60mm dish and cells were transfected 24 hours later using TransIT-2020 (Mirus) according to the manufacturer's instructions. A total of 2.5 μ g DNA was transfected: 1 μ g of Rb expression plasmid and either 1.5 μ g of pFC14a-HA-UL97 constructs or 0.1 μ g of pFN21a-HA-EBV-PK constructs²¹. Lysates were harvested 48 hours post transfection (hpt) and analyzed by Western blot with the following antibodies: Rb 4H1 (Cell Signaling, Cat#9309), Phospho-Rb Ser807/811 (Cell signaling, Cat#9308), HA (Covance MMS-101P), and Tubulin (Sigma, Cat#T9026). Western blot images were visualized using a LiCor Odyssey Fc. For immunofluorescence, coverslips were placed in 10cm dishes and 1×10^6 U-2 OS cells were seeded per dish. 24 hours later cells were transfected using a calcium phosphate co-precipitation method previously described²¹. A total of 25 μ g of DNA were transfected: 10 μ g pEGFP Φ LA-WT expressing GFP-lamin A and either 15 μ g of pCGN-HA-UL97 constructs or 2 μ g of pCGN-HA-EBV-PK constructs, pGEM7 (Promega) was used to balance total DNA levels. Cells were washed 3 times with DMEM 18 hours after DNA transfection, re-fed fresh DMEM with 10% FBS and 1% PSG. Glass coverslips with adherent U-2 OS cells were harvested 48 hours after transfection, washed in PBS (Thermo) and fixed in 1% paraformaldehyde. Indirect immunostaining was performed as previously described²¹. The antibodies used were HA (Roche 3F10) and Alexa Fluorophore 456 (Life Technologies, Cat# A11081). DNA was stained with Hoechst and cells were visualized with a Nikon Eclipse TE2000-S microscope. For the luciferase assays, 2.5×10^5 Saos-2 cells were seeded in a well of a six well plate transfected with TransIT-2020 as previously described²⁶. A total of 1.27 μ g DNA was transfected: 0.25 μ g FLAG-Rb, 1 μ g-kinases, 0.02 μ g E2F1-luc constructs, pCGN-HA as empty vector. Luciferase assays were analyzed using a luciferase reporter system (Promega) and measured on a Veritas microplate luminometer (Turner Biosystems).

Protein purification and *in vitro* reactions

Kinases were purified by following the manufacturer's instructions for HaloTag technology (Promega) as previously described⁵⁷. For the *in vitro* kinase reactions kinases were thawed on ice and aliquoted into their respective 5x kinase reaction buffers in the presence or absence of DMSO (Sigma) or 3-MB-PP1 (Calbiochem, Cat#529582). The final 1x concentration of each kinase buffer used contained: For UL97 50mM Tris pH 8.0, 5mM β -glycerophosphate, 10mM MgCl₂, 2mM DTT; for EBV-PK 100mM Tris pH 7.4, 150mM NaCl, 10mM MgCl₂, 0.5mM DTT, 0.2mM Na₃VO₄, 0.1mM NaF; for Cdk2 50mM Tris pH 7.4, 10mM MgCl₂, 0.1mM EDTA, 2mM DTT, 5mM β -glycerophosphate; for Src 50mM MOPS pH 7.2, 25mM β -glycerophosphate, 10mM EGTA, 4mM EDTA, 40mM MgCl₂, 25mM MnCl₂, 1mM DTT. Each reaction had 1mM of ATP (Sigma-Aldrich) or ATP analog (BioLog). For HCMV-UL97, EBV-PK, and Cdk2 the substrate was an Rb peptide (EMD Millipore, Cat#12-439), for Src the substrate was HaloTag purified Cdk1. All reactions were incubated for 30 minutes, UL97 was incubated at 37°C, and all other kinases at 30°C. Reactions were stopped by addition of SDS solution (1% SDS, 2% β -mercaptoethanol), boiled and analyzed by Western blot with the following antibodies: Rb 4H1 (Cell Signaling, Cat#9309), Phospho-Rb Ser807/811 (Cell signaling, Cat#9308), HA (Covance MMS-101P), Cdk1 (BD Biosciences, Cat# 610038), Cdk2 (Cell Signaling, Cat#2546), Src (Cell

Signaling, Cat#2108), phospho-tyrosine (Cell Signaling, Cat#8954). Western blot images were visualized and quantified where specified using a LiCor Odyssey Fc.

Covalent Capture

The method for lysate labeling was adapted from a previously described protocol⁸. Briefly, 6mg of NHDFs were harvested, lysed, and submitted to a 30 min *in vitro* kinase reaction at 30°C with 60ul of purified kinase in the presence of 1mM N6-ATP- γ -S and 0.5mM ATP to reduce background. Protein samples were then acidified for overnight digestion with Trypsin (Promega, cat# V5113), desalted using C18 Sep-Pack cartridges (Waters, cat# WAT020515), concentrated in a vacuum centrifuge to ~1ml remaining volume per sample, flash frozen in liquid nitrogen and lyophilized overnight on a table top lyophilizer. Reconstituted samples were bound to iodoacetyl SulfoLink beads (Thermo/Pierce, cat# 20401) with rotation overnight in the dark at 4°C. Phosphopeptides were eluted from the beads by oxidation with potassium peroxymonosulfate (oxone) (Sigma-Aldrich, cat# 228036). Eluted peptides were concentrated to ~10ul final volume in a vacuum centrifuge. Final samples were submitted to the University of Wisconsin Biotechnology Center for LC-MS/MS analysis with a Thermo Fisher Scientific Orbitrap Elite. Results were analyzed using SEQUEST and Proteome Viewer software. Peptides reported had a 1% FDR and minimum 2 X-Correlation values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Gatekeeper mutants of EBV-PK and HCMV-UL97

(A–C) Structural models for Cdk2, EBV-PK and HCMV-UL97 showing ATP (purple) docked into the active site. Gatekeeper residues are shown in blue. Residues mutated to make AS3 kinases are shown in green. (D) Sequence alignment of domain V and VII in the ATP binding pocket of the indicated viral and cellular kinases. The gatekeeper residue converted to glycine to make AS1 kinases is shown in the blue box. The residue preceding the DFG motif (shown in bold) that can be converted to alanine to make an AS3 kinase is shown in the green box. Additional residues tested as potential gatekeepers are highlighted in red. (E–F) Saos-2 cells were transfected for 48 hours with plasmids expressing Rb and HA-tagged kinase mutants in the presence of DMSO (–) or 10 μ M 3-MB-PP1 (3-MB) (+). Lysates were analyzed by Western blot with the indicated antibodies. WT, wild type; numbers represent the amino acid residue mutated to glycine (see text for details); KD, kinase dead; p-Rb, phosphospecific antibody for residues S807/811 on Rb; HA, hemagglutinin epitope; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase as a loading control. Image is representative of three independent biological replicates.

Figure 2. Gatekeeper mutants of EBV-PK and HCMV-UL97 phosphorylate known substrates
 (A) Lysates from Saos-2 cells transfected for 48 hours with plasmids expressing Rb and HA-tagged kinases in the presence of DMSO (–) or 10 μ M 3-MB-PP1 (3-MB) (+) were analyzed by Western blot with the indicated antibodies. Images are representative of three independent biological replicates. KD, kinase dead; p-Rb, phosphospecific antibody for residues S807/811 on Rb; HA, hemagglutinin epitope; Tub, tubulin as a loading control. (B) U-2 OS cells were transfected with plasmids expressing GFP tagged lamin A/C and HA tagged kinases and visualized by immunofluorescence microscopy 48 hours later. DNA was stained with Hoechst. Images are representative of three independent biological replicates.

Figure 3. AS-v-Cdks are sensitive to 3-MB-PP1

(A) *In vitro* kinase reactions with purified v-Cdk kinase, Rb peptide as the substrate and the indicated concentrations of 3-MB-PP1 were analyzed by Western blot. Bands corresponding to phosphorylated Rb S807/811 were quantified using the Li-COR Odyssey Fc and normalized to no inhibitor. Each point represents the average of three independent biological replicates. Bars represent the standard error. (B) Values from (A) at 0 μ M (DMSO) and 10 μ M 3-MB-PP1 for each kinase are displayed as analyzed by a two tailed unpaired Student t-test. *, $p < 0.05$; **, $p < 0.002$; n.s., not significant. (C) Saos-2 cells were transfected with Flag tagged Rb, Halo/HA tagged kinases and an E2F1-luciferase reporter plasmid. Lysates were harvested 48 hours later and analyzed for luciferase activity and for protein expression by Western blot. The relative luciferase activity of three independent biological replicates was plotted with error bars representing standard deviation. *, $p < 0.05$; **, $p < 0.01$ by a two tailed unpaired Student's t-test. A representative Western blot image is shown.

Figure 4. AS kinases utilize analog ATPs

(A) Table of bio-orthogonal ATPs used in this study. ATP is shown in box 0. The N⁶ position of the adenine ring is marked by an asterisk. The small box denotes the area of the derivitized ATP molecules shown in panels 1–10. (B) *In vitro* kinase reactions with purified kinases, Rb peptide as the substrate, and the indicated ATP molecule (0–10) were analyzed by Western blot with the indicated antibodies. A representative image of three independent biological replicates is shown. (C) *In vitro* cellular kinase reactions with Rb peptide (Cdk2) or Cdk1 protein (Src) as substrates, in the presence of DMSO, 0.5 μ M 3-MB-PP1 (0.5–3MB), 5 μ M 3-MB-PP1 (5-3MB), 5 μ M 1-NM-PP1 (5-1NM), or the absence of ATP (-ATP) were analyzed by Western blot with the indicated antibodies. p-Tyr is a pan phosphotyrosine antibody. A representative image of three independent biological replicates is shown. (D) *In vitro* kinase reactions with purified cellular kinases, and the indicated ATP molecule (0–10) were analyzed by Western blot with the indicated antibodies. A representative image of three independent biological replicates is shown.

Figure 5. Bio-orthogonal ATP usage by AS kinases

(A) Western blots (three independent biological replicates) from Figure 3B and Figure 3D were quantified using a Li-COR Odyssey Fc. Values from phosphospecific antibodies were used to calculate fold change of AS kinase over wild type for each ATP molecule (0–10 from Figure 3A). The error bars represent standard error. *, $p < 0.1$ using a Wilcoxon ranked sum test comparing AS to wild type.

Figure 6. EBV-PK-AS3 utilizes N6-Phe-ATP- γ -S to selectively label cell lysates

(A) Normal human dermal fibroblast (NHDFs) lysates were incubated with purified EBV-PK-AS3 or EBV-PK-WT in the presence (+) or absence (-) of N6-Phe-ATP- γ -S for 30 min at 30°C. Aliquots were labeled (+) or not (-) with p-nitrobenzyl mesylate (PNBM) for two hours and analyzed by staining with SyproRuby stain for total protein labeling and blotting with a thiophosphate ester (TPE) specific antibody. (B) Thiophosphorylated proteins from two independent biological replicates of *in vitro* reactions with EBV-PK-AS3 (AS3_1 and AS3_2) or EBV-PK-KD (KD_1 and KD_2) were covalently captured (see Methods) and analyzed by mass spectrometry. The number of proteins with phosphorylated peptides for

each replicate is shown with the overlap between samples in a Venn Diagram. (C) Amino acid predominance analysis for phosphorylated peptides present in two independent biological replicates of EBV-PK-AS3 labeled samples. (D) STRING V10.5 interaction network of proteins with phosphopeptides specific to EBV-PK-AS3 labeled samples.

Table 1
EBV-PK substrate identification

Proteins identified in both independent biological replicates of the EBV-PK-AS3 *in vitro* thiophosphorylation, covalent capture, mass spectrometry analysis. The list is arranged from highest to lowest confidence score based on the number of overall peptides detected. Phosphorylation sites detected in only one biological replicate and absent from KD samples are in parenthesis. Phosphorylation sites in bold have been identified previously ⁴⁵.

UniProt ID	Gene	Protein name	Phosphorylation sites	Other UniProt ID
P06400	RB1	Retinoblastoma-associated protein	S888 (S795)	
P02545	LMNA	Prelamin-A/C	S18, T19	
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	S5110	
A0A087WVQ9	EEF1A1	elongation factor 1-alpha1	T240	P68104 (T261)
Q99459	CDC5L	Cell division cycle 5-like protein	T424	
H0YFY6	NUMA1	Nuclear mitotic apparatus protein 1	T2106	Q14980
Q5TDI3	NUP133	Nuclear pore complex protein Nup133	S27 , T28 (S45)	Q8WUM0
Q9UQ35	SRRM2	Serine/arginine repetitive matrix protein 2	T2367, S2368 (T367, S2365, T2381)	
F5GWX5	CHD4	Chromodomain-helicase-DNA-binding protein 4	T1646	Q14839 (T1653)
F8W9Y9	MTA1	Metastasis-associated protein MTA1	T100	Q13330 (T564)
H0Y5I5	ZNF687	Zinc finger protein 687	T503	Q8N1G0 (T900)
H0YF21	MAP7D1	MAP7 domain-containing protein 1	S43	Q3KQU3 (S460)
O75533	SF3B1	Splicing factor 3B subunit 1	T326 (T248, T278)	
Q03252	LMNB2	Lamin-B2	T34	
P46821	MAP1B	Microtubule-associated protein 1B	T1788	
O43432	EIF4G3	Eukaryotic translation initiation factor 4 gamma 3	T678	
C9J830	PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit	T54	P13861
Q9Y2W1	THRAP3	Thyroid hormone receptor-associated protein 3	S243	
Q8WWQ0	PHIP	PH-interacting protein	T1480 (S1479)	
H3BQZ7	HNRNPUL2-BSCL2	Heterogeneous Nuclear Ribonucleoprotein U Like 2	T165	Q1KMD3
Q9HCK8	CHD8	Chromodomain-helicase-DNA-binding protein 8	T2051	