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Fluorescent Protein Biosensor for Probing CDK/Cyclin Activity in vitro and in Living Cells

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Cyclin-dependent kinases (CDKs) play an essential role in the coordination of cell cycle progression and transcriptional regulation; hyperactivation is associated with cancer. However there are few means of measuring their activity in a physiological context or their inhibition in response to therapeutics. To this aim we engineered a modular fluorescent protein biosensor that reports on phosphorylation by CDK/cyclins through real-time changes in fluorescence intensity. This allowed a comparison of enzymatic activity of recombinant kinases, monitoring inhibition by small molecules, and probing endogenous activities in lysates from healthy and cancer cell lines in a sensitive and quantitative fashion. This versatile tool was further implemented to probe the oscillatory activity of these kinases throughout the cell cycle by time-lapse imaging and ratiometric fluorescence quantification, following delivery of a red fluorescent protein fusion mediated by cell-penetrating peptides.

Introduction

Cyclin-dependent kinases (CDKs) are heterodimeric serine/threonine protein kinases that play major, established roles in coordinating cell growth and division; they are also recognized to be involved in wide variety of biological functions, including transcription regulation, developmental processes, and the differentiation of specific tissue types.[1–3] In mammalian cells three major CDK/cyclin complexes coordinate progression through the cell cycle. CDK4/6–cyclin D complexes are responsible for coordinating progression through G1, whereas CDK2–cyclins E and A are responsible for regulation of the S phase, and CDK1–cyclins A and B control the G2M transition (entry into mitosis) and progression through mitosis.[4,5] The CDK subunit performs the catalytic function, but it only becomes fully active and capable of binding its substrates efficiently following association with a cyclin subunit; this induces conformational changes in the CDK thus leading to alignment of the ATP-binding site with the catalytic cleft and favoring substrate access.[6–8] In addition, as cyclins exhibit periodic expression and degradation, they play a major role in determining the formation of CDK/cyclin complexes and modulating their subcellular localization. Finally, CDK/cyclin complexes are subject to post-translational modifications that either maintain the kinase in the inactive state or contribute to its activation, in particular phosphorylation of the CDK on its T-loop by CDK-activating kinase; this stabilizes substrate binding through direct interactions with positively charged amino acids[7–10] These kinases are frequently found to be hyperactivated in pathological disorders, including viral infection, neurodegenerative diseases, ischemia, and cancer, through mutation, overexpression, or amplification of the CDK or cyclin subunit.[11–13] As such, they constitute attractive pharmacological targets for the development of anticancer, antiviral, and antiparasitic drugs.[14–18] However there are few means of measuring their activity in a continuous and sensitive fashion (either in vitro or in living cells) and of assessing their inhibition by therapeutics. Detection of CDK activity remains essentially confined to endpoint radioactive assays and to approaches based on antigenic recognition of phosphorylated substrates.

Technologies that enable probing protein kinase activity in a sensitive and continuous fashion are essential to circumvent the limitations associated with endpoint radioactive and antibody-based approaches. In this respect, the development of fluorescent biosensors has provided new horizons and countless perspectives for basic research, biomedical applications, and drug discovery programmes. Over the past decade a broad family of fluorescent biosensors has been developed to probe protein kinases.[19] Among these, genetically encoded single-chain FRET biosensors afford countless applications for studying the dynamics of protein kinase activities in living cells,[20–23] but their expression can be lengthy and heterogeneous, varying from one cell to another. For example, a genetically encoded FRET activity biosensor was developed to probe CDK1/cyclin B activity in living cells.[24] This biosensor relies on ectopic expression in mammalian cells, and requires fine analysis of the fluorescence signals in single cells to quantify changes in FRET efficiency between a CFP and YFP pair (cyan and yellow fluorescent proteins) in individual cells. An alternative approach has been developed thanks to the chemical biol-

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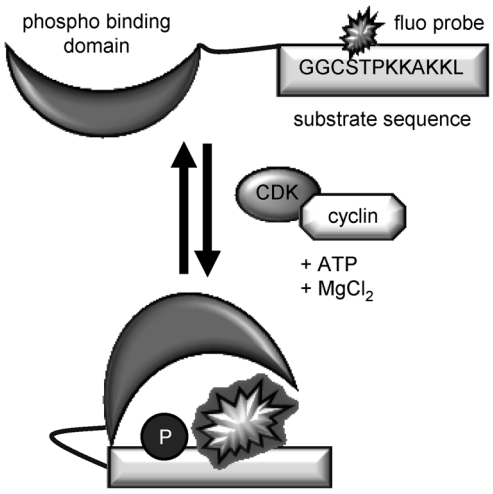
ogy toolbox of non-genetic biosensors: peptide, protein, and polymeric scaffolds conjugated to synthetic fluorescent probes.[20,25–35] Peptide biosensors developed to report on protein kinase activity rely on probes whose spectral properties are sensitive to phosphorylation, or to biosensor conformational changes that directly alter the environment of the probe and thus its fluorescent properties.[36] These biosensors constitute attractive tools with high sensitivity, but their application in living cells has not been widely developed due to their inability to cross cellular membranes.

Results and Discussion

In order to generate a sensitive and simple fluorescent biosensor with wide applicability and versatility, we engineered a modular protein biosensor that could be expressed in *Escherichia coli*, easily purified, and labeled with virtually any fluorescent probe. This biosensor comprises a peptide substrate derived from histone H1 (a natural substrate of CDK/cyclins, and which bears a threonine/proline (TP) phosphorylation site), into which we introduced a unique cysteine at position −2 for site-specific labeling with a fluorescent probe. The substrate sequence is linked to a phosphor-amino-acid binding domain (PAABD) derived from Plk1 (from which we removed all native cysteine groups) by a short proline/glycine-rich sequence. In this design, phosphorylation of the peptide substrate sequence by CDK/cyclins naturally favors its interaction with the PAABD, thereby inducing an intramolecular conformational change that alters the environment of the fluorescent probe. Scheme 1 depicts the modular structure of the biosensor and the conformational change induced by phosphorylation, thus altering exposure of the probe to the solvent.



Scheme 1. The CDKACT biosensor consists of a CDK substrate sequence (derived from histone H1) labeled with a fluorescent dye at −2 relative to the TP phosphorylation site, and a phospho amino-acid-binding domain that recognizes the substrate sequence phosphorylated by CDK/cyclin kinase. Phosphorylation of the substrate sequence induces intramolecular binding between the substrate and the PBD. This reaction is a reversible: equilibrium depends on the balance between kinases and phosphatases.

As this biosensor, which we termed CDKACT, was efficiently phosphorylated by recombinant CDK/cyclin complexes in a standard endpoint kinase assay with radioactive γ-ATP (Figure S1 in the Supporting Information), we labeled the unique cysteine proximal to the phosphorylation site in the substrate sequence with a Cy3 dye, and established a continuous fluorescence-based kinase assay based on changes in fluorescence intensity over time. CDKACT-Cy3 responded to phosphorylation by CDK2/cyclin A with a net increase in fluorescence (25%) over 25 min, whereas incubation with monomeric CDK2 (which is inactive as a kinase) did not result in any changes in CDKACT-Cy3 fluorescence (Figure 1A). Inhibition of CDK2/cyclin A kinase treated with the CDK inhibitor roscovitine[37] led to a significant reduction of this fluorescence enhancement (Figure 1A), whereas roscovitine had no significant effect on CDKACT-Cy3 fluorescence (Figure S2A). Incubation of the Cy3-labeled substrate sequence of CDKACT alone (no PAABD sequence) with recombinant CDK2/cyclin A did not promote any significant changes in Cy3 fluorescence, thus suggesting that phosphorylation alone is insufficient to promote interactions of the phospho group with the Cy3 probe, which would lead to fluorescence enhancement (Figure S2B). Addition of an excess of histone H1 substrate to the reaction led to a significant decrease in CDKACT-Cy3 fluorescence, thus indicating competition between the fluorescent biosensor and unlabeled substrate (Figure S2C). In comparison, when incubated with active CDK2/cyclin A, a Cy3-labeled nonphosphorylatable CDKACT mutant (phosphorylatable threonine in the substrate replaced by an alanine) underwent partial fluorescence enhancement (10%; Figure 1A). As CDK2/cyclin A has been reported to bind histone H1 substrate but not monomeric CDK2,[10] these experiments imply that CDK/cyclin binding alone to CDKACT is likely to contribute to partial fluorescence enhancement of the Cy3 probe (independently of a phosphotransfer reaction). We further examined the ability of other CDK/cyclins to phosphorylate CDKACT-Cy3 and found that both CDK1/cyclin B and CDK4/cyclin D promoted significant fluorescence enhancement of CDKACT-Cy3 (40 and 20%, respectively); this was inhibited by RO3306 (CDK1 inhibitor)[38] and PD-0332991 (CDK4,CDK6-specific inhibitor;[39] Figure 1B). In contrast, serine/threonine kinases CIV, Plk1, and Plk3 did not induce any significant changes in the fluorescence of CDKACT-Cy3 (Figure 1C).

We next asked whether CDKACT could be applied to probe CDK/cyclin kinase activity in cell extracts. Incubation of 20 μg extract of HeLa cells with CDKACT-Cy3 yielded a very similar fluorescence enhancement profile to that obtained with recombinant CDK/cyclins (Figure 2A). Incubation of the cell extracts with phosphatase inhibitors (to prevent dephosphorylation of CDKACT-Cy3 by phosphatases that counterbalance the effect of protein kinases) further increased the fluorescence of CDKACT-Cy3, thus implying that biosensor response was indeed associated with phosphorylation (Figure S3). The signal decreased upon addition of CDK inhibitors roscovitine and PD-033299 (Figure 2A). In contrast, neither U0126 (MAPK inhibitor) nor H89 (PKA inhibitor) prompted a significant reduction of CDKACT-Cy3 fluorescence (Figure 2B), thus implying that the

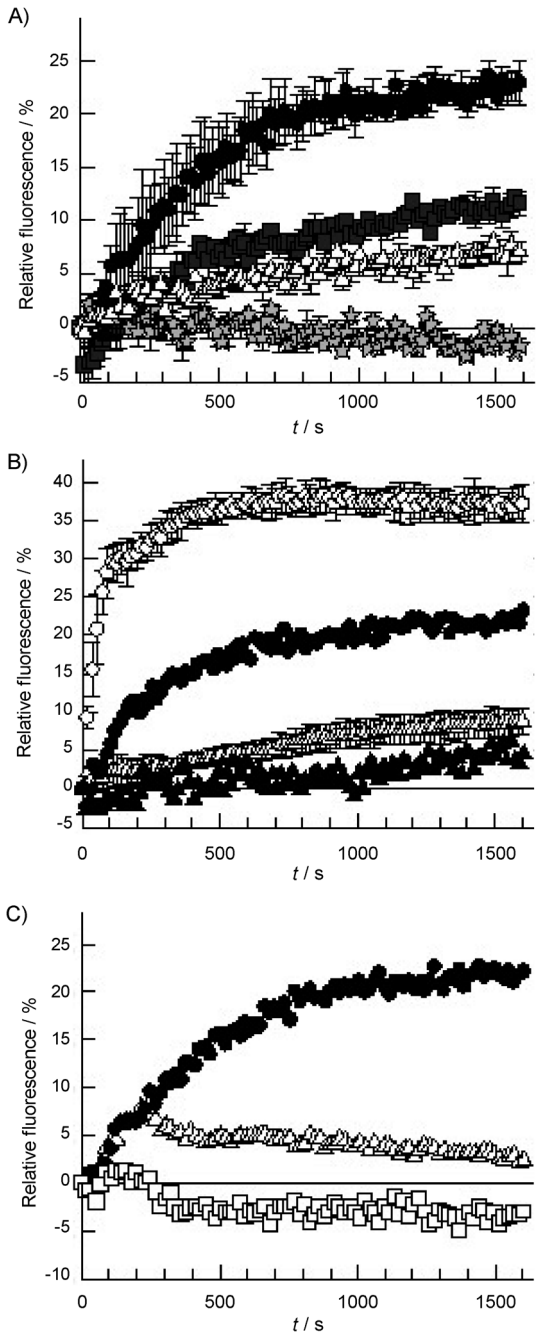


Figure 1. CDKACT-Cy3 (25 nM) is phosphorylated by active CDKs. A) CDKACT + CDK2/cyclin A (●); unphosphorylatable mutant + CDK2/cyclin A (■); CDKACT + monomeric CDK2 (★); CDKACT + 20 μM roscovitine (△). B) CDKACT + CDK1/cyclin B (○); CDKACT + CDK1/cyclin B + 20 μM RO3306 (△); CDKACT + CDK4/cyclin D (●); CDKACT + CDK4/cyclin D + 20 μM PD-0332991 (▲). C) CDKACT-Cy3 incubated with active CDK2/cyclin A (●), ClV (△) or Plk3 kinase (□).

biosensor response was associated specifically with CDK/cyclin activities. We therefore applied CDKACT-Cy3 to compare the activity of CDK/cyclins in extracts of the HT2-19 fibrosarcoma cell line in which CDK1 was ectopically expressed under control of an IPTG-inducible promoter, compared to 90% depletion of CDK1 protein levels after four days in the absence of IPTG.[40,41] HT2-19 cells were grown with and without IPTG, and 20 μg extracts from these were assayed in a fluorescence-based kinase assay. CDKACT-Cy3 fluorescence was reduced by 50% when incubated with extracts prepared from cells that did not overexpress CDK1, compared to extracts from cells expressing high levels of CDK1. This implies that in this cell line, CDK1 activity is responsible for at least half of all CDK/cyclin kinase activity (Figure 2C).We next compared overall CDK/cyclin kinase activity between the fibroblast cell line HS68 and the cervical cancer cell line HeLa with normalized concentrations of total cell extracts. The weak enhancement of CDKACT-Cy3 fluorescence upon incubation with HS68 cell extracts (relative to HeLa cell extracts; Figure 2D) is consistent with the differences in CDK and cyclin levels determined by western blotting.[16] This assay was further employed to compare total CDK/cyclin kinase activity between different cancer cell lines (HeLa, U2OS, A549, and MCF7; Figure 2E). CDKACT-Cy3 fluorescence enhancement was greatest upon incubation of the biosensor with HeLa cell extracts, closely followed by U20S; much weaker maxima were achieved upon incubation with A549 and MCF7 lysates, thus suggesting that CDK/cyclin activity is much lower in these cell lines, in agreement with the lower protein levels of CDKs and cyclins in A549 and MCF7 relative to HeLa and U20S.[16] Taken together these experiments show that CDKACT is a useful tool to measure CDK/cyclin activity in cell extract; it also allows characterization of the efficacy of a CDK inhibitor on endogenous kinase activity, and comparison of differences in CDK/cyclin activity between cell lines expressing different levels of CDKs and cyclins.

In order to apply CDKACT to probe CDK/cyclin kinase activity in living cells, we engineered a red fluorescent protein (RFP)–CDKACT construct, which we labeled with a Cy5 probe proximal to the phosphorylation site. This variant still responded to CDK/cyclin activity efficiently: the robust enhancement of Cy5 fluorescence (60%) in response to active recombinant CDK2/cyclin A was significantly reduced in the absence of ATP/$MgCl_2$ (Figure 3A). In contrast, the fluorescence of the RFP moiety did not exhibit any variation upon biosensor phosphorylation by the kinase (Figure S4A), thus indicating that it could be used as an intramolecular standard for ratiometric quantification of the relative changes in Cy5 fluorescence associated with CDKACT phosphorylation. RFP-CDKACT-Cy5 was introduced into living cells following complexation with the cell-penetrating peptide Pep1, which we previously demonstrated to efficiently deliver proteins, including RFP, into living cells.[42,43] (Figure S4B). Following internalization, HeLa cells were imaged by time-lapse microscopy: the fluorescence signals corresponding to RFP and Cy5 were acquired separately and normalized to determine the Cy5/RFP ratio over time. These experiments revealed a significant increase in the Cy5/RFP ratio at the onset of mitosis in dividing cells, concomitant with cell rounding in early prophase (Figure 3B). In contrast, when cells were imaged over a period of time when they did not divide, or when cells were treated with the CDK/cyclin inhibitor roscovitine, the fluorescence ratio did not vary (Figure 3C and D). When they were treated with H89 (PKA inhibitor), oscillations of Cy5/RFP fluorescence were observed again (Figure 3E). When the same experiment was performed with

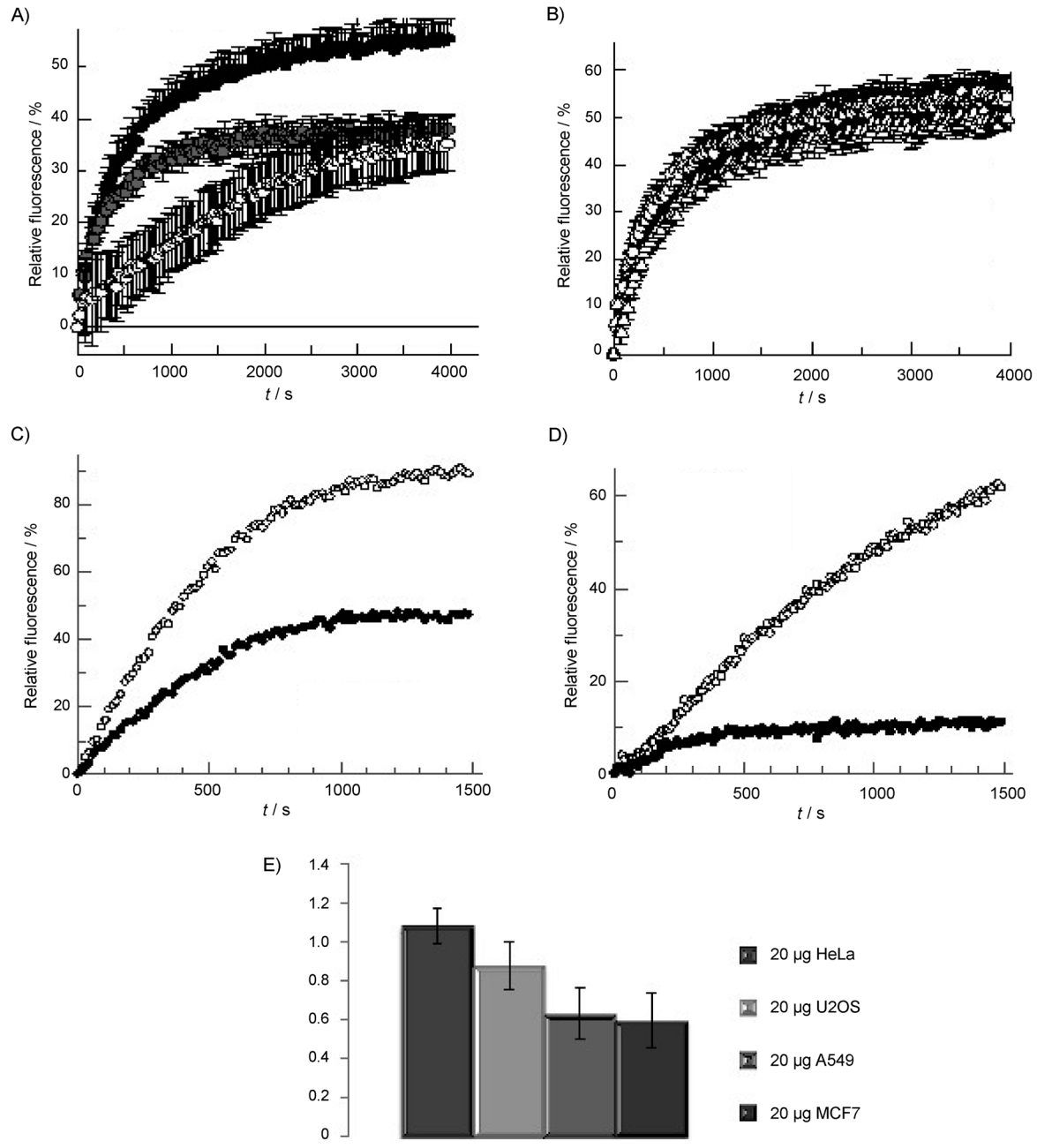


Figure 2. CDK/cyclin activity in mammalian cell extracts. Fluorescence of 25 nM CDKACT-Cy3 was monitored over time upon incubation with cells. A) HeLa cell extract (20 μg) alone (●) and with 20 μM CDK inhibitors roscovitine (●) or PD-0332991 (○). B) HeLa cell extract (20 μg) alone (●) and with 20 μM MAPK inhibitor U0126 (○) or 20 μM PKA inhibitor H89 (△). C) HT2-19 cell lysate (40 μg) with (○) or without IPTG (●; to overexpress CDK1). D) HeLa (○) and HS68 (●) cell extracts (20 μg). E) Maximal fluorescence enhancement of CDKACT-Cy3 for 20 μg extracts from HeLa, U2OS, A549, and MCF7 cells after 50 min.

a non-phosphorylatable mutant of RFP-CDKACT-Cy5, no significant variation in the Cy5/RFP ratio was observed before or throughout mitosis (Figure 3F). Ratiometric values corresponding to representative examples plotted in Figure 3 are summarized in Table 1. (All fluorescence values are reported in Tables S1–S5). Taken together, these results indicate that the RFP-CDKACT-Cy5 biosensor reports on CDK/cyclin activity in living cells and responds primarily to activities which are inhibited by roscovitine at the onset of mitosis.

Conclusion

In this study we describe a fluorescent protein biosensor that reports on CDK/cyclin activity through changes in fluorescence intensity, which allows continuous fluorescence-based kinase assays to be performed in vitro with recombinant kinases or cell extracts, as well as monitoring CDK/cyclin activities in living cells. Our assay circumvents the use of radioactivity or antibodies, and follows CDK/cyclin kinase activity in a highly sensitive and reversible fashion: only 10–25 nM biosensor is re-

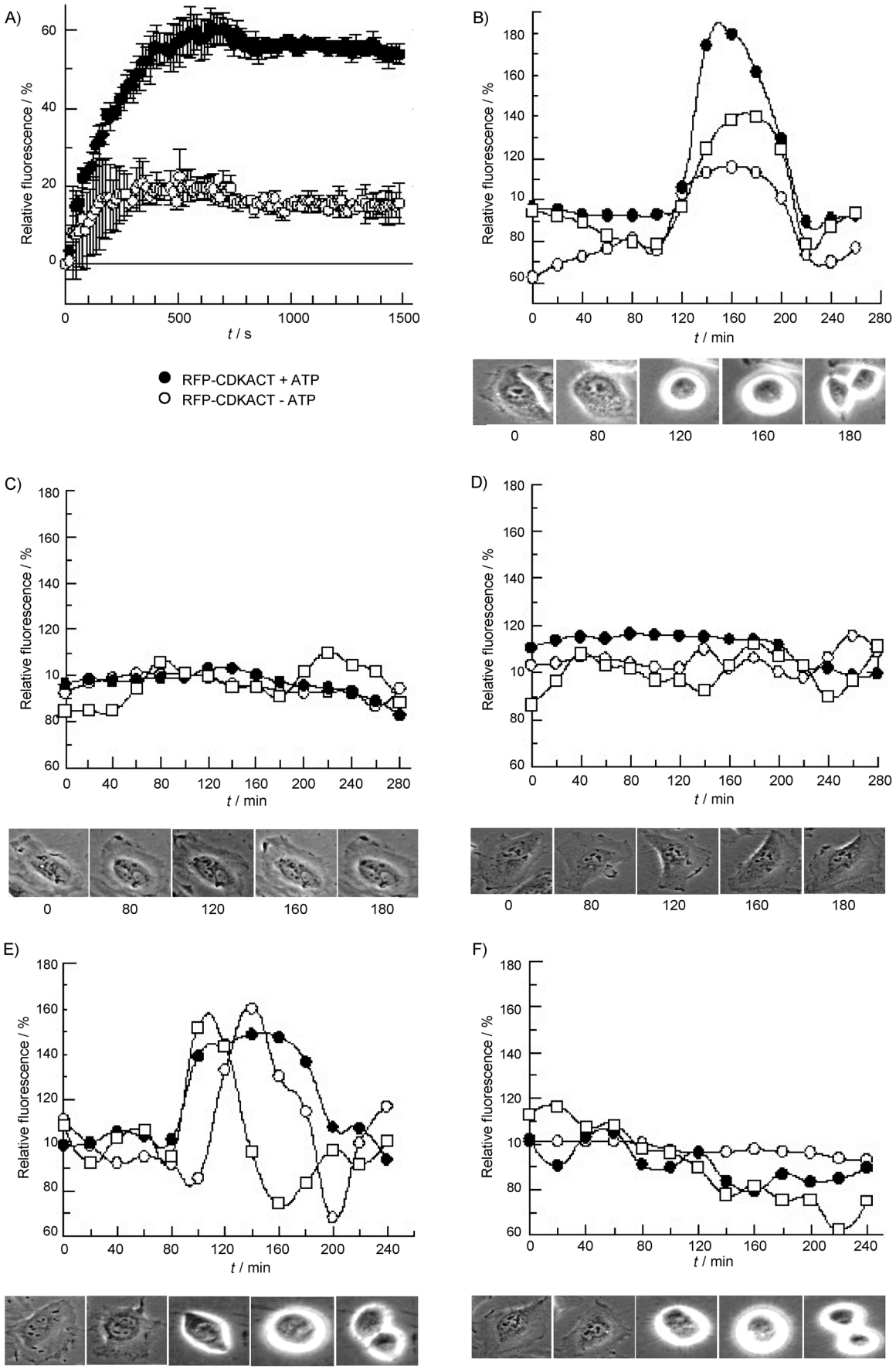


Figure 3. CDK/cyclin activity in living cells. A) Fluorescence enhancement of 25 nM Cy5-labeled RFP-CDKACT upon incubation with 25 nM active recombinant CDK2/cyclin A, with or without ATP/$MgCl_2$. B)–E) Cy5/RFP fluorescence intensity ratio profiles of RFP-CDKACT-Cy5 acquired from single cells 1 (○), 2 (●) or 3 (□) undergoing division (B) or Cy5/RFP fluorescence intensity ratio profiles of RFP-CDKACT-Cy5 acquired from non-dividing cells (C). Cy5/RFP fluorescence intensity ratio profiles of RFP-CDKACT-Cy5 acquired from cells treated with roscovitine (D). Cy5/RFP fluorescence intensity ratio profiles of RFP-CDKACT-Cy5 acquired from cells treated with the PKA inhibitor H89 (E) and Cy5/RFP fluorescence intensity ratio profiles of a non-phosphorylatable mutant of RFP-CDKACT-Cy5 acquired from cells undergoing division (F). Phase contrast micrographs acquired at different time points throughout the time-lapse experiment show the changes in cell morphology, such as cell rounding when cells enter and undergo mitosis.

Table 1. Fluorescence ratio (Cy5/RFP) of RFP-CDKACT-Cy5 and its mutant in HeLa cells.

t [min]	Div. cell.[a]	N-div. cell[b]	Rosco[c]	H89[d]	Mutant[e]
0	95.9	99.9	101.1	99.9	100.8
20	95.4	101.8	99.8	100.1	100.9
40	93.0	104.0	98.0	100.9	100.8
60	92.6	103.6	98.4	106.1	100.9
80	92.7	103.5	101.3	103.8	100.1
100	93.1	100.5	101.4	102.3	97.1
120	105.8	101.3	102.1	138.8	96.1
140	174.2	101.6	102.6	144.3	96.3
160	179.9	98.6	101.2	148.6	97.5
180	161.5	99.9	100.8	147.2	96.4
200	128.8	99.3	99.5	136.5	95.9
220	89.5	100.4	98.9	108.1	93.5
240	90.9	99.9	100.8	107.5	92.9

[a] Dividing HeLa cells. [b] Nondividing HeLa cells. [c] Roscovitine-treated HeLa cells. [d] H89-treated HeLa cells. [e] Dividing HeLa cells–RFP-CDKACT-Cy5 non-phosphorylatable mutant.

quired, two or three orders of magnitude lower than the substrate concentrations required in radioactive assays (2–20 μM). We have shown that CDKACT reports on enzymatic activity profiles, highlights differences between different CDK/cyclin complexes, and allows analysis of the efficiency of inhibitors of kinase activity. We have further successfully applied CDKACT to probe CDK/cyclin activities in cell extracts, thus demonstrating that it can report on differences associated with expression levels of CDKs and cyclins, as well as differences in CDK/cyclin activities in extracts from healthy and cancer cell lines. Finally, we applied this biosensor to probe CDK/cyclin activity in living cells and showed that we could monitor periodic oscillations associated with CDK/cyclin activity at the onset of mitosis. CDKACT therefore constitutes a versatile tool for probing CDK/cyclin activity in a continuous fashion, both in vitro and in living cells.

This system can directly probe kinase activity through changes in fluorescence intensity of an environmentally sensitive probe conjugated to the substrate sequence, and the same molecular species (i.e., purified recombinant protein biosensor) can be applied to report on recombinant kinase activity and on CDK/cyclin activity from cell extracts, and can be introduced into living cells for time-lapse imaging. Thus, it offers straightforward biosensing with broader applicability and versatility than genetically encoded biosensors, as the latter require expression and purification (for in vitro use) yet ectopic expression can be lengthy and vary from one cell to another if stable transfectants have not been established. This technology offers countless opportunities by conjugating fluorescent probes with very different spectral properties to the unique cysteine within the substrate sequence: from fluorescein derivatives to rhodamine, cyanine or infra-red probes, as long as they exhibit sensitivity to the environmental changes associated with the intramolecular conformational change that occurs upon phosphorylation of CDKACT. However, this nongenetic system depends on conjugation or complexation with a cell-penetrating moiety or formulation for cellular internalization and imaging CDK/cyclins in living cells.

CDK/cyclins constitute attractive pharmacological targets in several human disorders, including cancer, yet there is no means of probing their hyperactivity in a sensitive and quantitative fashion. The technology we describe here allows countless applications in vitro and in living cells. We therefore anticipate that it will be of great utility for identifying high CDK/cyclin kinase activity in pathological situations, thereby offering an alternative to antibody-based approaches for establishing diagnostic assays; it will provide essential information for tailored therapeutic intervention, and for monitoring responses to therapeutics targeting CDK/cyclin kinases. Finally, CDKACT technology provides a sensitive means of assessing the efficacy of inhibitors, and is therefore well suited for drug discovery programmes based on real-time fluorescence assays for screening complex libraries of compounds that interfere with CDK/cyclin activity in cell extracts or in living cells.

Experimental Section

Design and engineering of GST-CDKACT: GST-CDKACT was engineered by cloning the PBD domain of Plk1 (residues 367–603; GenBank accession no. NM_005030) into BamHI and EcoRI sites of the GST fusion vector pGEX-6P-1 (GE Healthcare), followed by a linker (PGAGGTGGLPGG) and a substrate sequence derived from histone H1 (GGCSTPKKAKKL) cloned at the EcoRI site. All six cysteine residues within the PBD domain were replaced by serine residues by mutagenesis. The complete sequence of CDKACT is as follows:

GEVVDSHLSDMLQQLHSVNASKPSERGLVRQEEAEDPASIPIFWVSKWVD-YSDKYGLGYQLDNSVGVLFNDSTRLILYNDGDSLQYIERDGTESYLTVSSH-PNSLMKKITLLKYFRNYMSEHLLKAGANITPREGDELARLPYLRTWFRTRSA-IILHLSNGSVQINFFQDHTKLILSPLMAAVTYIDEKRDFRTYRLSLLEEYGSSK-ELASRLRYARTMVDKLSSRSASNRLKASPGAGGTGGLPGGGGCSTPKKA-KKL

GST-CDKACT was expressed in *E. coli* BL21pLyS by induction with IPTG (0.5 mM) at 20 °C overnight, then purified on High Performance Glutathione Sepharose (GE Healthcare) in Tris·HCl (50 mM, pH 7.4) with NaCl (150 mM), and eluted with glutathione (50 mM in the same buffer) followed by gel filtration chromatography on Superdex75 (GE Healthcare) in the same buffer. Note that the GST tag was necessary to ensure solubility and function of the biosensor, as its removal lead to complete precipitation of the bio-

sensor. RFP-CDKACT was engineered by cloning the mRFP sequence from the pRSETB-mRFP vector[44] into pGex6P1, immediately upstream from CDKACT. GST-RFP-CDKACT was then expressed in *E. coli* BL21pLyS and purified as above. The GST tag was cleaved from GST-RFP-CDKACT with Prescission Protease (GE Healthcare). Following purification, GST-CDKACT and RFP-CDKACT were labeled with tenfold molar excess of Cy3- and Cy5-maleimide, respectively, then further purified on illustra NAP-5 Sephadex columns (GE Healthcare) in Tris·HCl (50 mM, pH 7.4) with NaCl (150 mM) to remove excess label. Non-phosphorylatable GST-CDKACT and RFP-CDKACT mutants were generated by site-directed mutagenesis of the phosphorylatable threonine to alanine.

Protein expression and purification of CDK: Recombinant CDKs and cyclins were expressed in *E. coli* by IPTG induction and purified by chromatography as described previously.[45] GST-CDK2/cyclin A, GST-CDK1/cyclin B and GST-CDK4/cyclin D were co-purified as complexes on a single GSTTrap column (GE Healthcare), then further incubated with recombinant GST-CIV, the CDK-activating kinase of *Saccharomyces cerevisiae* purified on GST-Trap column (GE Healthcare) by using the same protocol as for other GST-CDKs to ensure their complete activation through phosphorylation of the T-loop. The kinase activity of these complexes was verified in a radioactive endpoint assay. GST–CIV was expressed in *E. coli* and purified by chromatography on GST-Trap columns.

Radioactive kinase assays: Phosphorylation of CDKACT (3 μM) or histone H1 (5 μM; H4524 Sigma–Aldrich) by recombinant kinases (100 nM) was performed in Tris (50 mM, pH 7.5) with $MgCl_2$ (5 mM), cold ATP (50 μM), and [^{33}P]γ-ATP (0.15 μCi, (3000 Ci mmol^{-1}; GE Healthcare) for 30 min at 30 °C. The reaction was stopped by addition of Laemmli buffer, and samples were run on SDS-PAGE then exposed by autoradiography.

Real-time fluorescence experiments: Fluorescence kinase assays were performed in 96-well plates in a temperature-controlled chamber (POLARstar; BMG Labtech, Ortenberg, Germany) at 30 °C in potassium phosphate (200 μL, KH_2PO_4/K_2PO_4 (50 mM, pH 7.4) with NaCl (150 mM)), supplemented with $MgCl_2$ (5 mM) and ATP (0.5 mM) except when stated otherwise. The fluorescence of Cy3-labeled CDKACT with recombinant CDK/cyclin complexes or with cell extracts was monitored over time (Cy3 and RFP: λ_{ex} 544 nm, λ_{em} 590 nm; Cy5: λ_{ex} 645 nm, λ_{em} 675 nm). In all experiments, relative fluorescence was calculated following subtraction of CDKACT-Cy3 fluorescence from values obtained in the presence of protein kinases, inhibitors, or cell extracts. Data analysis was performed with GraFit (Erithicus Software, Horley, UK). Experiments were performed in triplicate; error bars indicate standard deviation.

Inhibitors: Roscovitine, PD-0332991, H89, and U0126 were purchased from Euromedex. RO3306 (CalbioChem) was purchased from Merck Millipore. All stock solutions were made in DMSO and diluted into PBS to the desired concentration immediately prior to use.

Cell culture and extract preparation: Cell culture media, sera and antibiotics were purchased from Invitrogen. HeLa, HS68, A549, MCF-7 and U2OS cells were cultured in DMEM with GlutaMAX supplemented with FCS (10%), penicillin (1 mM) and streptomycin (1 mM) at 37 °C in an atmosphere containing 5% CO_2. HT2-19 cells, provided by Dr. Andrew Porter, were cultured in DMEM with NEAA, antibiotics, sodium pyruvate and glutamine, and supplemented with FCS (10%).[40] Cell extracts were prepared in PBS lysis buffer (KH_2PO_4/K_2PO_4 (50 mM, pH 7.4), NaCl (150 mM), NP40 (0.1%), deoxycholate (0.1%), EDTA (2 mM), PMSF (1 mM), Complete Protease Inhibitor Cocktail (Roche)) and data were normalized following spectrophometric dosage at 280 nm; phosphatase inhibitors: NaF (50 mM), β-glycero-phosphate (40 mM), Na_3VO_4 (1 mM).

Cell-penetrating peptide delivery into living cells: For introduction into living HeLa cells, RFP-CDKACT-Cy5 (1–3 μM) was complexed with Pep1 (1:20, molar ratio) and overlaid onto cultured cells grown to subconfluency for 1 h, as described previously.[41–43]

Time-lapse imaging and fluorescence quantification: Live-cell imaging acquisitions of RFP and Cy5 fluorescence from RFP-CDKACT-Cy5 in HeLa cells were substracted for background signal (minimal fluorescence) by using Metamorph software (Molecular Devices, Sunnyvale, CA) followed by image analysis. Regions of interest (ROIs) corresponding to single cells in which fluorescence appeared homogeneous were defined, and the mean gray levels of fluorescence for each channel (RFP and Cy5) were quantified within these ROIs throughout time-lapse images. Fluorescence intensity values of both RFP and Cy5 were then normalized to the stabilized signal reached following internalization. For each experiment, the mean ratiometric value of Cy5/RFP was calculated from three or four different ROIs.

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Keywords: biosensors · CDK/cyclin · fluorescence · kinase activity · live-cell imaging

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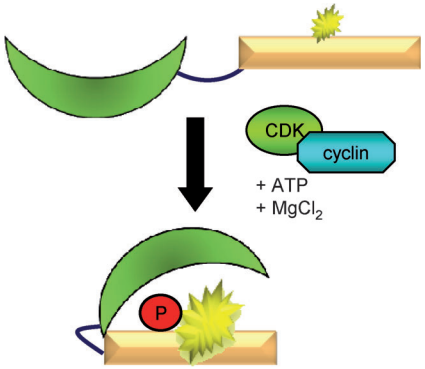
A modular protein biosensor reports CDK/cyclin kinase activity in vitro and allows assessment of CDK inhibitors in a continuous and quantitative fashion. Kinase activity was compared in cell lysates of healthy and cancer cell lines, and CDK/cyclin activity was monitored throughout the cell cycle by time-lapse imaging and ratiometric fluorescence quantification.



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Fluorescent Protein Biosensor for Probing CDK/Cyclin Activity in vitro and in Living Cells