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METHODS AND APPLICATIONS

Reconstitution and use of highly active human CDK1: Cyclin-B:CKS1 complexes

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Abstract

As dividing cells transition into mitosis, hundreds of proteins are phosphorylated by a complex of cyclin-dependent kinase 1 (CDK1) and Cyclin-B, often at multiple sites. CDK1:Cyclin-B phosphorylation patterns alter conformations, interaction partners, and enzymatic activities of target proteins and need to be recapitulated in vitro for the structural and functional characterization of the mitotic protein machinery. This requires a pure and active recombinant kinase complex. The kinase activity of CDK1 critically depends on the phosphorylation of a Threonine residue in its activation loop by a CDK1-activating kinase (CAK). We developed protocols to activate CDK1:Cyclin-B either in vitro with purified CAKs or in insect cells through CDK-CAK co-expression. To boost kinase processivity, we reconstituted a ternary complex consisting of CDK1, Cyclin-B, and CKS1. In this work, we provide and compare detailed protocols to obtain and use highly active CDK1:Cyclin-B (CC) and CDK1:Cyclin-B:CKS1 (CCC).

KEYWORDS

CDK1, cell cycle, CKS1, cyclin, cyclin dependent kinase, cyclin-B, phosphorylation, phostag, processivity, recombinant protein

1 | INTRODUCTION

A human cell that sets out to divide needs to condense DNA into chromosomes, break down the nuclear envelope, and form a mitotic spindle. Key for the initiation of these transformations is the rise in the activity of a complex between Cyclin-dependent kinase 1 (CDK1) and Cyclin-B, the main mitotic cyclin in vertebrates.[1,2] This results in the posttranslational modification of hundreds of proteins, including other mitotic kinases, on specific serine and threonine side-chains and often at multiple sites.[3–5] Tight spatiotemporal regulation of these substrates and of their role in mitosis is then enabled by the intricate balance between mitotic kinase activities and counteracting phosphatases.[6]

Phosphorylation regulates the conformation, the activity, and the binding partners of the mitotic protein machinery. CDK1 is primed to initiate mitosis after Cyclin-B replaces Cyclin-A as the major CDK1-associated Cyclin and after the side-chains of Thr14 and Tyr15 in CDK1 are dephosphorylated. This is promoted by a shift in balance between the kinases Wee1 and Myt1 and the

Cdc25 phosphatase which ultimately results in dephosphorylation of the glycine-rich loop (GEGTYG), unleashing its essential contribution to phosphotransfer.[1,7] The activity of CDK kinases also critically depends on the configuration of its activation loop. The phosphorylation of this loop, at position Thr161 in CDK1, is required to dock the activation loop at the base of the active site, enabling efficient substrate binding and phosphate transfer[8–10] (Figure 1a,b). Active CDK1 is thus phosphorylated at Thr161 and dephosphorylated at positions Thr14 and Tyr15.

Phosphorylation of the activation loop of CDK1 occurs in *trans* by a CDK-activating kinase (CAK). In metazoans, CDK1 is activated by the CDK7:Cyclin-H kinase,[11,12] which is also implicated, as a module of TFIIH, in the phosphorylation of the C-terminal domain of RNA polymerase II, as recently depicted.[13–16] Whereas metazoan CAK consists of a CDK7:Cyclin-H complex, activation of the budding yeast CDK1 (Cdc28) is mediated by the distantly related and monomeric CAK1 (hereafter scCAK1).[17–19]

Besides phosphorylation, the CDK1:Cyclin-B kinase activity and substrate binding preferences are also regulated by various binding proteins. An important regulator is CKS1, a small adaptor protein that binds to the large helical lobe of the CDK1 kinase fold (Figure 1c). CKS1 contains a binding pocket for phosphorylated threonine residues and guides substrates with proximal phosphothreonine residues to the active site of CDK1, resulting in multi-site phosphorylation.[20,21]

With a persistent interest in the functional and structural characterization of proteins that act in cell division, and attempts to reconstitute in vitro fundamental aspects of the cell division process, it is essential to recapitulate CDK1:Cyclin-B phosphorylation patterns in vitro with a recombinant kinase complex that is pure, active, and processive. We have developed and compared ways to activate recombinant CDK1 with yeast or human CAK

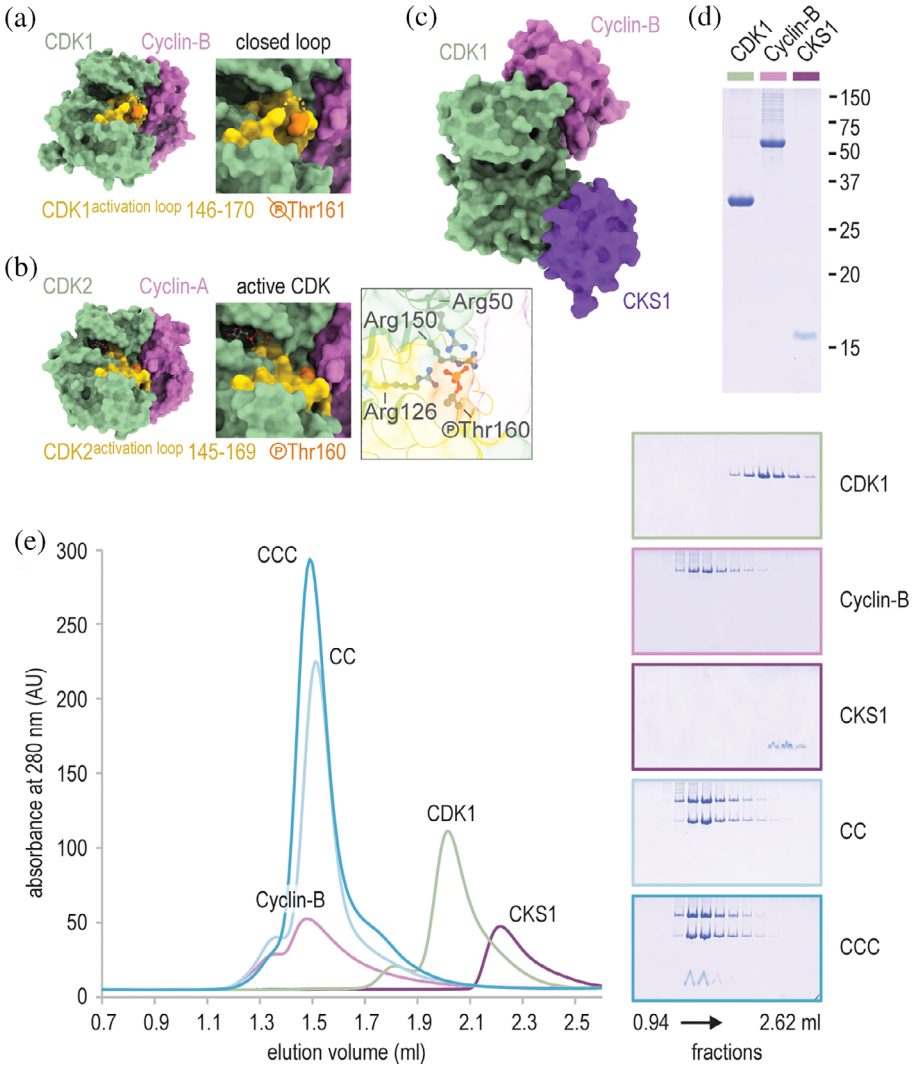

FIGURE 1 Reconstitution of stoichiometric CDK1:Cyclin-B and CDK1:Cyclin-B:CKS1 complexes. (a) Surface view of a complex between CDK1 (green) and Cyclin-B (purple) (PDB 4YC3).[10] The CDK1$^{162\text{-}173}$ activation loop is shown in gold and Threonine 161 in orange. Within the closed activation loop, residues CDK1$^{162\text{-}164}$ (HEV) are not visible and replaced with a dashed line. (b) Surface view of CDK2:Cyclin-A with bound ATP (PDB 1JST).[9] The side chains of Arginines 50, 126, and 150 coordinate the activation loop with a phosphorylated Threonine 160. (c) Surface view of a tripartite CDK1:Cyclin-B:CKS1 complex (PDB 4YC3). Compared to Panel (a), the structure is rotated 45° along the x-axis. (d, e) Analysis of purified CDK1, Cyclin-B, CKS1, CDK1:Cyclin-B (CC), and CDK1:Cyclin-B:CKS1 (CCC) by SDS-PAGE followed by Coomassie staining and size exclusion chromatography using a Superdex 200 increase 5/150 column

in vitro or in insect cells during protein expression. To boost the processivity of reconstituted CDK1:Cyclin-B complexes, we also set out to include CKS1 in our recombinant kinase complexes. Taken together, our analysis demonstrates how to reconstitute CDK1:Cyclin-B (CC) and CDK1:Cyclin-B:CKS1 (CCC) complexes, how to phosphorylate the activation loop of CDK1 efficiently, and how to reconstitute multi-site CDK1 phosphorylation patterns in vitro.

2 | RESULTS

2.1 | Reconstitution of stoichiometric CDK1:Cyclin-B and CDK1:Cyclin-B:CKS1 complexes

We set out to reconstitute CDK1:Cyclin-B (CC) and CDK1:Cyclin-B:CKS1 (CCC) complexes from their purified individual constituents. We therefore expressed full-length CDK1, Cyclin-B1, and CKS1 in insect cells and purified these proteins to homogeneity using size-exclusion chromatography after affinity tags were proteolytically removed (Figure 1d). Stable and stoichiometric CC (83 kDa) and CCC (93 kDa) complexes formed when CDK1, Cyclin-B, and CKS1 were mixed and were further purified using size-exclusion chromatography (Figure 1e). These results confirm that the formation of CDK1:Cyclin-B or CDK1:Cyclin-B:CKS1 complexes does not require the phosphorylation of the CDK1 activation loop (see below).

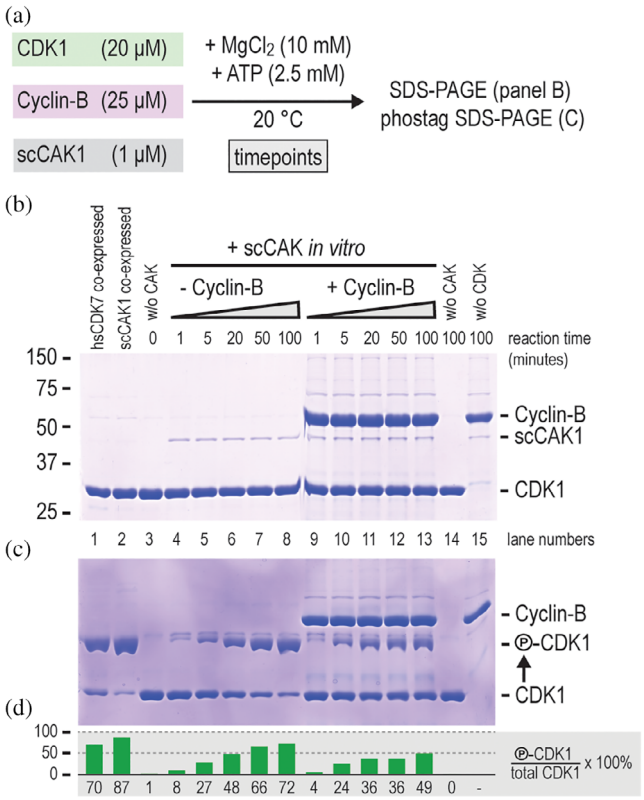

FIGURE 2 Phosphorylation of CDK1 in insect cells and in vitro by a CDK activating kinase. (a) Reaction scheme for the phosphorylation of CDK1 with scCAK1. (b, c) SDS-PAGE analysis of CDK1, Cyclin-B, and scCAK1. The presence of phostag-acrylamide (Panel c) slows the migration of phosphorylated CDK1. (d) Quantification of the phosphorylated and non-phosphorylated forms of CDK1 from the phostag gel shown in Panel c

2.2 | Phosphorylation of CDK1 in insect cells and in vitro by a CDK activating kinase

Since the phosphorylation of CDK1^{Thr161} by the CDK-activating kinase (CAK) promotes substrate binding and phosphorylation, we set out to co-express CDK1 and CAK in insect cells. Co-expression of CDK1 with budding yeast CAK (scCAK1) or with the catalytic subunit of the human CAK (hsCDK7) did result in effective CDK1 phosphorylation, as judged by its retarded mobility on a phostag-containing acrylamide gel[22] (Figure 2c, Lanes 1–3).

We next used purified scCAK1 (39 kDa) to phosphorylate CDK1 in vitro. Analysis by phostag-PAGE showed effective phosphorylation of CDK1 in a reaction of 100 min at room temperature (Figure 2c, Lanes 4–8). CDK1 associated with Cyclin-B is also a CAK substrate, although CDK1 phosphorylation appeared to be slower in the Cyclin-bound state (Figure 2c,d, Lanes 4–13). A preference of scCAK1 for a Cyclin-free substrate was previously also observed for CDK2 in the absence or presence of a five-fold excess of Cyclin-A.[23] The similar migration of Cyclin-B throughout the course of the experiment confirms that Cyclin-B is neither a CDK nor a CAK substrate.

To compare CDK1 activation in insect cells and in a purified system, we purified scCAK1 and hsCDK7 and generated a range of CDK1:Cyclin-B complexes (Figure 3a). As expected, scCAK1 activates CDK1 efficiently in vitro (Figure 3c, reaction g). Although purified hsCDK7 does phosphorylate CDK1:Cyclin-B in vitro, phosphorylation levels were markedly lower than for scCAK1 (Figure 3c, reactions f and g) and lower than during co-expression of hsCDK7 and CDK1 in insect cells (Figure 2, Lane 1).

2.3 | Phosphorylation of CDK1 threonine 161 activates recombinant CDK1:Cyclin-B

We next used semi-quantitative mass spectrometry to assess if the CAK-induced shift in CDK's phostag-PAGE

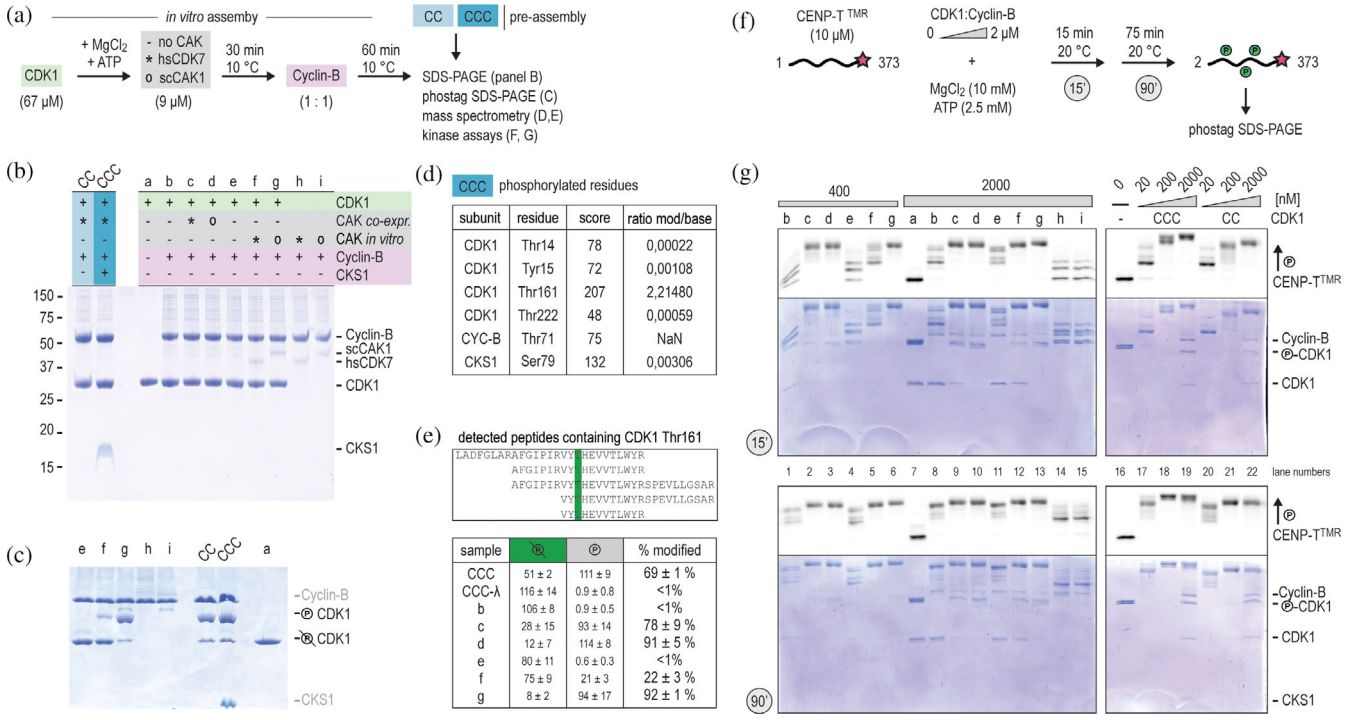

FIGURE 3 Phosphorylation of CDK1 Threonine 161 activates recombinant CDK1:Cyclin-B. (a, b) Preparation and SDS-PAGE analysis of CDK1:Cyclin-B complexes that were either in vitro assembled from purified components or pre-assembled into dimeric or trimeric complexes. Reactions (a) and (e) are technical replicates. (c) CDK1:Cyclin-B complexes were analyzed by phostag SDS-PAGE. The effect of scCAK1 or hsCDK7 co-expression on the phostag-migration of CDK1 (reactions b, c, d) is shown in Figure 2c, Lanes 1–3. (d) Six phosphorylated residues were detected by mass spectrometry in the CCC complex. Andromeda scores of the best-identified phosphopeptides and the ratio of the intensities of phosphorylated (mod) over non-modified (base) peptides are shown. (e) Five different peptides containing CDK1 Threonine 161 were detected after trypsin digestion. From three technical replicates, the average summed intensities (AU) were combined for peptides with phosphorylated Thr 161 and for peptides with non-phosphorylated Thr 161. The intensity of phosphopeptides as a fraction of the total peptide intensity is shown in the last column. The CCC sample that was exposed to lambda-phosphatase is marked CCC-λ. (f, g) Fluorescently labeled CENP-T was exposed to different kinase complexes and analyzed for multisite phosphorylation using phostag SDS-PAGE. Samples were analyzed after 15 min (upper gels) and after 90 min (lower gels). In-gel fluorescence (CENP-T) was recorded before Coomassie staining the same gels. Reactions in Lanes 1 and 4 as well as 8 and 11 are technical replicates

migration reflected phosphorylation of Threonine 161. Analysis of CDK1 that was exposed to human or yeast CAK in insect cells or in vitro confirmed the efficient phosphorylation of Threonine 161 and demonstrated that very few additional CDK1 residues were phosphorylated, and grossly substoichiometrically (Figure 3d). Whereas CDK1 was efficiently phosphorylated by scCAK1 both in vitro (92%) and upon co-expression in insect cells (91%), the phosphorylation by hsCDK7 was markedly lower in vitro than in insect cells (22 vs. 78%; Figure 3e). The overall degree of Thr161 phosphorylation detected by mass spectrometry matches the intensities of the modified and unmodified CDK on phostag-PAGE (Figures 2c and 3c) and exposure to lambda-phosphatase reversed Thr161 phosphorylation (Figure 3e).

To test and compare the activity of CDK1:Cyclin-B complexes, we used a 373-residue fragment encompassing the N-terminal region of the kinetochore protein CENP-T. This is an ideal multi-site phosphorylation model substrate as it is predominantly disordered and is phosphorylated by CDK1:Cyclin-B on multiple sites in vivo and in vitro.[24–27] CENP-T$^{1\text{-}373}$, expressed in bacteria and therefore unphosphorylated, was initially purified, labeled at its carboxy-end with tetramethylrhodamine (TMR) using Sortase-mediated conjugation (Guimaraes et al., 2013; Hirakawa et al., 2015), and finally purified to homogeneity using size-exclusion chromatography.

We used CENP-T at a concentration of 10 µM and varied the concentration of the CDK1:Cyclin-B complexes between 20 nM and 2 µM. To monitor CENP-T phosphorylation, we used in-gel fluorescence after phostag-PAGE (Figure 3f,g). CENP-T was not phosphorylated after 90 min in the absence of kinase or when incubated with CDK1 without Cyclin-B, highlighting the purity of the CENP-T and CDK1 samples and the strict Cyclin-dependency of CDK1 activity (Figure 3g, Lanes 7 and 16). Sparse CENP-T phosphorylation occurred in the absence of CDK1, probably caused by traces of kinase in the Cyclin-B sample (Figure 3g, Lanes 14 and 15). Relative to this control sample, phosphorylation appeared

enhanced when CENP-T was incubated with CDK1:Cyclin-B that had not been exposed to a CAK. At CDK1:Cyclin-B concentrations of 0.4 or 2 μM, CENP-T underwent a progressive increase in phosphorylation between 15 and 90 min (Figure 3g, Lanes 1, 4, 8, and 11). By contrast, apparently homogeneous multi-site CENP-T phosphorylation patterns were already visible after 15 min of phosphorylation with 0.2 or 0.4 μM CDK1:Cyclin-B complexes generated with kinase co-expressed with scCAK1 or hsCDK7 in insect cells (Figure 3g, Lanes 2, 3, and 21). After a 90-min reaction, comparable CENP-T phosphorylation levels were observed with concentrations of 20 nM of CAK-treated or 2 μM of CAK-untreated CDK1:Cyclin-B complexes (Figure 3g, Lanes 8, 11, and 20). CAK exposure thus increases the activity of recombinant CDK1:Cyclin-B complexes on CENP-T by at least two orders of magnitude (or possibly more, as we cannot exclude a fraction of basal phosphorylation of CDK1 in insect cells). Consistent with a modest but marked increase in the phosphorylation levels of CDK1 Thr161, the in vitro activation of CDK1:Cyclin-B with hsCDK7 resulted in increased CENP-T phosphorylation (Figure 3g, Lanes 4 and 5). However, CDK1 activation in vitro with scCAK1 was more effective (Figure 3g, Lanes 5 and 6), indicating that the phosphorylation of Thr161 correlates with kinase activity.

2.4 | CKS1 boosts the processivity of CDK1:Cyclin-B

A side-by-side comparison of CDK1:Cyclin-B (CC) and CDK1:Cyclin-B:CKS1 (CCC) complexes suggested the existence of a slower migrating form of CENP-T in the CCC reactions (Figure 3g, Lanes 17–22). To investigate putative effects of CKS1 on CDK1:Cyclin-B processivity, we followed the phosphorylation of CENP-T with 100 nM of CC or CCC over time (Figure 4). These complexes contained CDK1 that was activated by scCAK1 co-expression and that were reconstituted from individual constituents. We tested CC and CCC complexes directly after assembly from individual components, or that were further purified after assembly using an additional size-exclusion chromatography step (Figure 4a,b).

Following the phosphorylation of CENP-T over time revealed that the presence of CKS1 sped up multi-site phosphorylation approximately two-fold (Figure 4c,d). In addition to faster phosphorylation, CENP-T migration appeared to continuously decrease over time in the presence of CCC. This suggests that low-affinity CENP-T sites become CDK1 substrates when proximal phosphorylated threonine residues dock to CKS1.[20,21] The difference between high (∼5–10 sites) and hyper (10+ sites) phosphorylation on CENP-T was most apparent when the phostag concentration in the polyacrylamide gel was lowered five-fold (Figure 4e). Taken together, we demonstrate that the presence of CKS1 enables CDK1:Cyclin-B to efficiently establish multi-site phosphorylation patterns.

3 | DISCUSSION

Over the last decades, genetic, biochemical, and structural studies provided detailed insights into the regulation of the cell cycle by CDK:Cyclin combinations and

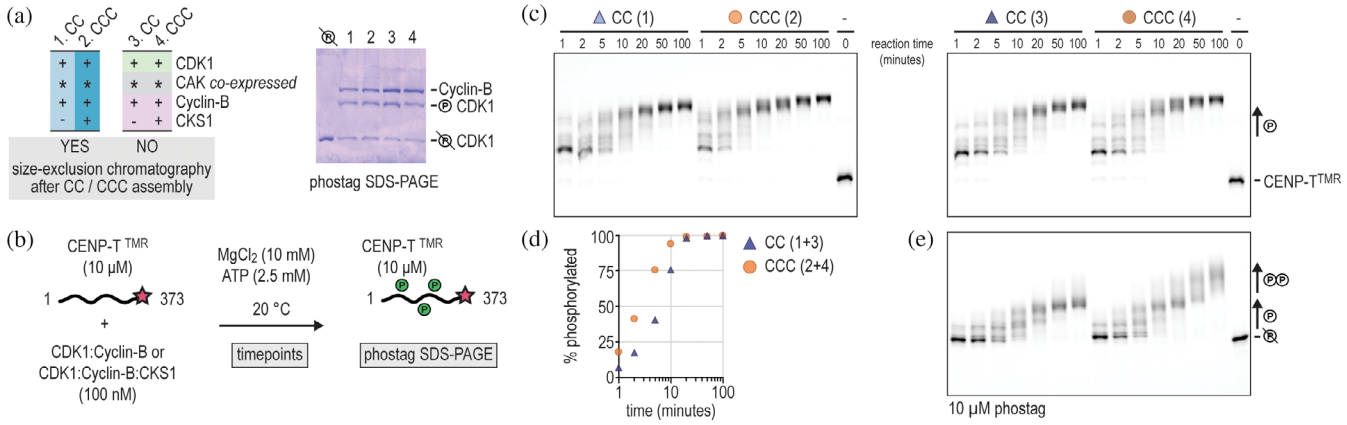

FIGURE 4 CKS1 boosts the processivity of CDK1:Cyclin-B. (a) Preparation and phostag SDS-PAGE analysis of CDK1:Cyclin-B and CDK1:Cyclin-B:CKS1 complexes that were either assembled from purified components or pre-assembled into dimeric or trimeric complexes. (b, c) CENP-T was exposed to different kinase complexes and analyzed for multi-site phosphorylation using phostag SDS-PAGE and in-gel fluorescence (CENP-T^{TMR}). (d) Quantification of phosphorylated/non-phosphorylated signals from the gels shown in Panel (c). (e) SDS-PAGE analysis of samples CC (3) and CCC (4) as in Panel (c) in the presence of 10 μM phostag acrylamide (all other gels contain 50 μM phostag-acrylamide)

the activation of Cyclin-dependent kinases by CAKs.[1,7] Since hundreds of CDK:Cyclin phosphorylation events are required to rewire the protein machinery in dividing mitotic or meiotic cells, in vitro reconstitution of phosphorylation patterns for the functional and structural characterization of simplified, smaller subsets of components is mandatory. To facilitate this type of experiments, the availability of pure and active recombinant CDK1:Cyclin-B complexes is essential, and here we have contributed to the development of a strong pipeline towards this goal. A previous study demonstrated how to obtain a pure and active CDK1:Cyclin-B complex using purified scCAK1 (expressed in bacteria) and CDK1 (expressed in insect cells), in vitro phosphorylation of CDK1 with scCAK1, and subsequent assembly of the active CDK1:Cyclin-B complex.[10] In this study, we built on this previous work and took a systematic approach to optimize protocols for the generation of highly active and processive recombinant CDK1:Cyclin-B:CKS1 complexes. These robust and easy-to-implement protocols and the corresponding expression plasmids, publicly available through Addgene (https://www.addgene.org/), will be a valuable resource for researchers with a range of backgrounds and an interest in investigating the protein machinery orchestrating cell division. We demonstrate how in-gel fluorescence and general protein staining after phostag-PAGE is a cost-effective and swift combination to analyze single and multi-site phosphorylation. Whereas mass spectrometry and site-directed mutagenesis of putative phosphorylation sites are needed to specify modified residues, phostag-PAGE provides a clear overview of phosphorylation coverage—for example to follow the amounts of phosphorylated and non-phosphorylated CDK1 Thr161—and can readily be combined with immunofluorescence. The latter is especially useful to study more complex reaction mixtures.

Here, we describe how we reconstituted highly active and stochiometric CAK-activated CC and CCC complexes after the separate expression and purification of CDK1, Cyclin-B, and CKS1. In previous years, we used CDK1:Cyclin-B complexes for in vitro studies that were purified after co-expression in insect cells from a single baculovirus.[27–30] Since these complexes were not explicitly CAK-activated and since CDK1 that is expressed without Cyclin-B is poorly active (Figure 3f), the activity of these complexes probably relied on the activation of a subset of CDK1:Cyclin-B by CAK activity in the host-cells (ovary cells from the cabbage looper moth *Trichopulsia ni*). Co-expression of CDK1, Cyclin-B, and CKS1 in insect cells was also used in a very recent study that revealed the structural basis for the formation of a complex between CCC and Separase.[31] In that study, a clear density for the phosphorylated Thr161 residue of CDK1 demonstrates that a significant subset of CDK1 was targeted by an endogenous CAK activity in insect cells.[31] These interpretations are consistent with classic studies revealing that an insect cell CAK activates CDK1:Cyclin-B complexes, but not CDK1 alone, during co-expression or when lysates expressing CDK1 and Cyclin-B are mixed[32] and that CDK7:Cyclin-H phosphorylates CDK1:Cyclin-B complexes, but not CDK1 alone.[11]

In agreement with these studies, we find that monomeric CDK1 was very little (<1%) phosphorylated on Thr161 when expressed as an isolated subunit and is thus not a good substrate of the endogenous CAK activity of insect cells. Interestingly, co-expression of scCAK1 or hsCDK7 results in the efficient phosphorylation of CDK1 in insect cells (Figure 2C, Lanes 1–3). This is interesting since an active human CAK module, in contrast to the monomeric scCAK1 in yeast, contains hsCDK7 and the co-factors Cyclin-H and MAT1, suggesting that host factors contribute to CAK activity in the absence of over-expressed Cyclin-B. Such factors could include a hsCDK7 activating kinase (CAKAK) or Cyclins that associates with hsCDK7 and CDK1. In fission yeast, it was, for example, demonstrated that the kinase Csk1 acts as a CAK and as a CAKAK.[33,34] Since scCAK1 activates CDK1 efficiently without the need for such additional factors, we recommend to use scCAK1 for the production of highly active recombinant CDK1 and to reconstitute full CDK7:Cyclin-H:MAT1 modules for further in vitro studies of human CDK activation mechanisms. Robust and efficient activation of CDK1 through co-expression of scCAK1 is, therefore, recommended for most applications. However, the option to activate CDK1:Cyclin-B in vitro with purified scCAK1 may be beneficial in reconstituted systems, for example to distinguish between kinase-dependent and -independent roles when CDK1:Cyclin-B is a stoichiometric component of larger complexes, or to modulate phosphorylation landscapes in reaction mixtures that contain other kinases and phosphatases.

How multisite phosphorylation rewires proteins that govern cell division was highlighted in a study showing that reconstituted APC/C could only bind its activator CDC20 after a region in the APC1 subunit was hyperphosphorylated and released from a CDC20 binding site on APC8.[35] In that work, APC/C was phosphorylated in vitro with PLK1 kinase and a CDK2:Cyclin-A:CKS2 complex in a manner that reflects APC/C phosphorylation in mitosis.[5,35] Although the constituents of the CDK2:Cyclin-A:CKS2 complex were individually expressed in bacteria and had not been exposed to a CAK, and were therefore most probably not phosphorylated, the resulting kinase did effectively reconstitute multi-site phosphorylation patterns on APC/C. CDK2:

Cyclin-A:CKS2 has been previously shown to be strongly dependent on the phosphorylation of the activation loop in CDK2.[36] Thus, we speculate that the docking of CDK2:Cyclin-A:CKS2 on APC/C results in CAK-independent substrate phosphorylation.

In summary, we describe the discrete effects of activation loop phosphorylation and CKS binding on the activity and processivity of a recombinant CDK1:Cyclin B complex, together with protocols for expression and access to the relevant expression vectors. These tools will support work of in vitro reconstitution of crucial aspects of cell division.

4 | MATERIALS AND METHODS

4.1 | Baculovirus generation and protein expression in insect cells

CDK1, Cyclin-B1, CKS1B, and hsCDK7 cDNA constructs were codon-optimized for insect cell expression and obtained from GeneArt (Life Technologies). The scCAK1 (CIV1) construct was kindly provided by David Barford. Expression cassettes of CDK1, Cyclin-B, CKS1B, scCAK1, and hsCDK7 were inserted into pLIB vectors[37] with N-terminal GST-3C (CDK1) or Polyhistide-TEV (others) tags and baculoviruses were generated following standard protocols.[38] For expression, *Sf9* cells were infected for 3–4 days and added (1:20 dilution) to logarithmically growing *Hi5*-derived *Tnao38* insect cells.[39] For co-expressions, *Sf9* cultures expressing CDK1 (1:20 dilution) and scCAK1 or hsCDK7 (1:40 dilution) were simultaneously added to the expression culture. All insect cells were kept at 27°C. Cells were harvested after 3 days by centrifugation at 1000*g* at room temperature, washed once with ice-cold PBS, pelleted by centrifugation at 1000*g* at 4°C, flash-frozen in liquid nitrogen, and stored at −80°C.

4.2 | CDK1 purification

All protein purification steps were performed on ice or at 4°C. Pellets from 2 L of insect cells expressing GST-3C-CDK1 (with or without CAK co-expression) were resuspended in 160 ml buffer A (50 mM HEPES pH 7.4, 250mNaCl, 2 mM TCEP, 5% vol/vol glycerol) supplemented with HP plus protease inhibitor mix (Serva) and DNase I (Roche). Cell lysates were prepared by sonication and cleared by centrifugation at 80,000*g* at 4°C for 30–45 min. The soluble lysate was passed through a 0,8 μm filter and loaded onto a column with 20 ml Gluathione Sepharose 4 Fast Flow resin (Cytiva). After washing with 25 column volumes of buffer A, CDK1 was cleaved with in-house generated 3C protease for 16 hr. The eluate was concentrated to 2 ml through centrifugation with a 30 k Amicon filter (Millipore) and separated on a Superdex 200 16/600 column equilibrated in buffer A. To remove GST, uncleaved GST-CDK1, and GST-3C-PreScission, a 5 ml GSH column (GE Healthcare) was mounted after the size-exclusion column. Selected fractions were concentrated to concentrations well above 100 μM. Purification from 2 L of insect cells yielded approximately 4 mg of CDK1.

4.3 | Cyclin-B purification

His-TEV-Cyclin-B containing lysates were prepared as described above with 15 mM imidazole in lysis and wash buffers. After loading onto a 5 ml or 10 ml Talon (Clontech) or Ni-NTA (GE Healthcare) column, and washing with approximately 50 column volumes, Cyclin-B was eluted in buffer A with 250 mM imidazole and concentrated to 2 ml through centrifugation with a 30 k Amicon filter (Millipore). To remove the polyhistidine tag, Cyclin-B was exposed to TEV protease for 16 hr. Cyclin-B was further purified on a Superdex 200 16/600 column equilibrated in buffer A. To remove His-TEV protease and uncleaved His-Cyclin-B, a 5 ml Talon column (GE Healthcare) was mounted after the size-exclusion column. Selected fractions were concentrated to concentrations well above 100 μM. Purification from 1 L of insect cells yielded approximately 15 mg of Cyclin-B.

4.4 | CKS1 purification

His-TEV-CKS1 was purified as described for Cyclin-B but using 5 k Amicon filters and a Superdex 75 16/600 size-exclusion column. Approximately 5 mg of CKS1 was obtained from 0.5 L of insect cells.

4.5 | CDK1:Cyclin-B (CC) and CDK1:Cyclin-B:CKS1 (CCC) formation

Purified CDK1, Cyclin-B, and CKS1 were mixed in a 1:1:0 (CC) or 1:1:2 (CCC) ratio for 1–2 hr on ice with concentrations above 100 μM. After size-exclusion chromatography on a Superdex 200 16/600 column in buffer A, fractions containing CC or CCC were concentrated through centrifugation with a 30 k Amicon filter (Millipore), flash-frozen in liquid nitrogen, and stored at −80°C. Analytical size-exclusion chromatography (Figure 1) was performed using a Superdex 200 5/150 column.

4.6 | scCAK1 and hsCDK7 purification

The CDK1 activating kinases scCAK1 and hsCDK7 were purified as described for Cyclin-B above and yielded approximately 0.5 mg and 5 mg, respectively.

4.7 | CENP-T purification

CENP-$T^{2\text{-}373}$ was expressed, purified, and fluorescently labelled as described.[27] In brief, the expression of GST-3C-CENP-$T^{2\text{-}373}$ with a C-terminal -LPETGG extension was induced in *Escherichia coli* BL21(DE3)-codon-plus-RIL cells through the addition of 0.35 mM IPTG for ~14 hr at 20°C. Subsequent steps were performed at 4°C or on ice. Cleared lysates were prepared by sonication and centrifugation and bound to a Glutathione-Agarose resin (Serva). GST-3C-CENP-T was cleaved off the beads with in-house generated 3C protease for 16 hr. After further purification using a Heparin HP column (GE Healthcare), CENP-T was fluorescently labeled with a GGGGK-TMR (5-Carboxytetramethylrhodamine) peptide (GenScript) using the Calcium-independent Sortase 7 M.[40] After size-exclusion chromatography using a Superdex 200 16/600 column (GE Healthcare) in a buffer containing 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM TCEP, CENP-$T^{2\text{-}373\ TMR}$ was concentrated to 118 μM and stored at −80°C.

4.8 | In vitro phosphorylation

Phosphorylation reactions were performed in the presence of $MgCl_2$ (10 mM) and ATP (2.5 mM) and monitored on standard denaturing 10% polyacrylamide gels[41] in the presence of 50 μM phos-tag-acrylamide (Fujifilm Wako Chemicals).[22] The in vitro phosphorylation of CDK1 by scCAK1 and hsCDK7 is described in detail in Figures 2 and 3. The in vitro phosphorylation of CENP-$T^{2\text{-}373\ TMR}$ by CDK1:Cyclin-B and CDK1:Cyclin-B:CKS1 complexes is described in detail in Figures 3 and 4.

4.9 | Mass spectrometry of CDK1

To analyze the phosphorylation of CDK1 by mass spectrometry, samples were reduced, alkylated and digested with LysC and Trypsin and prepared as previously described.[42] Obtained peptides were separated on an U3000 nanoHPLC system (Thermo Fisher Scientific). Samples were injected onto a desalting cartridge, desalted for 5 min using water in 0.1% formic acid, backflushed onto a Pepmap C18 nanoHPLC column (Thermo Fisher Scientific) and separated using a gradient from 5 to 30% acetonitrile with 0.1% formic acid and a flow rate of 300 nl/min. Samples were directly sprayed via a nano-electrospray source in an Orbitrap type mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in a data-dependent mode acquiring one survey scan and subsequently up to 15 MS/MS scans. To identify phospho-sites, the resulting raw files were processed with MaxQuant (version 1.6.17.0) searching for CDK1, Cyclin-B, and CKS1 sequences with acetylation (N-term), oxidation (M) and phosphorylation (STY) as variable modifications and carbamidomethylation (C) as fixed modification. A false discovery rate cut-off of 1% was applied at the peptide and protein levels and as well on the site decoy fraction.[43]

4.10 | Structures

Surface views of CDK1:Cyclin-B:CKS1 (PDB 4YC3)[10]and of CDK2:Cyclin-A with bound ATP (PDB 1JST)[9] were prepared using Chimera X (version 0.9).[44]

4.11 | Reagent availability

The following plasmids are available through Addgene (addgene.org/Andrea_Musacchio/): pLIB-GST-3C-CDK1 (177011), pLIB-HIS-TEV-Cyclin-B (177012), pLIB-HIS-TEV-CKS1(177013), and pLIB-HIS-TEV-scCAK1(177014).

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AUTHOR CONTRIBUTIONS

Pim J. Huis in 't Veld: Conceptualization (equal); formal analysis (lead); investigation (lead); methodology (equal); project administration (lead); supervision (equal); visualization (lead); writing – original draft (lead); writing – review and editing (supporting). **Sabine Wohlgemuth:** Methodology (equal); resources (equal). **Carolin Koerner:** Methodology (equal); resources (equal). **Franziska Mueller:** Investigation (supporting). **Petra Janning:** Investigation (supporting). **Andrea Musacchio:** Conceptualization (equal); funding acquisition (lead); supervision (equal); writing – review and editing (lead).

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