Human glioma PKC- α and PKC- β II phosphorylate cyclin-dependent kinase activating kinase during the cell cycle

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Abstract. Cell cycle phase transition is regulated in part by the trimeric enzyme, cyclin-dependent kinase activating kinase (CAK) which phosphorylates and activates cyclin-dependent kinases (cdks). Protein kinase C (PKC) inhibitors prevent cell cycle phase transition, suggesting a fundamental role for PKCs in cell cycle regulation. We report that in glioma cells, CAK (cdk7) is constitutively associated with PKC- α . *In vitro* phosphorylation, co-immunoprecipitation, and analysis of phosphorylated proteins by autoradiography indicate that CAK (cdk7) is a substrate for PKC- α and PKC- β II hyperphosphorylation. These results establish a role for PKC- α and PKC- β II in the activation of CAK during the glioma cell cycle.

INTRODUCTION

The rapid proliferation rates of glioma cells have been attributed to inherently high levels of protein kinase C (PKC; Couldwell *et al.* 1990; Pollack *et al.* 1990). It is possible that PKC may interact with cyclins and their cyclin-dependent kinases (cdks) during cell cycle progression (Levin *et al.* 1990). The normal mammalian cell cycle is controlled by a complex composed of cdks, their regulatory subunits called cyclins (cyclins A to E), inhibitors of cyclin kinases, i.e. p16, p21, p57 and p27, and proliferating cell nuclear antigen, a subunit of DNA polymerase- δ (reviewed in Elledge & Harper 1994). In transformed cells, the quaternary complex is not present and the primary cell cycle effectors are the cyclin/cdk complexes. Activation of the cdks is through phosphorylation of a threonine residue by cdk-activating kinase (CAK). CAK itself must be activated by phosphorylation before it can phosphorylate cdks. CAK is composed of cdk7, cyclin H and a 36-kDa polypeptide (p36 or MAT1; Fisher *et al.* 1995). In the absence of MAT1, cdk1, cdk2 and cdk4 activation requires cyclin association and phosphorylation by CAK at the conserved threonine residue (T160–T176). However, when MAT1 is present it promotes the stable binding of cdk7-cyclin H and circumvents the need for T160–T176 phosphorylation. CAK also associates with the general transcription factor TFIIF and phosphorylates the carboxyl-terminal domain of RNA polymerase II (Fisher *et al.* 1995).

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A link between PKC isozyme expression, CAK activation and cell cycle regulation has been implicated in recent studies. In addition to the ubiquitous cdks, cell cycle progression is controlled in part by oscillations in inositol 1,4,5,-trisphosphate which induce transient changes in intracellular calcium leading to calcium release from intracellular stores and mitosis (Kao *et al.* 1990; Tombes *et al.* 1992; Ciapa *et al.* 1994). In the budding yeast *Saccharomyces cerevisiae*, PKC depletion blocks cell division (Levin *et al.* 1990) and depending on the mammalian cell type, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced PKC activation results in either terminal growth arrest (Levin *et al.* 1990), mitogenesis (Rozengurt 1986), differentiation (Rovera *et al.* 1979), or transient growth arrest (Gescher & Reed 1985; Coppock *et al.* 1992; Skouv *et al.* 1994). Furthermore, depletion of cellular PKC levels by TPA pretreatment decreases the influence of platelet derived growth factor and serum on cyclin D1 mRNA expression (Winston & Pledger 1993). Transfection and overexpression of PKC isozymes with constitutive expression vectors affect cell proliferation and promote neoplastic transformation (Watanabe *et al.* 1992; Cacace *et al.* 1993; Mischak *et al.* 1993; Hans *et al.* 1995). PKC activity and PKC isozyme content fluctuate during cell cycle progression and PKC- α may function in inhibition of G₁ to S transition (Kosaka *et al.* 1993; Sasaguri *et al.* 1993). Recent studies suggest that reduced cdk phosphorylation post-staurosporine (a non-specific PKC inhibitor; STSP), TPA or phorbol-12,13-dibutyrate treatment may be due to inhibition of CAK (Coppock & Nathanson 1993; Schiner *et al.* 1994; Asidu *et al.* 1995; Gadbois *et al.* 1995; Hamada *et al.* 1996), implicating CAK as a possible PKC substrate.

MATERIALS AND METHODS

Cell culture

The U-373MG cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). Cells were seeded (1×10^6) and grown as monolayers on 75 cm² flasks containing 90% Dulbecco's modified Eagle's minimal essential medium (DMEM), 10% fetal calf serum (FCS), 2 mM L-glutamine, 4.5 gm/l glucose, and antibiotics (penicillin 10 U/ml and streptomycin 10 µg/ml) according to Ponten & MacIntyre (1986).

Cell cycle analysis

Cell cycle analysis was performed as previously described (Acevedo-Duncan *et al.* 1997). Confluent cell cultures were semi-synchronized by contact inhibition and serum starvation for 48 h. Subsequently, cells were incubated in serum and removed at specific times, washed in Dulbecco's phosphate-buffered saline (DPBS), trypsinized, and fixed by dropwise addition (while vortexing) of cold ethanol until a concentration of 60% ethanol was reached. At 4 h post serum addition, cells were labelled sequentially with bromodeoxyuridine (BrdUrd; 5 µM, 0.5 h) and thymidine (5 µM; 0.5 h). Nuclei were analysed for DNA content using a BrdUrd/propidium iodine double staining protocol and flow cytometry (Carlton *et al.* 1991). Nuclear BrdUrd staining was detected with anti-BrdUrd monoclonal antibody (Becton Dickinson, San Jose, CA) and fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse IgG (Sigma Chemicals, St. Louis, MO). The distributions of 40 000 nuclei were quantified using a FAC STAR^{Plus}, flow cytometer (Becton Dickinson) and ModFitLT Cell Cycle Analysis program (Version 2.0; Verity Software House, Inc., Topsham, ME). Statistics: Statistical determination was by Student's *t*-test using the Minitab program (Mininc. State College, PA).

Metabolic labelling

U-373MG cells were grown to confluency on 75 cm² flasks, serum starved for 48 h, and washed twice with phosphate-free DMEM. Cells were incubated for 4 h in 5 ml phosphate-free DMEM containing 0.1 mCi/ml [³²P]-orthophosphate (cat.# 64014; ICN Biochemicals, Irvine, CA). Subsequently, cells were washed twice with PBS. Immediately following the DPBS washes (at T₀) cells were incubated in DMEM containing 10% serum and either DMSO or calphostin C. [³²P]-orthophosphate labelled cells were subfractionated as described below and used for immunoprecipitation of cdk7 and cdk2.

Light activation of calphostin C

Cells were treated with calphostin C [0.5 μ M; dissolved in dimethyl sulphoxide (DMSO)] and flasks containing cells were placed under ordinary fluorescent light for 5 min to photoactivate calphostin C. Brief (min) photoactivation of calphostin C has been shown to produce irreversible inhibition of PKC in glioma cells with prolonged abrogation of total PKC activity (Gopalakrishna *et al.* 1992).

Cell fractionation and Western blot analysis

Following treatments cells were placed on ice to terminate the incubation. Cell extracts were prepared by washing twice with ice cold DPBS. Monolayers were scraped at 4 °C, resuspended and sonicated in 2 ml homogenization buffer (50 mM HEPES (pH 7.5) 150 mM NaCl, 0.1% Tween-20, 1 mM EDTA and 2 mM ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.1 mM orthovanadate, 1 mM NaF, 2 mM PMSF, 2.5 μ g/ml leupeptin, 1 mM DTT, 0.15 U/ml aprotinin; Agrawal *et al.* 1995). Cell suspensions were centrifuged at 100 000 *g* for 30 min to obtain cell extracts. Protein content was measured according to Bradford (1976). Cell extracts (50–80 μ g protein) were electrophoresed according to Laemmli (1970). Proteins were transblotted according to Towbin *et al.* (1979). Immunoreactive bands were visualized with enhanced chemiluminescence according to manufactures instructions (ECL; Amersham, Piscataway, NJ).

Immunoprecipitations and PKC activity assay

U-373MG cells were subfractionated and lysates (150 μ g or 300 μ g) were immunoprecipitated with affinity purified antibodies (4–6 μ l). Immune complexes were rocked overnight at 4 °C and incubated at 4 °C for 1 h with 100 μ l of protein A/G. Subsequently, protein A/G immune complexes were washed twice with lysis buffer. PKC activity assay was initiated by suspension of pelleted immunoprecipitates in 127 μ l of PKC kinase buffer (Davis & Clark 1983) using [γ -³²P]-ATP (20 μ Ci/reaction Neg-002Z; NEN Dupont, Boston, MA) to test the ability of PKC isozymes to phosphorylate cdk7. The PKC kinase buffer consisted of 20 mM Tris-HCl, pH 7.5, 6 mM magnesium acetate, phosphatidylserine (PS; 5 μ g), diacylglycerol (DAG; 0.2 μ g) and 0.5 mM CaCl₂. Basal kinase activity was measured in the absence of CaCl₂ and phospholipid and in the presence of 1 mM EGTA.

RESULTS

Calphostin C delays S phase progression

The consequences of cell cycle modulators on the cell cycle depend on the position of the cell cycle phase in which they are applied (Sinclair 1968; Allalunis-Turner *et al.* 1997). To establish

the cell cycle effects of calphostin C on U-373MG cells in G_1 , we used confluent cultures which have a high Quiescence-Gap1 (G_0/G_1) cell population and attempted a reversible G_0/G_1 arrest by serum starvation. Calphostin C was used since it is a selective, potent and irreversible PKC inactivator with an IC_{50} of $0.05 \mu\text{M}$ (Kobayashi *et al.* 1991). It inhibits both the phorbol ester binding and phosphotransferase activity by binding to the regulatory domain of all PKC isozymes without competing for phospholipids (Kobayashi *et al.* 1991; Gopalakrishna *et al.* 1992). To establish a relationship between PKC isozyme expression and cell cycle regulation, cells were grown to confluence and serum starved for 48 h. The cell cycle was initiated by serum addition in combination with either DMSO (vehicle control) or calphostin C ($0.5 \mu\text{M}$; dissolved in DMSO). This concentration of calphostin C ($0.5 \mu\text{M}$) has been shown to inhibit overall PKC activity in glioma U-373MG cells (Acevedo-Duncan *et al.* 1997). Following addition of serum and DMSO, or calphostin C, cells were fixed at 2 h intervals for analysis of total DNA by flow cytometry. Figure 1a illustrates representative FACS profiles from a single experiment in which serum deprivation for 48 h produced a cell population (at time zero in culture; T_0) consisting of 87% G_0/G_1 , 9% S and 4% $G_2 + \text{M}$. Since the cells are aneuploid, there are two G_0/G_1 peaks. The first dark-shaded peak is the 2N (diploid) population and the second equal-sized unshaded peak is the 4N (tetraploid) population. The average T_0 obtained from four experiments in duplicate was 81% G_0/G_1 , 12% S and 7% $G_2 + \text{M}$. Since serum starvation did not produce a synchronous G_0/G_1 cell population, we examined the cell cycle effects of calphostin C on a high G_1 asynchronous cell population. These results are consistent with published data indicating that it is difficult to completely arrest transformed cells (Hans *et al.* 1995). Calphostin C did not produce a G_1 arrest which is expected since the p53 tumour suppressor is mutated in U-373MG and there is no functional p53 pathway (Russell *et al.* 1995). However, as previously reported (Acevedo-Duncan *et al.* 1997), calphostin C affected S phase progression and provoked a 2-h S phase delay (Fig. 1b). These results may indicate that calphostin C was turning over at this time since calphostin C inhibits PKC protein synthesis for 12 h (Gopalakrishna *et al.* 1992).

Modulations of cell cycle markers and PKC isozymes throughout the cell cycle

To identify which PKC isozymes are involved in G_1/S transition or S phase, cells from each of the time points were harvested and prepared for Western blotting. As a control for protein loading, we probed Western blots of total cell lysates for the protein actin using mouse anti-actin (SC-8432, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1/200. Actin Western blots showed actin immunoreactive bands at a molecular weight of 43 kDa. The actin immunoreactive bands were of equal intensity indicating that equal amount of protein were loaded into each lane (data not shown). Lysates were probed for cell cycle markers (cyclins, cdks and cdk inhibitors) and the presence of PKC- α , βI , βII , γ , δ , ϵ , ζ , η , θ , ι , and μ . Cell cycle stage was confirmed by checking cell cycle progression markers (Fig. 2a). U-373MG cells exhibited normal cell cycle progression as judged by induction of G_1 cyclins and the G_1/S cdk inhibitor, p21. In serum/DMSO treated cells, p21 protein levels peaked at 4 h and subsequently decreased between 8 and 20 h. The decline in p21 coincides with escape of cells from G_1 arrest (Bae *et al.* 1995). In contrast, the cdk inhibitor p27 exhibited an abnormal protein profile. Instead of a sharp decrease concomitant with entry into G_1 (Hengst & Reed 1996), p27 was expressed throughout the cell cycle, and only slightly declined from 2 to 16 h with a reaccumulation at 18–24 h. In comparison to controls, calphostin C-treated cells expressed smaller amounts of p27 at 2–10 h and p27 gradually increased from 12 to 24 h. The control p27 results are inconsistent with cell cycle progression and normal p27 status. However, these results may not be unique since aberrant, constant and biphasic p27 levels during cell cycle progression have been reported (Poon *et al.* 1995; McIntyre *et al.* 1999).

Figure 1. Effects of calphostin C on U-373MG cell cycle distribution as a function of time. (a) FACS analysis of DNA replication during a mitotic time course. Cells were stimulated with 10% FCS and either 4 μ l of DMSO (controls; left column) or calphostin C (0.5 μ M; right column). Light activation of calphostin C was as described in Materials and Methods. To establish DNA flow cytometry settings, U-373MG cells were karyotyped (Genzyme Genetics, Tampa, FL) and found to be aneuploid. The DNA histograms are from one representative experiment and illustrate two cycling populations with a G_0/G_1 peak at 2N (first dark-shaded peak) and another at 4N (second equal-sized unshaded peak). Total DNA content for G_0/G_1 , S and $G_2 + M$ was quantified by addition of each of the phases in both populations. (b) Percentage of cells in S phase. Open symbols represent control (DMSO)-treated cells. Solid symbols represent cells treated with calphostin C (0.5 μ M). Asterisk represent significant differences between control and calphostin C-treated Cells at 14 h ($P = 0.05$) and 16 h ($P = 0.01$). Data is the mean of two independent experiments. Number of events collected was 40 000 per time point and treatment group.

Figure 1. Continued

Next, we examined the G_1 cyclins, D1, D2 and D3. Western blots of either cyclin D1 and D2 depicted two bands representing two phosphoproteins (Matsushime *et al.* 1994). The slower migrating species is constitutively expressed while the more intense lower band is known to be more pronounced at the G_1/S boundary (Matsushime *et al.* 1994). With the exception of high cyclin D3 levels at T_0 , induction of cyclin D1, D2 and D3 in control and calphostin C-treated cells were concomitantly regulated with a maximum increase at 4 h, and a decline thereafter. Western blots of cdk2, cdk7, MAT1 and cyclin H showed expected invariant levels of these proteins (Tassan *et al.* 1994; Fisher *et al.* 1995). However, in cells treated with calphostin C, the faster migrating form of cdk7 was virtually undetectable. Since cdk7 phosphorylation results in formation of a lower molecular form of cdk7 (Labbe *et al.* 1994), the absence of the lower molecular form of cdk7 in calphostin C treated cells, suggest that PKC is directly or indirectly involved in cdk7 phosphorylation. Immunoblots of cyclin A depicted its constitutive expression instead of its normal induction during S phase and mitosis. Cyclin B1 was also present throughout the cell cycle. Since most of the cell cycle markers were expressed in a synchronous manner, abnormal accumulation of cyclin A or B1 may be due to point mutations within the D box preventing cyclin degradation by ubiquitin-mediated proteolysis (Luca *et al.* 1991; Bastians *et al.* 1998).

The relative levels of control PKC isozymes were as follows: greatest abundance PKC- η , ζ , θ , δ , γ ; moderately present PKC- α , βI , βII , ι , μ , and minimally present PKC- ϵ . PKC- α levels in

Figure 2. Western blot analysis of cell cycle markers (a) and PKC isozymes (b) in U-373MG cells throughout the cell cycle. Shown are representative immunoblots from four independent experiments. Duplicate cultures from the experiment depicted in Fig. 1 were harvested at the indicated times and prepared for Western blots. Equal amounts of cellular protein (80 μ g) were loaded per well. Standards and antibodies (used at a 1/2000 dilution) against the following antigens were purchased from Santa Cruz Biotech. (Santa Cruz, CA); p21 (SC-397, SC-4077WB); p27 (SC-1641, SC-4091WB); MAT1 (SC-6234, SC-4237WB); cdk7 (SC-7344, SC-4103WB); cdk2 (SC-6248, SC-4069WB); cyclin A (SC-751); cyclin B1 (SC-752); cyclin D1 (SC-8396); cyclin D2 (SC-593); cyclin D3 (SC-182); cyclin H (SC-855, SC-4102WB); PKC- α (SC-8393); PKC- βI (SC-8049); PKC- βII (SC-210); PKC- δ (SC-937); PKC- ϵ (SC-214-G); PKC- μ (SC-936); PKC- θ (SC-1875); PKC- η (SC-1875); Cdk2 (SC-6248). Standards for cyclin A (C4312), cyclin B1 (C4437), cyclin D1 (C615), and cyclin E (C6440) were obtained from Sigma (St. Louis, MO). All standards for cell cycle markers were used at a concentration of 30–50 ng. The standard for PKC isozymes was rat brain lysate (2 ng). Anti-PKC- ι (P20520; Transduction Lab., Lex., KY) was used at 1 : 12 000 dilution. Secondary antibodies were obtained from Accurate (JOZ000003, JOM000003, Westbury, NY) and used at 1 : 15 000 dilution. Antibodies to PKC- ζ and PKC- γ were produced and characterized by Dr Rayudu Gopalakrishna (USC, Los Angeles, CA).

control and calphostin C-treated cells cycled during the 24 h time period (Fig. 2b). Maximum PKC- α immunoreactivity was observed at T_0 . In control cells, PKC- α levels remained constant from 2 to 10 h, gradually declined at 12–18 h, and subsequently increased from 20 to 24 h. However, constant PKC- α levels were not observed at 2–10 h in calphostin C-treated cells. Instead, PKC- α immunoreactivity declined earlier (at 8 h) and lasted until 20 h. Western blots of control PKC- β I showed maximal levels at T_0 followed by a continuous reduction in PKC- β I. Compared to controls, calphostin C initially reduced PKC- β I at 2–10 h and PKC- β I returned to control levels at 12–24 h. PKC- β II, PKC- ϵ , PKC- ι and PKC- μ remained constant in control and calphostin C-treated cells. PKC- ζ was also essentially invariant with the exception of a slight reduction of PKC- ζ in calphostin C-treated cells. Control PKC- η levels increased at 2–12 h and 22–24 h. Additionally, in control cells there was an increase in abundance of a slow-migrating form of PKC- η from 4 to 12 h. The slow-migrating form of PKC- η was less abundant in calphostin C treated cells and the pattern of PKC- η recapitulated control PKC- η . However, its immunoreactivity was less suggesting that PKC- η is calphostin C sensitive. Irregular levels of control PKC- δ and PKC- γ in control or calphostin C lysates were observed, probably due to differing phosphorylation states of the PKCs. PKC- η levels remain fairly constant in calphostin C lysates, with the exception of diminished levels at 10 h.

Calphostin C abrogates CAK (cdk7) and cdk2 phosphorylation

To evaluate whether PKC was directly involved in CAK phosphorylation, the phosphorylation of CAK and cdk2 was monitored throughout the cell cycle following *in-vivo* $^{32}\text{P}_i$ metabolic cell labelling and calphostin C treatment. Silver stained PAGE gels of either immunoprecipitated cdk7 or cdk2 demonstrated the presence of various bands including IgGs (55 and 50 kDa), Fab fragments (25 kDa), cdk7, 42 kDa; cyclin H, 37 kDa and cdk2, 33 kDa (data not shown). Autoradiograms showed phosphorylation of CAK (cdk7; Fig. 3a) and cdk2 (Fig. 3b) in control cells at 13 h post serum addition. In contrast, calphostin C-treated cells fail to accumulate hyperphosphorylated CAK or cdk2 at 13 h post serum addition, suggesting that PKC is directly or indirectly affecting CAK at the G_1/S transition. The precise timing of the cdk7 phosphorylation may vary a little between experiments due to variations in the synchrony of the cell cycle.

Cdk7 is a substrate for PKC- β II and PKC- ι

To further understand the mechanism by which PKC isozymes regulate CAK phosphorylation and activation, aliquots were taken from the same cell extract for co-immunoprecipitation of CAK and PKC isozymes throughout the cell cycle to determine if CAK is an *in-vitro* PKC substrate as judged by PKC activity assays in SDS-PAGE autoradiography. Autoradiograms of PKC- α , β I, γ , δ , ϵ , ζ , η , θ , μ , and cdk7 co-immunoprecipitates showed minimal cdk7 background kinase activity, and no obvious relationship between these PKC isozymes and cdk7 phosphorylation was detected. However, co-immunoprecipitates of cdk7 and PKC- β II or PKC- ι subjected to an *in-vitro* PKC activity assay, and autoradiography demonstrated that in control cell extracts, cdk7 served as a PKC- β II or PKC- ι substrate as judge by either phosphatidylserine/diacylglycerol/ Ca^{2+} (PS/DAG/ Ca^{2+})- or PS-dependent phosphorylation of cdk7, respectively.

To determine whether a functional link existed between the phosphorylation status of PKC- β II, PKC- ι , cdk7 and cdk2 throughout the cell cycle, we tested the ability of these proteins in individual immunoprecipitated complexes to serve as PKC physiological targets. Autoradiograms of control (DMSO) PKC- β II or PKC- ι immunoprecipitates depicted high PKC activity levels as judged by potent phosphorylation of cdk7 and cdk2 at 14 h post serum addition

Figure 3. Calphostin C inhibits phosphorylation of CAK and cdk2. U-373MG cells were grown to confluency, serum starved for 48 h, and washed twice with phosphate-free DMEM. Cells were incubated for 4 h in phosphate-free DMEM containing 0.1 mCi/ml [32 P]-orthophosphate and washed twice with PBS. Immediately following the washes with PBS (at T_0) cells were incubated in DMEM containing 10% serum and either the PKC inhibitor calphostin C (0.5 μ M) or equivalent volumes of DMSO (vehicle control). Lysates (50 μ g) prepared as described in Fig. 2 were immunoprecipitated with 5 μ l of rabbit antibodies against cdk7 (CAK; Fisher & Morgan 1994) or cdk2 (SC-748) and rocked overnight at 4 $^{\circ}$ C. Immune complexes were incubated for 1 h with 50 μ l of protein A-agarose beads, washed and resolved by silver stained SDS-PAGE (10% acrylamide) and autoradiography. Autoradiograms and arrowheads indicate (a) cdk7 (CAK) and (b) cdk2 protein phosphorylation at 13 h post-serum treatment in control but not calphostin C-treated cells. Autoradiograms were exposed for 5 days. Position of molecular weight markers are indicated in kilodaltons and shown on the left.

(Fig. 4a–d). Phosphorylation of cdk7 in these immunoprecipitates increased its electrophoretic mobility (Labbe *et al.* 1994).

This data supports a model in which PKC- ι / β II phosphorylate and activates CAK, CAK subsequently phosphorylates and activates cdk2.

In control PKC- β II and PKC- ι immunoprecipitates, additional reproducible cdk7 phosphorylation was present at 4 h and 6 h, respectively (Fig. 4a,b). However, no obvious mechanism was found that can explain the cdk7 phosphorylations at 4 and 6 h. Cdk2 was also phosphorylated at 4, 8, and 16 h in control PKC- β II immunoprecipitates (Fig. 4c) and at T_0 in PKC- ι immunoprecipitates (Fig. 4d). In contrast, calphostin C inhibited or reduced (Fig. 4c) the phosphorylation of cdk7 and cdk2 in PKC- β II or PKC- ι immunoprecipitates. When comparing cdk2 phosphorylation in Figs 3 and 4 there is a labelled cdk2 peak at T_0 in Fig. 4d but not at T_0 in Fig. 3b. The most probable reason as to why cdk2 was labelled in the PKC- ι immunoprecipitates (Fig. 4d) and not labelled at T_0 in Fig. 3b is that the experimental design in Figs 3 and 4 are different. In Fig. 3, cdk7 and cdk2 were immunoprecipitated from 32 P-labelled cells. In Fig. 4, PKC- ι was immunoprecipitated from non-radioactive cell lysates and subjected to a PKC activity assay. Therefore, the labelled cdk2 shown in Fig. 4c is associated with PKC- ι and the cdk2 was probably phosphorylated by the components (cdk7) present in the PKC- ι immunocomplex.

Figure 4. Phosphorylation of cdk7 and cdk2 in PKC- β II and PKC- ι immunoprecipitates. Cell treatments, fractionations and immunoprecipitations were performed as described in Figs 2 and 3. U-373MG lysates (150 μ g) were immunoprecipitated with affinity purified rabbit antibodies against PKC- β II (4 μ l; SC-210) or PKC- ι (4 μ l; SC-727) and rocked overnight at 4 °C. Immune complexes were then incubated at 4 °C for 1 h with 100 μ l of protein A/G and washed twice with lysis buffer. PKC activity assay was initiated by suspension of immunoprecipitates in 127 μ l of PKC kinase buffer (Davis & Clark 1983) using γ - 32 P] ATP (20 μ Ci/reaction Neg-002Z; NEN Dupont, Boston, MA) to test the ability of PKC- β II or PKC- ι to phosphorylate cdk7. Immunoprecipitates of control or calphostin C-treated cell extracts were assayed for PKC activity in the presence of PS/DAG/ Ca^{2+} (shown) or EGTA (not shown) as described in Materials and Methods. Reactions proceeded for 5 min at 30 °C and were terminated by addition of SDS sample buffer. Labelled proteins were subjected to SDS-PAGE and autoradiography. Control mock immunoprecipitations were assayed in the presence of PS/DAG/ Ca^{2+} (e, g) or EGTA (f, h) and included U-373MG lysates and protein A/G alone (lanes 1e and 1g), anti-PKC- β II (lane 2e) or anti-PKC- ι (lane 2g) plus protein A/G, or anti-cdk7 plus protein A/G (lane 3e and 3g). The autoradiograms typify three independent experiments and PKC activity assays.

PKC- β II and PKC- ι are physically associated with cdk7

The physical association of cdk7 with PKC- β II or PKC- ι throughout the cell cycle was established by individual immunoprecipitation of cdk7 and detection of PKC- β II, or PKC- ι in immunocomplexes. Western blots of cdk7 immunoprecipitates detected the presence of small amounts PKC- β II associated with cdk7 (Fig. 5a). Abundant PKC- ι was complexed with cdk7 at T_0 and from 6 to 22 h (Fig. 5b). The presence of cdk7 in PKC- β II immunoprecipitations as a function of time is not shown in Fig. 5 because, despite the fact that phosphorylated cdk7 can be generated in PKC- β II (Fig. 4a), Westerns of cdk7 did not detect cdk7 in the PKC- β II immunoprecipitations. However, PKC- β II was detected in PKC- ι immunoprecipitates at 14 h (Fig. 5c).

We also utilized the MDA-MB-468 breast cancer cell line to investigate whether cdk7 was immunocomplexed with PKC- ι in a different cell type. Western blots of PKC- ι demonstrated that PKC- ι is present in cdk7 immunoprecipitates derived from the MDA-MB-468 breast cancer cells (Fig. 5g). These results indicate that the association between cdk7 and PKC- ι may be a generalized cellular phenomena.

Purified PKC- β II phosphorylates baculovirus HA-tagged cdk7

To further establish the involvement of PKC in cdk7 phosphorylation, we tested whether purified PKC- β II (which unlike PKC- ι is commercially available) would phosphorylate cdk7. Baculovirus

Figure 5. Western blot analysis of the physical association between cdk7, PKC- β II and PKC- α . Lysates from U-373MG (150 μ g) or MDA-MB-468 breast cancer cells (300 μ g) were prepared at the indicated times as described in Fig. 4, immunoprecipitated with rabbit antibodies to cdk7 (6 μ l; SC-529), PKC- α (4 μ l; SC-727) or PKC- β II (4 μ l; SC-210) and rocked overnight at 4 °C. Immune complexes were incubated at 4 °C for 1 h with 100 μ l of protein A/G and washed twice with lysis buffer. Proteins in the cdk7 immunoprecipitates were subjected to Western blotting with antibodies against PKC- β II (SC-210; a) or PKC- α (P20520; Transduction Lab., Lexington, KY) (b). Secondary antibodies were obtained from Accurate and used at 1 : 15 000 dilution. Control mock immunoprecipitations included U-373MG cell extracts and protein A/G alone (lane 1), rabbit IgGs (2.4 μ g/6 μ l; 02-6102, Zymed, San Francisco, CA) plus protein A/G (lane 2), or mouse IgGs (2.4 μ g/6 μ l; SC-2025) and protein A/G (lane 3). The MDA-MB-468 cell line was obtained from the American Type Tissue Culture Collection. Western blots represent results from a minimum of three independent experiments.

HA-tagged human cdk7 protein was produced in Sf9 insect cells and used for *in-vitro* phosphorylation studies. Autoradiograms of cdk7 immunoprecipitates subjected to PKC activity assays in the presence of PS/DAG/ Ca^{2+} show no cdk7 phosphorylation in uninfected Sf9 cells (Fig. 6b; lane 1). However, some phosphorylation of cdk7 was evident in cells expressing HA-tagged cdk7 (Fig. 6b; lane 2). Addition of exogenous PKC- β II (1 ng) readily phosphorylated immunoprecipitated baculovirus HA-tagged cdk7 (Fig. 6b; lane 5). In contrast, PKC- β II phosphorylation of cdk7 was reduced when assayed in the presence of EGTA (Fig. 6a; lane 5), and no phosphorylation was detected in uninfected Sf9 cells incubated with PKC- β II (Fig. 6a; lane 6). Next, the inhibitory action of a different PKC inhibitor, RO-31-7549 on cdk7 immunoprecipitates from Sf9 cells was assessed in PKC activity assays to confirm that PKC- β II inhibition would block cdk7 phosphorylation. Treatment of cdk7 immunoprecipitates from uninfected (Fig. 6; lane 7) or infected Sf9 insect cells expressing HA-tagged cdk7 (Fig. 6; lane 8) with PKC- β II (1 ng) and RO-31-7549 (1 μ M) abrogated cdk7 phosphorylation.

DISCUSSION

In conclusion, our experiments established that at least two PKC isozymes control cell cycle progression by directly or indirectly regulating the phosphorylation of CAK (cdk7). Evidence supporting this conclusion includes the calphostin C induced 2 h S phase delay (Fig. 1a,b). Except for diminution of the faster migrating cdk7 band in calphostin C-treated cells, this delay

Figure 6. Phosphorylation of immunoprecipitated baculovirus HA-tagged cdk7 by PKC- β II and its inhibition with RO-31-7549. The human MO15/cdk7 protein baculovirus containing a carboxyl-terminal HA epitope tag (Fisher & Morgan 1994) was expressed in Sf9 insect cells according to Turkson *et al.* (1999). Sf9 cells (39 μ g) were immunoprecipitated with rabbit anti-HA (6 μ l; sc-805). PKC- β II phosphorylation of baculovirus HA-tagged cdk7 immunoprecipitates was assayed in the presence of EGTA (a) or PS/DAG/ Ca^{2+} (b). Anti-HA immunoprecipitates were treated with purified PKC- β II (1 nanogram/reaction; Upstate Biotech., Lake Placid, NY) and subsequently incubated in the absence (lanes 4–5) or presence (lanes 7–8) of 1 μ M RO-31-7549 (CalBioChem, San Diego, CA). Immunoprecipitates were then subjected to PKC activity assays (Davis & Clark 1983). Proteins were analysed by SDS–PAGE and autoradiography. Control and mock immunoprecipitations included extracts of uninfected Sf9 insect cells (lane 1) and Sf9 cells expressing baculovirus HA-tagged cdk7 (lane 2) plus anti-HA and protein A/G, PKC- β II plus protein A/G (lane 3), and PKC- β II plus protein A/G and RO-31-7549 (lane 6).

occurred without affecting the constitutive protein expression of PKC- β II, PKC- α , MAT1, cyclin H, and cdk2 (Fig. 2a,b).

Although, PKC- α and β II protein concentration remained invariant throughout the cycle, it is possible that formation of complexes between these PKC isoforms and their target(s) does vary as a function of the cell cycle as demonstrated in Fig. 3.

Furthermore, incubation of cells with calphostin C prevented hyperphosphorylation of CAK (cdk7) and cdk2 at 13 h post serum addition (Fig. 3a,b). With the exception of PKC- β II and PKC- α , co-immunoprecipitation of CAK (cdk7) and different PKC isozymes ruled out the participation of other PKC isozymes in the phosphorylation and activation of CAK. As shown in PKC activity assays, the physical association between PKC- β II, PKC- α and cdk7 serves to facilitate their interaction leading to cdk7 phosphorylation, cdk7 activation and subsequently cdk2 phosphorylation (Fig. 4). Western blots of cdk7 immunoprecipitates demonstrated the temporal and constitutive association of PKC- β II and PKC- α with cdk7 (Fig. 5), respectively. The phosphorylation of baculovirus HA-tagged human cdk7 with purified PKC- β II further confirms a role for PKC- β II in cdk7 phosphorylation (Fig. 6). Collectively, these results demonstrate that the interaction of PKC- β II and PKC- α is involved in the phosphorylation and activation of cdk7. The sequence of events and functional link between other intermediaries, PKC- β II, and PKC- α , ensuing cdk7 activation, are being investigated.

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