Regulation of Microfilament Organization by Kaposi Sarcoma-associated Herpes Virus-cyclin-CDK6 Phosphorylation of Caldesmon

Maria Emanuela Cuomo, Axel Knebel, Georgina Platt, Nick Morrice, Philip Cohen, and Sibylle Mittnacht

From the Cancer Research UK Centre for Cell and Molecular Biology, Chester Beatty Laboratories, The Institute of Cancer Research, 237 Fulham Road, SW3 6JB London, United Kingdom, MRC Protein Phosphorylation Unit, University of Dundee, MSI/WTB Complex, Dow Street, DD1 5EH Dundee, United Kingdom, and KinaseSource, Laboratory 4.21, MSI/WTB Complex, Dow Street, DD1 5EH Dundee, United Kingdom

Kaposi sarcoma-associated herpes virus (KSHV) encodes a D-like cyclin (K-cyclin) that is thought to contribute to the viral oncogenicity. K-cyclin activates cellular cyclin-dependent kinases (CDK) 4 and 6, generating enzymes with a substrate selectivity deviant from CDK4 and CDK6 activated by D-type cyclins, suggesting different biochemical and biological functions. Here we report the identification of the actin- and calmodulin-binding protein caldesmon (CALD1) as a novel K-cyclin-CDK substrate, which is not phosphorylated by D-CDK. CALD1 plays a central role in the regulation of microfilament organization, consequently controlling cell shape, adhesion, cytokinesis and motility. K-cyclin-CDK6 specifically phosphorylates four Ser/Thr sites in the human CALD1 carboxyl terminus, abolishing CALD1 binding to its effector protein, actin, and its regulator protein, calmodulin. CALD1 is hyperphosphorylated in cells following K-cyclin expression and in KSHV-transformed lymphoma cells. Moreover, expression of exogenous K-cyclin results in microfilament loss and changes in cell morphology; both effects are reliant on CDK catalysis and can be reversed by the expression of a phosphorylation defective CALD1. Together, these data strongly suggest that K-cyclin expression modulates the activity of caldesmon and through this the microfilament functions in cells. These results establish a novel link between KSHV infection and the regulation of the actin cytoskeleton.

Nearly all cellular responses are controlled through protein phosphorylation and the protein kinases catalyzing this process represent the largest single family of enzymes (1). The choice of substrates determines the selectivity of the kinase action and its cellular impact. Therefore, the nature of the substrates of a kinase can provide important clues as to its physiological role and function (2).

Recently, several oncogenic gamma herpesviruses have been described to contain within their genome a cyclin-like activator for cyclin-dependent kinases (CDKs) (3). The Kaposi sarcoma herpes virus (KSHV) or human herpes virus 8 (HHV8), a human tumor virus associated with the development of Kaposi sarcoma and several lymphoid malignancies in immunocompromised individuals (4–6), encodes a cyclin (K-cyclin) that is thought to have descended from cellular D-type cyclins based on co-linearity and sequence identity. Strong evidence from transgenic mouse models suggests that K-cyclin contributes significantly to the oncogenic process elicited by this virus (7, 8).

D-type cyclins are recognized for their involvement in human oncomogenesis (9) and K-cyclin shares their ability to activate the closely related cellular CDK4 and CDK6 and hence phosphorylate and inactivate the retinoblastoma tumor suppressor protein (Rb) (10).

In addition to Rb, K-cyclin-CDK complexes can phosphorylate proteins that are not substrates for those CDKs when activated by cellular cyclin D. These include the CDK inhibitor p27CKI, which is normally phosphorylated by cyclin E-CDK2, targeting it for degradation by the proteasome (11, 12). They also include cdc6 and orc1, through which K-cyclin may initiate DNA replication in a manner analogous to cyclin A (13, 14). A further substrate for K-cyclin-CDK is Bcl2, with consequent loss of its anti-apoptotic function (15). In KSHV negative cells Bcl2 phosphorylation is facilitated by c-Jun N-terminal kinase in response to mitotic checkpoint activation (16). These observations suggest that K-cyclin-CDK6 complexes mimic the activity of a range of other cellular kinases with an impact on cellular functions distinct from cyclin D. The extent to which K-cyclin-activated CDKs phosphorylate noncanonical substrates is currently unknown.

To systematically approach this question, we undertook a kinase substrate tracking and elucidation (KESTREL) screen (17) in which we searched for proteins phosphorylated by K-cyclin-CDK6. Here, we report the identification of human caldesmon (hCALD1) as a novel substrate for CDK6 kinase in complex with K-cyclin.

CALD1 regulates microfilament organization and activities in complex ways (18, 19). In its active form it associates with and cross-links actin microfilaments. This assists their bundling and stability (20, 21), possibly through interference with actin-severing and -capping activities (22). Other evidence indicates that CALD1 inhibits binding of Arp2/3 to actin, opposing the accelerated filament growth and branching that arise in conjunction with ruffling movement and membrane protrusion (23). CALD1 can also bind to myosin and in its actin-bound form inhibits the actin-activated ATPase activity of myosin (24). Caldesmon functions are regulated by Ca2+/calmodulin binding and by assisted laser desorption ionization time-of-flight; MS/MS, tandem mass spectrometry; CalM, calmodulin; EGFP, enhanced green fluorescent protein; KESTREL, kinase substrate tracking and elucidation; GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; hCALD1, human caldesmon.
phosphorylation, which inhibits actin association and actin-myosin ATPase inhibition (25–31). Modulation of CALD1 activity facilitates the control of a complex array of cell features including cell shape, cytohesin, cell adhesion, cell-cell contact, motility, contraction, and the internal movement of cell organelles (27, 29, 31–35).

The results presented here provide novel insight into substrate phosphorylation by K-cyclin-associated kinases and further implicate this cyclin in the regulation of microfilament-associated functions via the ectopic phosphorylation and inactivation of caldesmon.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Related Procedures—*U2OS, NIH3T3, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 4.8 mM l-glutamine at 37 °C and 5% CO2-KSHV positive PEL cell lines BC-3 (36) and BCP-1 (37) and the EBV immortalized lymphoblastoid LCL3 were maintained in RPMI 1640 medium with 10% fetal calf serum. Frozen HeLa cell pellets were purchased from 4C Biotech. NIH3T3 and U2OS cells were transfected using GenePORTER™ (Gene Therapy Systems, Inc.). Total cell lysates were produced in 0.25 M NaCl, 10 mM HEPES-KOH, pH 7.0, 5 mM β-mercaptoethanol, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride, 1 mM aprotinin, 2.5 μg/ml leupeptin, and 0.5% v/v Triton X-100.

*Plasmids and Molecular Cloning—*pGEX2T-Rb (763–928) for the expression of GST-Rb Ct (the recombinant carboxyl-terminal fragment of the human retinoblastoma protein) in bacteria and the construction of hCALD1 vectors for cyclinCDK expression have been described (38, 39). A full-length cDNA for hCALD1 was produced by reverse transcription-PCR from HeLa cell using RNAzol B (Biogenesis) for RNA preparation and the cDNA cycle kit (Invitrogen) for cDNA synthesis. The PCR primers used were 5’-CGCGGATCCTAGGATTTGGACGAGTTGACGAGTTGACGAGTTGAG-3’ (forward) and 5’-CGCCTCGAGTCAAACCTCCGCTCGAGTCAAACCTCCGCTCGAGTCAAACCTCCGCTCGAGTCAAACCTCCGCTCGAGTCAAACCTCCGCTCGAGTCAAACCTCCGCTCGAGTCAAACCTCCGCTCGAGTCAAACCTCCGCTCGAG-3’ (reverse), covering the full reading frame of human hCALD1 (Swiss-Prot/GenBank™ accession number Q05682). To facilitate directional cloning, primers were designed to introduce a restriction consensus for BamHI adjacent to the start and one for XhoI adjacent to the stop codon (underlined). Following amplification, a major product was obtained, gel-purified, and inserted into BamHI/XhoI-restricted pcDNA3HisMAX C (Invitrogen) for mammalian expression or pGEX-6p1 (Amersham Biosciences) for bacterial expression. Clones containing inserts of the correct size were selected and their identity validated by DNA sequencing.

pcDNA-K-cyclin, pCMV CDK6 and CDK6DN, and expression plasmid for the phosphorylation-defective CALD1 (CALD1 7th) have been described (11, 27, 40). pCMV-enhanced green fluorescent protein (EGFP) vector was from Invitrogen.

*KESTREL Screen—*KESTREL screening was performed essentially as described (17). Briefly, cleared HeLa cell nuclear and cytosolic extracts (equivalent to 15 mg of total protein) were fractionated independently using sequential Mono-Q and Mono-S chromatography, and individual fractions were used as substrate for K-cyclin-CDK6 or various controls. Screening for substrates was performed for 5 min at 30 °C using 25 μl of each fraction and 2 milliunits of cyclin-CDK, or the equivalent amount of protein in the inactive monomeric CDK preparation, in a total volume of 30 μl of KESTREL kinase buffer (4 mM ATP, 5 × 104 cpm of [γ-32P]ATP, 40 mM Tris-HCl, pH 7.5, 2 mM MnCl2, 1 mM dithiothreitol, aprotinin 20 μg/ml, leupeptin 20 μg/ml). Reactions were terminated with 10 μl of SDS-loading buffer (320 mM Tris-HCl pH 6.8, 8% (v/v) SDS, 20 mM EDTA, 32% (v/v) glycerol, 1.14 mM β-mercaptoethanol, 0.02% w/v bromphenol blue) and analyzed using a 7% SDS-polyacrylamide gel followed by blotting onto a polyvinylidene difluoride membrane and exposure to x-ray film.

**Identification of p90 by Mass Spectrometry—**Purified p90 was incubated for 10 min in the absence or presence of 2 milliunits of K-cyclinCDK6 with 10 mM magnesium and 0.1 mM [γ-32P]ATP, denatured in SDS, alkylated, loaded on a NuPAGE 4–12% gradient Tris-glycine gel (Novex), and visualized using SYPRO Orange (Molecular Probes). The prominent 32P-labeled SYPRO Orange-stained protein band was excised, digested with trypsin, and analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF, Perseptive Biosystem Elite STR) as described (17, 41).

**Phosphorylation Site Mapping—**HeLa cell-derived p90 was phosphorylated for 30 min at 30 °C using 2 milliunits of K-cyclinCDK6 in 40 μl of KESTREL kinase buffer. 32P-Labeled proteins were digested with trypsin and the resulting peptides separated by HPLC on C18 resin as described previously. The relative amount of phosphorylation for each peptide was estimated from the amount of radioactivity associated divided by the amount of radioactivity loaded. Phosphopeptides were analyzed by MALDI-TOF-TOF mass spectrometry on an Applied Biosystems 4700 proteomics analyzer, with the peptide sequence and position of phosphorylation determined by MALDI-MS/MS fragmentation of selected phosphopeptide parent ions. Individual MALDI-MS/MS spectra were searched using the Mascot search engine (MatrixScience) run on a local server. Spectra were also annotated manually. In some instances phosphopeptides were identified using nanoelectrospray mass spectrometry on a Micromass Q-TOF-2 or an Applied Biosystems 4000 Q-Trap mass spectrometer. For routine confirmation, solid phase Edman degradation and one-dimensional phosphoamino acid analysis was performed as described (17, 42). Residue numbering used throughout relates to the sequence of hCALD1 (Swiss-Prot/GenBank™ accession number Q05682).

**Recombinant Protein Production and Related Procedures—**His-hCALD1 was purified from U2OS or NIH3T3 cells transiently transfected with pcDNA3HisMAX C-hCALD1 using TALON metal affinity resin (Clontech). Production and purification of GST-tagged proteins was as described (38). In *Vitro Protein Phosphorylation—*Production of K-cyclinCDK6, cyclin D1-CDK4, cyclin E1-CDK2, cyclin B1-CDK1, and monomeric CDK controls using recombinant baculoviruses was performed as described (38). Specific activities of the different kinase preparations (mol of ATP transfer/mol of substrate) were estimated using GST-Rb Ct as a substrate. Unless indicated otherwise, phosphorylation reactions were conducted in cyclin D kinase buffer as described previously (38). For routine radioactive reactions, substrates were exposed to kinase for 10 min at 27 °C in the presence of 10 μM ATP and 0.1 μCi of [γ-32P]ATP in a final volume of 20 μl. Radioactive products were separated on SDS-PAGE and visualized by autoradiography. Signals were quantified by PhosphorImager. Bulk phosphorylated GST-hCALD1 for biochemical experiments was produced by incubating 5 μg of substrate for 30 min at 27 °C in a 100-μl reaction containing 1 mM ATP. The amount of kinase used was optimized to give maximal recognition by the phospho-specific hCALD1 antibodies.

For phosphorylation of hCALD1 in the presence of F-actin, F-actin was preincubated for 30 min on ice with GST-hCALD1 at a 1:1 molar ratio in a final volume of 20 μl. 1 μl of kinase and 9 μl of reaction mix containing 30 μM ATP and 0.3 μCi of [γ-32P]ATP were added, and reactions were incubated for 10 min at 27 °C. Conditions for hCALD1 phos-
Caldesmon Phosphorylation by K-cyclin-CDK6

Phosphorylation in the presence of calmodulin (CaM) were identical, except that the ratio of CaM to GST-hCALD1 was 5:1 and the kinase buffer contained 2.5 mM CaCl₂.

**Antibodies**—The antibody reagents used were: mouse monoclonal α-hCALD1 pan antibody C56520 (Transduction Laboratories), mouse monoclonal 9E10 α-vec tag antibody (HybriDoma Unit at CBL, ICR), mouse monoclonal α-actin pan antibody Ab5 (NeoMarker), mouse monoclonal α-human Rb antibody 14001A (BD Pharmingen), rabbit polyclonal α-CALD6 antibody C-21 (Santa Cruz Biotechnology), mouse α-γ-tubulin (Sigma), horseradish peroxidase-conjugated secondary antibodies (Pierce). Sheep sera with selectivity for hCALD1 phosphorylated on Thr-730 (α-Ph-hCALD1 730) and Ser-789 (α-Ph-hCALD1 789) were produced by immunizing sheep with keyhole limpet hemocyanin-conjugated phosphopeptides (Thr723CSPTAAG(pT)PNKETA736 where pT is phosphorylated) and 782CSVDKVT(pS)PTKV793 (where pS is phosphorylated), respectively. Sera were affinity-purified by chromatography on resin-coupled phosphopeptides. For immunoblot analysis, they were used in the presence of 0.5 μg/ml unphosphorylated peptides.

**Calmodulin-Affinity Chromatography**—The p90-containing Mono-Q fraction was loaded onto a 0.5-ml CaM-Sepharose 4B column (Amersham Biosciences) and processed as recommended by the manufacturer. CaM binding assays using phosphorylated GST-hCALD1 were performed using CaM-Sepharose 4B, essentially as described (25). Fractions were examined by SDS-PAGE using SYPRO Orange staining or immunoblotting.

**Actin Binding Assays**—G-actin binding was assessed by incubating phosphorylated or mock-phosphorylated GST-hCALD1 (1 μg) with 6 μM G-actin (Cytoskeleton Inc.) for 30 min at 4 °C in 50 mM HEPES-KOH, pH 7.5, 0.15 mM NaCl, 10 mM MgCl₂, 1 mM ATP, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% (w/v) aprotinin, 2.5 μg/ml leupeptin, 0.5% (v/v) Triton X-100). GST-hCALD1 was subsequently recovered on 10 μl of packed glutathione-Sepharose 4B (Amersham Biosciences). Beads were washed three times with 1 ml of HEPES buffer and analyzed for associated actin and GST-hCALD1 by SDS-PAGE and immunoblot.

F-actin was prepared using the non-muscle actin-binding protein spun down assay kit (Cytoskeleton Inc.). F-actin/ligand binding was monitored by co-sedimentation following the instructions provided. Supernatant and pellet fractions were collected, resuspended, and analyzed by SDS-PAGE and immunoblot.

**siRNA**—BC-3 primary effusion leukemia cells were seeded at a density of 1 × 10⁵ cell/ml into 6-well dishes and transfected with small interfering RNA duplexes (siRNA) using HiPerFect™ agent (Qiagen) according to the manufacturer’s instructions. Sequences for targeting the major latency transcripts L1/L2 (43), which encode K-cyclin, were: 5′-AAGUGUAAUUGUUCUCUAA-3′, siRNA1; 5′-AATAGCATCAATGCTGACCATC-3′, siRNA2. A siRNA pool targeting an irrelevant, nonessential cell protein (PRK, Sigene SMART POOL M-003527-00, Dharmakon) and transfection agent alone were used as controls.

**Immunofluorescence Microscopy**—Cells were fixed 24 h following transfection in 4% paraformaldehyde for 5 min at room temperature and stained with Texas Red-X-labeled phalloidin (Molecular Probes) for 20 min at room temperature. Fluorescence images were acquired using a Bio-Rad MRC1024 confocal microscope. The mean phalloidin-associated fluorescence intensity, cell perimeter, and cell circularity of individual transfected cells was determined using the ImageJ program (rsb.info.nih.gov/ij/).

**RESULTS**

**KESTREL Screen for Novel Cellular Substrates of K-cyclin-CDK6**—The identification of novel target proteins is a powerful means to delineate the physiological significance and impact of a given kinase. The KESTREL methodology, which uses fractionated cellular extract as a substrate for exogenous purified kinases provides a powerful strategy for the identification of physiologically relevant protein kinase substrates (17, 44–47). We used this methodology to identify novel biological targets for K-cyclin-activated CDK6. Both nuclear and cytosolic extracts derived from exponentially growing HeLa cells were screened following fractionation on either cation or anion chromatography. All resultant fractions were assayed in parallel using K-cyclin in complex with human CDK6 (K-cyclin-CDK6), CDK6 alone (as a negative control), or human cyclin D1 in complex with human CDK4 (cyclin D1-CDK4) for a closely related cellular kinase. All kinase complexes were prepared from baculovirus-infected insect cells. Cyclin D activates both CDK4 and CDK6 to phosphorylate Rb to a similar extent, but cyclin D1-CDK4 complexes have a higher specific kinase activity when produced in insect cells, and thus this kinase complex was used throughout the KESTREL screen. Reaction conditions were chosen such that a common substrate for K-cyclin-CDK6 and cyclin D1-CDK4, Rb Ct (amino acids 763–928), was phosphorylated equally by both of the enzymes (Fig. 1A). No incorporation of phosphate into Rb Ct was observed with CDK6 alone, indicating that a kinase activity resembling K-cyclin-CDK6 is not generated in insect cells in the absence of K-cyclin expression. To distinguish phosphorylation products that may derive from contaminants in the kinase preparation or by autophosphorylation of the kinase itself, a set of reactions was run in the absence of exogenous substrates (see for example Fig. 1B, lanes 1–3). Lastly, all fractions were assayed without exogenous kinase preparation in order to identify signals arising from phosphorylation by HeLa cell-derived kinases (see for example Fig. 1B, lane 7). We note that omission of exogenous kinase often yielded phosphorylation activity that was not seen when kinase preparation had been added to the fraction, suggesting that one or more components of the insect cell-derived kinase preparations may inhibit the endogenous kinase activities present in the column fractions (compare for example lanes 4–6 with lane 7 in Fig. 1B).

In total, 80 different HeLa-derived fractions were screened yielding evidence for a minimum of four distinct substrates that were phosphorylated specifically in K-cyclin-CDK6- but not cyclinD1-CDK4-containing reactions (not shown). This confirms the known ability of K-cyclin to modulate CDK substrate selection toward a broader range of substrates when compared with cellular cyclin D. In addition, two substrates specifically phosphorylated in vitro by cyclin D1-CDK4 but not K-cyclin-CDK6 were discovered. This could suggest that diversion of K-cyclin from cyclin D generates a kinase activity that does not sufficiently phosphorylate other substrates in a manner that is not seen in the K-cyclin-CDK6 screening experiment (not shown).

**Fig. 1B shows the phosphorylation pattern of Mono-Q fraction 8 from nuclear extract, revealing a putative protein substrate with an apparent molecular mass of 90 kDa (p90) that is strongly phosphorylated in reactions containing K-cyclin-CDK6 (lane 5). Phosphorylation of p90 is not apparent in samples containing CDK6 alone (lane 4) or cyclin D-CDK4 (lane 6) or in the sample containing K-cyclin-CDK6 in the absence of added substrate, inferring that p90 is derived from HeLa cells and phosphorylated selectively by K-cyclin-CDK6.

**Identification of p90**—To determine the identity of p90, we purified this putative substrate from HeLa nuclear extracts employing sequential chromatography on Mono-Q, heparin-Sepharose, and Mono-S (see
supplemental Fig. S1) and phosphorylation by K-cyclin-CDK6 to track its location on each column. p90 substrate positive fractions from Mono-S were pooled and loaded on a gradient gel followed by SYPRO Orange staining. The major product in this preparation was a 90-kDa protein (Fig. 1C), which co-migrated with radiolabeled K-cyclin-CDK6-phosphorylated p90 (not shown). Tryptic fingerprinting of this protein yielded peptide masses matching with hCALD1 with sequence coverage of 25% (summarized in supplemental Fig. S2). This suggested that the 90-kDa K-cyclin-CDK6 substrate is human caldesmon.

To substantiate that the p90 substrate and caldesmon were the same protein, we tested whether p90 from the Mono-Q column (see Fig. 1B) was retained in a Ca2+-dependent manner on a CaM affinity column, as would be expected for CALD1, which is known to bind Ca2+/CaM. p90 effectively bound to the resin in the presence of Ca2+, being undetectable in either the flow-through (FT) or wash fractions (W1, W2) but readily detectable in the eluate when using EGTA-containing buffer (E1, E2, E3) (Fig. 1D). This finding strongly supported the assignment made on the basis of the tryptic mass fingerprinting.

**Identification of Phosphorylation Sites by Mass Spectrometry**—To confirm that K-cyclin-CDK6 phosphorylates p90-hCALD1 and to identify the sites of phosphorylation, we processed the HeLa-derived p90 after incubation with K-cyclin-CDK6 for identification of phosphorylated peptides. Separation on reverse phase chromatography of the tryptic digest of purified p90 substrate showed two major radioactive peaks (Fig. 2A, P1 and P2). Mass spectrometry analysis revealed that each peak contained a single phosphopeptide with masses consistent with hCALD1-derived tryptic peptides comprising residues 782–792 (P1) and residues 719–739 (P2). One-dimensional phosphoamino acid analysis demonstrated modification on serine for P1 and threonine for P2, and solid phase Edman degradation confirmed that the sites of phosphorylation were on residue 8 for peptide P2 and residue 12 for P2, corresponding to Ser-789 and Thr-730, respectively (Fig. 2, C and D). The more minor release of 32P at residue 8 for peptide P2 (Fig. 2D) could represent the phosphorylation of threonine 726, but there was no mass spectral evidence to suggest that the diphosphopeptide phosphorylated at both threonine 726 and threonine 730 was present. Both Thr-730 and Ser-789 comply with the (S/T)P consensus known to be required for the phosphorylation by CDK4/6 kinases, thus providing strong evidence that hCALD1 is a direct substrate for K-cyclin-CDK6. The radioactivity associated with the respective peaks accounted for 40% (P1) and 46% (P2) of the total incorporated radioactivity, suggesting that Thr-730 and Ser-789 are the major K-cyclin-CDK6 phosphorylated sites in hCALD1 purified from exponentially growing HeLa cells.

**K-cyclin Phosphorylation of CALD1 in Vitro**—To provide independent evidence that hCALD1 is a direct substrate for K-cyclin-CDK6, we generated two recombinant forms of human caldesmon. A construct was engineered for the expression in *Escherichia coli* of full-length hCALD1 as a GST-tagged protein, allowing purification of the product.
by affinity chromatography on glutathione-Sepharose. Furthermore, we generated a construct for mammalian cell expression of a hexahistidine (His)-tagged hCALD1, permitting purification of the product by immobilized metal affinity chromatography (IMAC). Using purified versions of these proteins as substrates, we probed for phosphorylation by K-cyclin/CDK6 together with other cyclin/CDKs (Fig. 3, A and B). Reactions containing GST-Rb Ct, a common substrate for these kinases, were run in parallel as a reference for the relative level of activity associated with each kinase (Fig. 3A, lower panels). Comparable amounts of GST-hCALD1 and His-hCALD1 were present in each sample as determined by Western blot (Fig. 3A, top right panel; Fig. 3B, bottom panel). Both forms of recombinant hCALD1 were avidly phosphorylated by K-cyclin-CDK6, corroborating the proposition that human caldesmon constitutes a genuine substrate for this kinase in vitro. Consistent with results from the KESTREL screen, recombinant hCALD1 was not phosphorylated by cyclinD1-CDK4, although this kinase effectively phosphorylated the GST-Rb Ct reference substrate. However, hCALD1 was phosphorylated by cyclin A2-CDK2, cyclin A2-CDK1, cyclin B1-CDK1, and to a lesser extent by cyclin E1-CDK2. It has previously been reported that purified mitotic HeLa cell CDK1 can phosphorylate CALD1 (34, 48–50) and that this may be critical for the induction of microfilament disassembly during mitosis (48, 51). However, phosphorylation of CALD1 by cyclin A and E-associated kinases, which gain activity during G1 and S phase, has not been reported previously.

We further assessed whether phosphorylation of recombinant hCALD1 arises at Thr-730 and Ser-798 by immunoblotting using phospho-specific antisera to these sites. Neither α-P-hCALD1 730 nor α-P-hCALD1 789 serum recognized GST-hCALD1 exposed to inactive, 

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monomeric CDK6, but both antibodies gave signals with hCALD1 phosphorylated in the presence of K-cyclin/CDK6, cyclin A/CDK2, cyclin E/CDK2, and cyclin B/CDK1, but not cyclin D1/CDK4, although comparable amounts of hCALD1 were present in each sample (Fig. 3C, top panel). Recognition of phosphorylated caldesmon by these antibodies is dependent on the presence of phosphate-accepting residues at their cognate recognition site (supplemental Fig. S3). This demonstrated the site-selective and phosphorylation-dependent recognition of hCALD1 by these reagents and established that K-cyclin/CDK6, as well as cellular cyclin A, E, and B-activated CDKs, can phosphorylate recombinant hCALD1 on Thr-730 and Ser-789 in vitro.

K-cyclin Phosphorylation of hCALD1 in Vivo—In vitro hCALD1 can be phosphorylated by several serine/threonine protein kinases, including cyclin B2/CDK1 (34), Ca²⁺/calmodulin kinase II (52), casein kinase II (53–55), p21-activated kinase (56), protein kinase C (57, 58), and extracellular signal-regulated kinases (ERK) (59, 60). However, evidence for CALD1 phosphorylation in cells has been provided only for the mitosis-activated CDK1 in non-muscle cells (25, 48) and ERK in smooth muscle (59–61).

To address whether hCALD1 is capable of being phosphorylated by K-cyclin/CDK6 in cells, we transiently co-expressed His-hCALD1, K-cyclin, and CDK6 in osteosarcoma-derived U2OS, which do not contain the KSHV sequence to express K-cyclin, and investigated the phosphorylation state of hCALD1. Analysis of cell lysates showed that His-hCALD1 was expressed adequately and to a similar level, regardless of the presence of K-cyclin/CDK6, and further, that K-cyclin and CDK6 were expressed correctly (Fig. 4A). After IMAC purification of lysates, analysis of the His-hCALD1 using phosphorylation-selective hCALD1 antibodies demonstrated that Thr-730 is not detectably phosphorylated in cells in the absence of K-cyclin/CDK6 but that a strong signal is observed when cells express K-cyclin/CDK6 (Fig. 4B, middle panel). By performing immunoblots of serial dilutions of each sample (2-fold lower each time), we showed that phosphorylation of Thr-730 increases by more than 8-fold, thus indicating a major effect of K-cyclin/CDK6 on the modification of this site (Fig. 4B, top and middle panels). In contrast, Ser-789 was detectably phosphorylated in cells that did not express K-cyclin/CDK6, in line with reports that Ser-789 is prominently modified in cells (60, 61). Despite this finding, some increase is observed in
cells containing K-cyclin/CDK6 (Fig. 4B, bottom panel). Taken together, these results support the notion that K-cyclin/CDK6 complexes can modulate phosphorylation of hCALD1 in cells and do it both quantitatively, by increasing the level of baseline phosphorylation, and qualitatively, by introducing a modification that is not readily found in the absence of this kinase complex.

To extend our analysis, we compared the phosphorylation state of endogenous hCALD1 in KSHV negative (LCL3) and KSHV positive (BC-3 and BCP-1) pre-B cell-derived lymphoblastoid cell lines. Both BC-3 and BCP-1 express K-cyclin mRNA and protein, whereas these are undetectable in LCL3 (62). Comparative analysis of lysates derived from these cell lines using α-P-hCALD1 730 antibodies revealed that this site was barely phosphorylated in LCL3 but was considerably modified in the KSHV positive BC-3 and BCP-1 cells (Fig. 4C, middle panel). Modification of Ser-789 did not rise as markedly, consistent with the notion that this site may already be modified constitutively at a high level in KSHV negative cells, and only a minor increase was apparent in the KSHV positive lines (Fig. 4C, bottom panel). Thus, in cells with natural K-cyclin expression, we detected a change of hCALD1 phosphorylation, which
in degree and appearance is greatly reminiscent of that seen with exogenous expression of K-cyclin-CDK6. To further confirm the link between the enhanced CALD1 phosphorylation and K-cyclin, we made use of RNA interference to diminish K-cyclin protein expression in BC-3 PEL cells (Fig. 4D). Both siRNA1 and siRNA2, designed to target K-cyclin-encoding transcripts, down-regulated K-cyclin protein by about 80 and 50%, respectively, and treatment with both reduced phosphorylation of CALD1 Thr-730 when compared with mock treated cells. In contrast, an irrelevant control siRNA (siRNA C) had no effect. None of the siRNA oligonucleotides used affected the levels of CALD1 protein itself. Phosphorylation of Ser-789 was also not detectably affected, in accordance with the previous observation that modification of Ser-789 may be largely K-cyclin-independent. Equal loading of protein was demonstrated with antibodies against γ-tubulin (Fig. 4D, bottom panel). Thus, down-regulation of K-cyclin in KSHV-infected cells results in a specific alteration of CALD1 phosphorylation status, providing additional confidence that K-cyclin can drive caldesmon modification in vivo. Together, the above experiments provide strong evidence that hCALD1 is a bona fide in vivo substrate for K-cyclin-activated kinase.

Saturation Mapping of Phospho-sites on Recombinant Human Caldesmon—Our previous results (Fig. 2) showed that K-cyclin-CDK6 phosphorylates HeLa-derived p90 substrate on two major sites. However, six sites complying with the canonical consensus (S/T)P are present in hCALD1, five of which are conserved in homologues from other mammalian species (see Fig. 5E) (48). Furthermore, previous work on mitotic CDK1-phosphorylated CALD1 using either Edman sequencing (49) or mutagenesis (63) provided evidence for in vitro phosphorylation of sites in addition to Thr-730 and Ser-789.

Therefore, using recombinant GST-hCALD1 as a substrate, we reevaluated the extent of possible phosphorylation by K-cyclin-CDK6. Phosphorylation of GST-hCALD1 resulted in near maximal incorporation of phosphate after 30 min (Fig. 5A). C$_{18}$ chromatography of tryptic digests derived from this material resolved into seven major $^{32}$P-labeled peaks (P1-P7) (Fig. 5B), together accounting for more than 90% of the total radioactivity incorporated and, hence, likely to account for the full complement of phosphorylated residues. Mass spectrometry analysis unambiguously located the peptides corresponding to six of the peaks within the hCALD1 sequence, together with the identification of the phosphorylated residues (for summary see Fig. 5C). P1 and P2 peptides were identical to P1 and P2 as observed and characterized in our analysis using cell-derived hCALD1 (see Fig. 2), thus confirming phosphorylation of the recombinant hCALD1 on Thr-730 and Ser-789. The identity of the peptide producing peak P3 could not be determined by either mass spectrometry or Edman sequencing, raising the possibility that it represents a very small peptide species that evades detection by these methods. P4 and P5 were found to contain peptides related to P1 and P2, phosphorylated on Thr-730 and Ser-789, respectively. P6 and P7 were related peptides with masses that predicted phosphorylation at both Thr-753 and Ser-759. Both of these peptides contain trypsin-missed cleavages between Lys-752 and Thr-753 and Lys-758 and Ser-759 due to the presence of a phosphorylated residue next to the site of tryptic cleavage. Both peptides also contain the oxidized form of tryptophan (kynurenin), and interestingly, the P6 peptide was generated by an unusual tryptic cleavage across the Lys-762–Pro-763 bond, which is normally trypsin-resistant. The sites of phosphorylation were confirmed by both MALDI-MS/MS (not shown) as well as solid phase Edman degradation, which revealed releases of radioactivity following cycle 8 and cycle 14 for each peptide (Fig. 5D).

Together, our analyses reveal that, as for the cell-derived hCALD1 substrates, Thr-730 and Ser-789 are major sites of phosphorylation in recombinant hCALD1. In addition, Thr-753 and Ser-759 are modified in recombinant material (see Fig. 5E for summary). It is notable that 45% of the total radioactivity was associated with peaks P6 and P7, which cover Thr-753 and Ser-759, indicative that phosphorylation on these sites occurs at a stoichiometry similar to that of Thr-730 and Ser-789. Why Thr-753 and Ser-759 are not apparently phosphorylated in cell-derived hCALD1 is not clear, but it may be because of prior phosphorylation of these sites by cell-derived kinases, dephosphorylation by phosphatases contaminating the cell-derived hCALD1 preparation, and/or incomplete phosphorylation of the substrate under the conditions used. We noted that all sites identified in the recombinant hCALD1 conformed to the known consensus for phosphorylation by CDK.

The array of sites modified by K-cyclin-CDK6 in recombinant hCALD1 closely resembles, but is not identical to, those reported for rat or chicken caldesmon phosphorylated by mitotic CDK1 (63). In these studies CDK1 was found to modify Ser-724, which apparently is not phosphorylated by K-cyclin-CDK6. Although Ser-724 is contained within peptides yielding peak P2 from both cell-derived and recombinant hCALD1 and P5 from recombinant hCALD1, it is always detected in an unphosphorylated state, suggesting that it may not be used as a site for modification by K-cyclin-CDK6. In chicken CALD1, mitotic CDK1 also targets a site corresponding to Thr-638 in the human sequence. It is possible that P3, which evaded identification in our analysis, contains a peptide with modification on this residue. The small size of this predicted tryptic peptide, CFTPK, is in line with such an assumption.

Effects of K-cyclin-CDK6 Phosphorylation on hCALD1 Function—The results presented above indicate that K-cyclin-CDK6 is capable of targeting the majority of sites known to become modified upon CALD1 phosphorylation by mitotic CDK1 in vitro. Importantly, these sites cluster within the regions known to facilitate binding of CALD1 to actin (see Fig. 5E) (64–67), and phosphorylation of CALD1 by mitotic CDK1 (25, 63) and ERK1 (28, 30) has previously been shown to affect this interaction.

We therefore investigated the impact of K-cyclin-CDK6 phosphorylation on binding of GST-hCALD1 to actin filaments using the known ability of CALD1 to associate and co-sediment with filamentous (F-) actin. Consistent with this, the majority of GST-hCALD1 was drawn into the pellet fraction when centrifuged in the presence of F-actin while remaining in the supernatant when on its own (Fig. 6A). Co-sedimentation with F-actin was unaffected when CALD1 alone was used during the phosphorylation reaction. In contrast, GST-hCALD1 phosphorylated in the presence of K-cyclin-CDK6 or cyclin B-CDK1 remained in the supernatant, thus providing evidence that phosphorylation by K-cyclin-CDK6 disables the association of hCALD1 with actin filaments as it does phosphorylation by cyclin B-CDK1.

In addition to its ability to interact with F-actin, which results in actin filament bundling and actin-myosin cross-linking, CALD1 can bind to monomeric (G-) actin (68). The latter is thought to facilitate nucleation, which is instrumental when rebuilding the actin cytoskeleton following mitosis and during cell movement. To monitor the interaction between CALD1 and G-actin, we performed pull-down assays using GST-hCALD1 bound to a glutathione-Sepharose matrix (Fig. 6B). The Sepharose bound GST-hCALD1 permitted recovery of G-actin from solution (lanes 5 and 6), whereas GST-Rb Ct used in the same conditions did not (lanes 7 and 8). Importantly, binding of G-actin to GST-hCALD1 was abolished after phosphorylation with K-cyclin-CDK6 or cyclin B-CDK1, but not monomeric CDK6 (Fig. 6C, top panel). Note that comparable amounts of hCALD1 protein were recovered on the beads, as shown by immunoblotting using a α-hCALD1 pan antibody.
FIGURE 5. Identification of residues phosphorylated by K-cyclin-CDK6 on recombinant caldesmon. A, phosphorylation of bacterially produced hCALD1 by K-cyclin-CDK6 in vitro. 0.5 μg of purified GST-hCALD1 produced in E. coli was incubated with K-cyclin-CDK6 in the presence of [γ-32P]ATP. After separation on SDS-PAGE, the full-length GST-hCALD1 was excised from the gel and the specific radioactivity determined by Cerenkov counting.

B, separation of 32P-labeled tryptic peptides from GST-hCALD1 using reverse phase HPLC. Purified GST-hCALD1 was phosphorylated by K-cyclin-CDK6 in the presence of [γ-32P]ATP and analyzed as described in the legend for Fig. 2A. C, analysis of phosphopeptides derived

| P | Observed Mass | Theoretical Mass | Amino Acid Sequence |
|---|---------------|------------------|---------------------|
| P1 | 1269.60 | 1269.60 | 782-QSVDKVTsPTK -792 |
| P2 | 2126.91 | 2127.01 | 719-GNVFSSPTAAGtPNKETAGLK -739 |
| P3 | not observed | | |
| P4 | 406.21# | 406.197# | 787-VTsPTKV -793 |
| P5 | 1527.637 | 1527.6845 | 719-GNVFSSPTAAGtPNK -733 |
| P6 | 2159.925 | 2160.01 | 746-lNEwLTKpDGNKsPAPK -762 |
| P7 | 3398.612 | 3398.640 | 746-lNEwLTKpDGNKsPAPKPSDLRPGDVSSK -774 |

D

E
Taken together, the above data demonstrate that phosphorylation by K-cyclin\(/\text{H18528}\)CDK6 abolishes the interaction of hCALD1 with both monomeric and filamentous actin, inferring that this kinase affects all known mechanisms by which caldesmon modulates the function of microfilaments.

**Effects of K-cyclin-CDK6 Phosphorylation on CALD1 Binding to Calmodulin**—CALD1 also interacts with Ca\(^{2+}\)/CaM, and this interaction is thought to constitute a regulatory event preventing de novo binding of free CALD1 to monomeric and filamentous actin. Yet other evidence exists that association of Ca\(^{2+}\)/CaM with filament-bound caldesmon by mass spectrometry. Shown are the masses [M\(+\text{H}\)]\(^{-}\) observed by MALDI-TOF-TOF mass spectrometry compared with the theoretical values calculated from the amino acid sequences. Peptide P4 was analyzed by nanoelectrospray mass spectrometry, and the mass values represent that of the [M\(+2\text{H}\)]\(^{2+}\) ion. s, phosphoserine; t, phosphothreonine; w, kynurein. D, identification of the phosphorylation sites in peptides P6 and P7. Peptides P6 and P7 from B were subjected to solid phase sequencing as described for Fig. 2, C and D. E, functional domains of hCALD1. The myosin-binding domain is shown as cross-hatched bars, the regions that bind actin (amino acids 630–734 and 749–790) and calmodulin (amino acids 715–722 and 744–752) are shaded gray and black, respectively. The (S/T)P consensus sequences are indicated. Positions phosphorylated by K-cyclin-CDK6 are marked with an asterisk.
Caldesmon Phosphorylation by K-cyclin-CDK6

Caldesmon (CALD1) may result in stabilization of existing filaments (31). Ca\(^{2+}\)/Calmodulin (CaM) binds to a discontinuous sequence that overlaps with the CALD1 actin binding sites (69) and is positioned adjacent to the residues modified by K-cyclin-CDK6 (see Fig. 5E).

We thus investigated whether phosphorylation by K-cyclin-CDK6 may affect the ability of hCALD1 to associate with calmodulin by measuring the retention of phosphorylated and unphosphorylated GST-hCALD1 on CaM-Sepharose in the presence of Ca\(^{2+}\) (Fig. 7). GST-hCALD1 was detected using either SYPRO Orange staining or pan- and phospho-site-selective hCALD1 antibodies. In its unphosphorylated form or when treated with nonmoneric CDK6, GST-hCALD1 bound firmly to the resin and was released only with EGTA-containing elution buffer. In contrast, when phosphorylated by K-cyclin-CDK6 or cyclin B/CDK1, GST-hCALD1 eluted during the initial wash. Thus, phosphorylation by K-cyclin-CDK6 or cyclin B/CDK1 substantially reduces the affinity of hCALD1 for calmodulin. In summary, phosphorylation of hCALD1 by K-cyclin-CDK6 broadly affects the biochemical properties of CALD1, disabling its interaction both with its effector (actin) and with its regulator (Ca\(^{2+}\)/CaM).

Effects of Actin and Calmodulin on K-cyclin-CDK6-mediated Phosphorylation of hCALD1—Previous work showed that association of CALD1 with F-actin or Ca\(^{2+}\)/CaM blocks the phosphorylation of CALD1 by mitotic CDK1 (50), suggesting that this kinase may primarily act on free CALD1 and does not affect filament- or Ca\(^{2+}\)/CaM-bound forms.

Because the pattern of hCALD1 phosphorylation by K-cyclin is similar to that by cyclin B/CDK1, we investigated whether F-actin or calmodulin binding affects phosphorylation by K-cyclin-CDK6. As shown in Fig. 8A, preincubation with F-actin reduced the phosphorylation of hCALD1 by K-cyclin-CDK6 by more than 60%, affecting this kinase more substantially than did cyclin B/CDK1. Similarly, pre-binding to Ca\(^{2+}\)/CaM also partially suppressed K-cyclin-CDK6 phosphorylation of hCALD1 (Fig. 8B), although the impact was less pronounced than that of actin and similar in degree for K-cyclin-CDK6 and cyclin B/CDK1. These results indicate that phosphorylation of hCALD1 by K-cyclin-CDK6 and cyclin B/CDK1 follows similar restrictions, indirectly suggesting that similar conformational requirements may exist for recognition or access to the phosphate-accepting amino acids for both cyclin-CDK complexes.

K-cyclin Expression Affects Microfilament Integrity and Cell Shape—Previous work has implicated CALD1 in the regulation of microfilaments integrity in vivo (27). We therefore probed for the effects of K-cyclin expression on actin cytoskeleton morphology using human osteosarcoma-derived U2OS cells. These cells, which display extensive stress fibers, were transiently transfected with a plasmid encoding K-cyclin or empty vector, together with a plasmid encoding EGFP to mark transfected cells. After 24 h, phallolidin staining of F-actin revealed remarkable alterations in microfilament appearance in K-cyclin-transfected but not in empty vector-transfected cells (Fig. 9A). These changes included the near absence of cortical actin and stress fibers in K-cyclin-expressing cells, which instead presented with short fiber fragments. Quantitative analysis (Fig. 9B) involving optical scoring of 100 cells per condition in three independent experiments revealed that more than 60% of K-cyclin-transfected cells lacked the normal, linear appearance of actin bundles but instead showed fragmented filaments. In contrast, nearly 95% of cells transfected with empty vector contained linear actin bundles, and cells with fragmented or undetectable filaments were rare in these samples. The effects on microfilament appearance elicited by K-cyclin expression were almost fully overcome when a kinase-defective, dominant-negative form of CDK6 (CDK6DN) or a mutant form of CALD1 with alanine substitutions in its proline-directed phosphorylation sites (CALD1 7th) was co-expressed but not when catalytically active CDK6 or wild-type caldesmon was used instead (Fig. 9B). Representative photomicrographs of cells transfected with the various plasmid combinations are shown in supplemental Fig. S4. Results in accord with these were obtained when phallolidin fluorescence in individual cells was quantified using the mean intensity algorithm provided by the ImageJ software (Fig. 9C). Cells from K-cyclin-transfected cultures displayed substantially lower mean fluorescence intensity, consistent with the absence of fibers or substantially decreased fiber density. The decrease in mean intensity was abolished by co-expression of CDK6DN or phosphorylation-defective CALD1, which partially, following expression of wild-type CALD1, provided direct evidence that K-cyclin can affect microfilament organization by a mechanism involving CDK activity and caldesmon phosphorylation. Consistent with previous results (27), both wild-type and phosphorylation defective CALD1 when expressed in isolation resulted in an increase in mean fluorescence intensity, in line with CALD1 stabilization of actin filament formation and density. Together, the above results strongly support the notion that K-cyclin through CDK-dependent phosphorylation of caldesmon affects microfilament organization and structure.

![Image](http://www.jbc.org/)

FIGURE 7. Reduced binding to calmodulin of caldesmon phosphorylated by K-cyclin-CDK6. 3 \(\mu\)g of either unphosphorylated GST-hCALD1 or GST-hCALD1 phosphorylated by the indicated cyclin-CDK complexes in vitro were chromatographed separately onto a calmodulin resin. Fractions collected from the washing and elution steps (indicated as W1 and W2, E1, E2, E3, and E4, respectively) were subjected to SDS-PAGE and stained for protein with SYPRO Orange. Further aliquots were also immunoblotted with \(\alpha\)-hCALD1 and \(\alpha\)-P-hCALD1 730 antibodies, as indicated. Seph, Sepharose.
The observed modification in the actin cytoskeleton was accompanied by changes in cell shape. U2OS cells normally feature a symmetrical polygonal shape and grow in tight, epithelium-like clusters. These features were unaltered in cells transfected with empty control vector. In contrast, cells expressing K-cyclin displayed highly irregular shapes with an increased number of membrane protrusions, an elongated appearance, and an overall loss of cell-cell contact (see Fig. 9A and supplement Fig. 4). These observations were confirmed by measurements based on the assessment of cell circularity and cell perimeter in individual transfectants. Such measurements indicate a consistent loss of circularity (Fig. 9D) in the K-cyclin-expressing cells but an increase in cell perimeter (Fig. 9E). As in the previous analysis, co-expression of either CDK6DN or phosphorylation-defective CALD1, but not the wild-type version of these proteins, abolished the K-cyclin associated effects, providing strong evidence that, like modulation of the microfilament structure, these shape responses also relate to the CDK-mediated phosphorylation of caldesmon.

Taken together, the above results support the notion that K-cyclin affects the integrity of actin stress fibers and, through this, cellular morphology by targeting caldesmon for CDK6 phosphorylation.

**DISCUSSION**

We provide evidence here that the actin- and calmodulin-binding protein caldesmon is a substrate for CDK6 when activated by K-cyclin, the D-like cyclin encoded by Kaposi sarcoma herpes virus. We demonstrate that K-cyclin-CDK6 complexes phosphorylate and modify the properties of recombinant hCALD1 in vitro and that K-cyclin expression promotes and qualitatively modulates hCALD1 phosphorylation in cells. Furthermore, expression of K-cyclin in cultured U2OS cells affects actin cytoskeleton integrity and cellular shape; CDK catalysis and phosphorylation of caldesmon are essential for both these responses. Together, these results provide strong evidence that K-cyclin has the capability to affect caldesmon activity and through this modulate microfilament functioning and associated events in cells.

Evidence for the phosphorylation of hCALD1 by K-cyclin-CDK complexes came from a KESTREL screen, an unbiased approach for the identification of kinase substrates within complex protein mixtures. The KESTREL method previously has proven to be a powerful tool to identify novel and physiological substrates for a diverse set of protein kinases (17, 45–47). P90-hCALD1 represents one of a handful of putative substrates detected in the screen reported here and is selectively phosphorylated by K-cyclin- but not cyclin D1-activated kinase.

Caldesmon is a known substrate for CDK1, a kinase that is activated specifically and selectively at the onset of mitosis. Unexpectedly, we found that cyclin E- and cyclin A-activated CDK2 can also phosphorylate hCALD1. These CDK2 complexes, which are formed and activated in cells during the late stages of the G1-phase, have not been implicated previously in caldesmon phosphorylation. Although phosphorylation of CALD1 by these kinase complexes has yet to be documented to arise in cells, our findings raise the possibility that a wider range of cyclin-dependent kinases may affect the cytoskeleton by modi-
FIGURE 9. Effects of K-cyclin expression on actin cytoskeleton and cell shape. A, K-cyclin expression affects actin stress fibers and cell shape in mammalian cells. U2OS cells were transiently transfected with K-cyclin or control plasmid and a plasmid encoding EGFP. After 24 h, cells were fixed and F-actin structures visualized using fluorescently labeled phalloidin. Representative photomicrographs depicting EGFP (left) and phalloidin fluorescence (right) of the same image are shown. B, K-cyclin mediated modulation of actin filament appearance depends on CDK6 activity and caldesmon phosphorylation. U2OS cells were transfected with EGFP plasmid and expression plasmids as indicated. F-actin structures were visualized using phalloidin as described in A. A minimum of 100 EGFP positive cells for each plasmid combination was scored as to the distribution and appearance of the phalloidin signal. The percentage of cells with contiguous fibers (white bars) or fragmented fibers (black bars) is depicted. Error bars represent standard deviation between three independent samples.
ulation of caldesmon activity. In vitro, caldesmon is phosphorylated by a diverse set of kinases, suggesting that this protein is tied into, and controlled by, a complex web of signaling events. Several of these kinases, such as calmodulin-dependent kinase II and casein kinase II, phosphorylate residues within the amino-terminal myosin binding region of CALD1 and abolish its interaction with myosin (54, 55). In contrast, mitotic CDK1 and MAPK/ERK both modify CALD1 within the carboxyl-terminal actin-binding region and affect its association with actin, although the impact of these kinases on CALD1 function appears to differ to some extent. Recent evidence suggests that in vitro MAPK/ERK phosphorylation does not abolish the association of CALD1 with F-actin, although it prevents the trans-filament linkage and thus filament bundling (70). In contrast, mitotic CDK1 leads to full dissociation of CALD1 from F-actin filaments (48). The functional significance of these differences is not clear, but it may be explained by the different array of phosphorylation catalyzed by the two kinases. MAPK/ERK is known to phosphorylate porcine CALD1 on two sites, corresponding to the human Ser-759 and Ser-789, both positioned at the extreme carboxyl terminus of the actin-binding region of CALD1. In contrast, mitotic CDK1 can phosphorylate additional sites, including two (Thr-730, Thr-753) positioned adjacent to a more internal actin-binding sequence. The results shown here reveal that K-cyclin/CDK6 phosphorylates Thr-730 and Thr-753 and thus may mirror the impact of CDK1 rather than ERK/MAPK phosphorylation.

Although similar, the sites reported to be phosphorylated by CDK1 and those determined by our work for K-cyclin/CDK6 are not identical, indicating that the two kinases differ in site preference and/or the mode of substrate recognition. A major difference relates to Ser-724 that does not apparently acts as acceptor for K-cyclin/CDK6-catalyzed phosphate transfer. It is unlikely that Ser-724 phosphorylation has evaded detection. Three different peptides, one generated from cell-derived hCALD1 and two derived from recombinant hCALD1, span the sequence containing this serine, showing it to be in a nonphosphorylated form. Previous work characterizing phosphorylation of chicken CALD1 by mitotic CDK1 also suggested mutually exclusive phosphorylation of the sites corresponding, in the human sequence, to Thr-753 and Thr-759 (49). Our analysis of K-cyclin/CDK6-phosphorylated hCALD1 however clearly documents the existence of a peptide that carries phosphate on both Thr-753 and Thr-759, indicating that phosphorylation of one site does not exclude phosphorylation at the other. At present, it is not clear whether phosphorylation of specific sites fulfills discernable functions, and conclusions as to whether functionally distinct effects may arise from the differences in phosphorylation by K-cyclin/CDK6 and mitotic CDK1 are currently not possible.

Our in vitro analysis indicated that phosphorylation by K-cyclin/CDK6 impacts hCALD1 functions in ways that are indistinguishable from cyclin B/CDK1. Activation of CDK1 arises specifically during mitosis, and CALD1 phosphorylation by this kinase is implicated in the disassembly of the microfilament at the beginning of prophase, providing for unhindered chromosome segregation and cytokinesis. In addition, recent work has linked the activation of CDK1 adjacent to the cell membranes in the promotion of cell movement during interphase (34). In contrast, K-cyclin expression and associated kinase activity is constant throughout the cell cycle, and K-cyclin/CDK6 complexes are present both in the nucleus and in the cytoplasm in KSHV-transformed cells (71). Furthermore, K-cyclin-activated kinases are known to evade regulatory loops that restrain the activity of cellular cyclin/CDKs in response to extracellular signaling cues (3). Together, these observations indicate that phosphorylation of caldesmon by K-cyclin-activated kinases may not be confined to a particular cell cycle position or signaling context but may arise throughout, leading to constitutive caldesmon hyperphosphorylation and consequential impairment of microfilament organization.

Several independent observations in KSHV-infected cells are consistent with aberrant modulation of actin cytoskeleton functions. Human vascular endothelial cells, which normally form cobblestone-like cell arrays, are known to respond to KSHV infection with a striking shape change leading to narrow, light-refractive cell bodies, loss of cell junctions, and decreased substratum adhesion (72, 73); this is quite reminiscent of the response that arises in the U2OS cells upon K-cyclin expression, as shown above (72, 73). This morphological transformation is also reproduced in transgenic mice with endothelium selective K-cyclin expression (74). The work presented here provides a possible molecular explanation of how these morphological alterations are achieved.

An apparently related question is why KSHV may have developed means to affect microfilament structure and function. Disintegration of the actin cytoskeleton is known to occur upon infection with a wide range of animal viruses, including human herpesvirus 1 and 2 and the human immunodeficiency virus (75). In most instances, the means by which viruses achieve this effect have not been defined, but the widespread association of this response with viral infection suggests a benefit for viral replication and/or virus spread.

Importantly, there is strong evidence for alterations of the microfilament architecture during cancer development (76) and that misregulation of microfilament functions contributes to cancer invasion (77, 78). Several reports link loss of CALD1 function to oncogenesis (79). For instance, v-Src-transformed cells display a reduced expression of CALD1 (80, 81), whereas v-ErbB2-transformed fibroblasts show enhanced tyrosine phosphorylation of CALD1 that correlates with stress fiber disassembly (82). Lastly, missplicing of the CALD1 gene has been observed in glioma microvasculature and is associated with tight junction breakdown between endothelial cells and vascular leakage (83). Thus, K-cyclin-induced phosphorylation of CALD1 could provide a cancer-promoting event, independent of, and in addition to, the impact of this cyclin on cell cycle progression and proliferation.

Acknowledgments—We thank the Post Genomics and Molecular Interactions Centre, University of Dundee, for the mass spectrometry facilities. Dr. David Campbell for assistance with protein sequencing and phosphoamino acid analysis, and Dr. Fumio Matsumura for making available the CALD1 7th expression construct. Phospho-specific antibodies were purified and characterized by the staff of the Division of Signal Transduction Therapy, School of Life Sciences, University of Dundee.

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Caldesmon Phosphorylation by K-cyclin/CDK6
Regulation of Microfilament Organization by Kaposi Sarcoma-associated Herpes Virus-cyclin·CDK6 Phosphorylation of Caldesmon

Maria Emanuela Cuomo, Axel Knebel, Georgina Platt, Nick Morrice, Philip Cohen and Sibylle Mittnacht

*J. Biol. Chem.* 2005, 280:35844-35858.
doi: 10.1074/jbc.M503877200 originally published online August 22, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503877200

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