βII Protein Kinase C Is Required for the G2/M Phase Transition of Cell Cycle*

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Entry into mitosis requires the coordinated action of multiple mitotic protein kinases. In this report, we investigate the involvement of protein kinase C in the control of mitosis in human cells. Treatment of synchronized HL60 cells with the highly selective protein kinase C (PKC) inhibitor chelerythrine chloride leads to profound cell cycle arrest in G2 phase. The cellular effects of chelerythrine are not due to either direct or indirect inhibition of the known mitotic regulator p34cdc2/cyclin B kinase. Rather, several lines of evidence demonstrate that chelerythrine-mediated G2 phase arrest results from selective inhibition and degradation of βII protein kinase C. First, chelerythrine causes dose-dependent inhibition of βII PKC in vitro with an IC50 identical to that for G2 phase blockade in whole cells. Second, chelerythrine specifically inhibits βII PKC-mediated lamin B phosphorylation and mitotic nuclear lamina disassembly. Third, chelerythrine leads to selective loss of βII PKC during G2 phase in synchronized cells. Fourth, chelerythrine mediates activation-dependent degradation of PKC, indicating that βII PKC is selectively activated during G2 phase of cell cycle. Taken together, these data demonstrate that βII PKC activation at G2 phase is required for mitotic nuclear lamina disassembly and entry into mitosis and that βII PKC-mediated phosphorylation of nuclear lamin B is important in these events.

In higher eukaryotes, entry into mitosis is characterized by a dramatic structural reorganization of the cell. Mitotic events include chromosome condensation, nuclear lamina disassembly, and cytokinesis. Recent studies have demonstrated that multiple protein kinases play key regulatory roles in mitosis (1-4). The p34cdc2/cyclin B kinase is required for the G2/M phase transition in both yeast and higher eukaryotes (5, 6). Introduction of p34cdc2/cyclin B kinase into mammalian fibroblasts leads to mitotic chromosome condensation and cytoskeletal rearrangements, but not to mitotic nuclear lamina disassembly (7). Consistent with its critical role in mitotic events, p34cdc2/cyclin B kinase directly phosphorylates many mitotic phosphoproteins (3). However, the fact that p34cdc2/cyclin B kinase is not sufficient to mediate all aspects of mitosis demonstrates that other mitotic regulators are required (7).

Increases in cytosolic free calcium have been implicated in the regulation of mitosis in a number of cell systems (8-12). Intracellular calcium release triggers nuclear envelope breakdown in sea urchin embryos, and calcium transients are required for nuclear envelope breakdown in mammalian fibroblasts (8-12). Potential targets for the increase in intracellular calcium at the G2/M phase transition include the calcium/calmodulin-dependent protein kinases and the calcium/phospholipid-dependent protein kinase (PKC).1 Accumulating evidence has implicated both of these protein kinases in the control of a number of mitotic events (reviewed in Ref. 4). PKC has been implicated in the regulation of mitosis in a number of systems (4, 13-19). Genetic evidence indicates that the yeast PKC homolog, PKC1, is an essential gene required for cell cycle (13). Deletion of PKC1 leads to recessive lethality and a mutant cell cycle phenotype characterized by a block in G2 phase (13).

PKC has also been implicated in cell cycle progression in mammalian cells (15, 16, 19). Staurosporine, a potent but relatively nonselective protein kinase C inhibitor, arrests cells at two cell cycle phases, G2 and G2/M phase, depending on the concentration used (15). Several staurosporine analogues and other structurally distinct PKC inhibitors also lead to inhibition of cell cycle at G2/M phase (16, 19). However, neither the individual PKC isotypes required during G2/M phase nor the mechanism by which inhibition of PKC leads to G2/M phase arrest have been elucidated. In the present study, we utilize the highly selective PKC inhibitor chelerythrine to demonstrate that PKC activity is required for entry of human cells into mitosis. Furthermore, we show that PKC activation is required for phosphorylation of key mitotic sites on nuclear lamin B previously implicated in mitotic nuclear lamina disassembly (17, 18). Finally, we find that βII PKC is a critical PKC isotype involved in the G2/M phase transition.

EXPERIMENTAL PROCEDURES

Cell Synchronization and Flow Cytometric Analysis—Human promyelocytic leukemia (HL60) cells were synchronized in G1/S phase with 2 μg/ml aphidicolin (Sigma) at a cell density of 1 x 10^6/ml for 18 h as described previously (18). Cells were released from the G1/S blockade (time = 0) by removal of aphidicolin and resuspended in Iscove’s medium (Life Technologies, Inc.) containing 10% iron-supplemented calf serum (HyClone). In some cases, nocodazole, chelerythrine, or staurosorine was added to the cells during mid to late S phase (5-7 h after release from aphidicolin) at the concentrations indicated in the figure legends. Cells were sampled at the indicated time points after release from aphidicolin, fixed in 90% methanol, stained with propidium iodide, and analyzed for cell cycle progression using flow cytometry as described previously (18).

Immunofluorescence Staining for Lamin B, and Detection of DNA—HL60 cells were fixed in 90% methanol and then incubated in phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA) at room temperature for one hour. Fixed cells were incubated for 2 h in

1 The abbreviations used are: PKC, protein kinase C; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; PAGE, polyacrylamide gel electrophoresis; dC8, dioctanoylglycerol.
Protein Kinase C Activity Is Required for Mitosis

A 1:100 dilution of a mouse monoclonal antibody to human lamin B1 (Matritech in PBS-BSA, washed three times with PBS-BSA, and incubated for 1 h with a 1:50 dilution of an fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Kirkegaard and Perry Laboratories in PBS-BSA. The stained cells were washed three times in PBS-BSA, once with PBS, and then incubated with 0.5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) in PBS. Doublet lamins (ECL, Amersham) as described previously (18, 22). Protein levels were quantitated by densitometry (Molecular Dynamics).

The effect of PKC activation on chelerythrine-induced degradation was assessed by treating asynchronous cells with either 20 μM chelerythrine, 20 μM dicyclohexylammonium chloride (DCC), or the combination of both compounds for up to 9 h. At the indicated times, total cell lysates were subjected to immunoblot analysis for α, βII, and γ PKC as described above.

RESULTS

Chelerythrine Induces Dose-dependent G2/M Phase Arrest in Synchronized Human Cells—Previous studies demonstrated that staurosporine, a potent protein kinase inhibitor, induces cell cycle arrest in both G2 and G2/M phase (15), suggesting a role for protein kinase C in cell cycle progression. However, staurosporine is a relatively nonselective protein kinase inhibitor, making it difficult to attribute its cellular effects to inhibition of PKC rather than any of a number of other cellular kinases. Of particular concern, staurosporine has also been shown to be an effective inhibitor of p34<sup>cdc2</sup>/cyclin B kinase, a kinase whose activity is required for entry into mitosis (15). Therefore, we chose to investigate the effects of the highly selective PKC inhibitor, chelerythrine chloride, on the cell cycle of human leukemia (HL60) cells. Chelerythrine exhibits little inhibitory effect on cAMP-dependent protein kinase, Ca<sup>2+</sup>/calmodulin-dependent kinase or tyrosine kinases (23) making it useful for these studies.

In order to assess the effects of chelerythrine on cell cycle progression, HL60 cells were synchronized in G1/S phase with aphidicolin and allowed to progress through cell cycle. These cells progress synchronously through cell cycle, returning to G1 phase by 12 h after removal of aphidicolin (Fig. 1A). In contrast, cells released into medium containing chelerythrine progressed to G2/M phase, but failed to return to G1 phase. A similar cell cycle arrest in the G2/M phase was seen in cells treated with either staurosporine, a nonselective protein kinase inhibitor that is known to arrest cells in G2/M phase (15), or with nocodazole, a mitotic spindle poison that arrests cells in mid-metaphase (Fig. 1A). As has been reported with staurosporine (15), chelerythrine leads to a secondary G2/M cell cycle arrest in G2 phase, as indicated by the accumulation of a smaller G2 phase population in cells treated with these compounds. Little or no cell cycle progression was observed at 15 and 24 h, demonstrating that these compounds induce cell cycle blockade at G2/M phase rather than slowed cell cycle progression (data not shown). The cell cycle arrest induced by chelerythrine is dose-dependent with an apparent IC<sub>50</sub> of about 16 μM (Fig. 1B).

Chelerythrine Blocks Cells in G2 Phase Prior to Mitosis—Flow cytometric analysis of DNA content revealed that both chelerythrine and nocodazole lead to significant arrest in G2/M phase (69 and 67% G2/M phase cells, respectively). However, flow cytometry does not discriminate between cells in G2 phase and those in mitosis. Therefore, we determined the mitotic index of cells arrested with either chelerythrine or nocodazole. In chelerythrine-treated cells, the mitotic index was 4%, while in nocodazole-treated cells, the mitotic index was 65%. Comparison of the mitotic indices and flow cytometric data revealed that whereas nocodazole arrested cells in mitosis (67% G2/M phase = 65% mitotic index + 2% G2 phase cells), chelerythrine led to arrest in G2 phase (69% G2/M phase = 65% G2 phase + 4% mitotic index).

A number of morphological hallmarks have been identified...
throughout the cytosol with little or no staining associated with cells reveals that lamin B1 is distributed in a vesicular pattern for the G2/M phase transition. These include the condensation we assessed cells treated with these agents for characteristics of the interphase nucleus, and the solubilization of the cellular chromatin into highly condensed, paired chromosomal bodies (Fig. 2). DAPI (Fig. 2). Staining of nocodazole-treated cells with DAPI reveals the presence of highly condensed metaphase chromatin (Fig. 2). In contrast, immunofluorescence staining of chelerythrine-treated cells reveals a nuclear rim staining pattern characteristic of the interphase nuclear lamina (Fig. 2D). The spatial relationship between the nuclear lamina and the interphase chromatin in chelerythrine-treated cells is revealed when both DNA and lamin B1 staining are visualized together (Fig. 2E). These results indicate that the nuclear lamina of chelerythrine-treated cells is intact and forms a continuous ring surrounding the nuclear contents. In contrast, the nuclear lamina in nocodazole-treated cells has undergone mitotic disassembly as evidenced by the vesicular, cytosolic distribution of lamin B1 (Fig. 2B).

In interphase cells, the nuclear lamina is highly polymerized, and the nuclear lamins are insoluble in buffers containing non-ionic detergents (24). During mitosis, however, the nuclear lamina disassembles, and the resultant lamin dimers are soluble in non-ionic detergents (24). Lamin B1 solubility was therefore used to determine the polymeric state of the nuclear lamina in chelerythrine- and nocodazole-treated cells (Fig. 3). Treated cells were fractionated into Nonidet P-40 soluble and insoluble fractions, and the presence of lamin B1 in these fractions was assessed by immunoblot analysis using a monoclonal lamin B1 antibody. As expected, lamin B1 is recovered in the Nonidet P-40 insoluble (nuclear) pellet from unsynchronized cells (Fig. 3A), reflecting the fact that the vast majority of these cells are in interphase. After nocodazole treatment, a significant proportion of the lamin B is recovered in the Nonidet P-40-soluble supernatant with the remainder found in the insoluble pellet (Fig. 3B). In contrast, essentially all the lamin B from chelerythrine-treated cells is recovered in the Nonidet P-40-insoluble pellet (Fig. 3C). Densitometric analysis of the immunoblots reveals that about 45% of the total lamin B in nocodazole-treated cells is Nonidet P-40-soluble with the remainder recovered in the insoluble pellet, whereas ~5% of the total lamin B from chelerythrine-treated cells is Nonidet P-40-soluble. These data are in good agreement with the observed mitotic indices of nocodazole- and chelerythrine-treated cells. Therefore, by both morphologic and biochemical criteria, chelerythrine-treated cells contain a structurally intact, highly polymerized nuclear lamina which has not undergone mitotic nuclear lamina disassembly. In contrast, nocodazole-treated cells exhibit metaphase morphology characterized by a depolymerized, vesiculized nuclear lamina that is soluble in Nonidet P-40, indicative of mitotic nuclear lamina disassembly. Therefore, in the presence of chelerythrine, cells complete S phase and arrest in G2 phase prior to mitosis.

Chelerythrine Directly Inhibits PKC but Not p34cdc2/Cyclin B Kinase in Vitro—Given the G2 phase cell cycle blockade induced by chelerythrine, we wished to assess the mechanism by which it elicits this effect. Chelerythrine has been reported to be a potent and highly selective inhibitor of protein kinase C (23). To confirm the specificity of this compound for inhibition of protein kinase C, we first assessed the effect of chelerythrine on purified recombinant α, β1, and ζ PKC (the three PKC isotypes expressed in HL60 cells; Ref. 21), and on cyclin-dependent kinases affinity-purified from mitotic cells with p13 agarose beads in vitro (Fig. 4A). Chelerythrine leads to dose-dependent inhibition of α, β1, and ζ PKC activity. Of the three PKC isotypes, β1 PKC is most sensitive to chelerythrine inhibition (IC50 ~15 μM), followed by α (IC50 ~25 μM) and ζ PKC (IC50 >40 μM). These IC50 values, particularly those for α and

![Fig. 1. Chelerythrine arrests synchronized HL60 cells in G2/M phase.](http://www.jbc.org/)

**A** DNA histograms of synchronized HL60 cells in the presence of various cell cycle agents. HL60 cells were synchronized in G0/S phase by treatment with aphidicolin and released into growth medium. Synchronized cells were treated with either diluent (Control, top panels), 40 ng/ml nocodazole (second panel), 200 nm staurosporine (third panel), or 20 μM chelerythrine (bottom panel) as described under "Experimental Procedures." Cell cycle distribution was assessed at the indicated times by flow cytometry as described under "Experimental Procedures." B, chelerythrine-induced cell cycle blockade is dose-dependent. HL60 cells were synchronized and treated with the indicated concentrations of chelerythrine as described under "Experimental Procedures." 15 h after release, cells were analyzed for cell cycle distribution by flow cytometry. Results are plotted as the percentage of maximal response (64.4% of cells in G2/M phase) versus chelerythrine concentration. The data are from triplicate experiments and are plotted as the mean ± S.E. Some error bars are masked by the data point symbols (squares).
**βII PKC**, are in close agreement with the IC50 for G2 phase blockade in whole cells (16 μM; Fig. 1B). In contrast, chelerythrine had little or no inhibitory effect on affinity-purified mitotic cyclin-dependent kinase activity even at concentrations of 40 μM. The predominant cyclin-dependent kinase activity from mitotic cells is p34cdc2/cyclin B (1). These results indicate that chelerythrine does not directly inhibit p34cdc2/cyclin B kinase activity at concentrations that cause profound G2 phase arrest in whole cells. Similar results were obtained with p34cdc2/cyclin B kinase immunopurified using antibody to the carboxyl terminus of p34cdc2 (data not shown). We conclude that the cell cycle effects of chelerythrine do not appear to be due to either direct or indirect inhibition of p34cdc2/cyclin B kinase.

**Chelerythrine Inhibits Mitotic Phosphorylation of Lamin B1 at Ser405**—Our recent studies using human erythroleukemia (K562) cells demonstrated that βII PKC is a lamin kinase that directly phosphorylates lamin B1 at a prominent mitotic phosphorylation site, Ser405 (18). Therefore, we assessed the phosphorylation status of lamin B1 in chelerythrine-treated G2 phase, and asynchronous (interphase) cells (Fig. 4B). p34cdc2/cyclin B kinase isolated from both nocodazole- and chelerythrine-treated cells exhibits similar high cyclin-dependent histone kinase activity, consistent with the low levels of p34cdc2/cyclin B kinase activity present during interphase (1). Identical results are obtained when p34cdc2/cyclin B kinase is isolated by p13suc1 affinity or by immunoprecipitation using p34cdc2 or cyclin B-specific antibodies (Fig. 4B). The p34cdc2/cyclin B kinase activity associated with chelerythrine-treated cells is slightly lower than that of mitotic cells, perhaps because chelerythrine-treated cells have not yet entered mitosis when p34cdc2/cyclin B kinase activity is at its peak. These data demonstrate that the effects of chelerythrine do not appear to be due to either direct or indirect inhibition of p34cdc2/cyclin B kinase.
Protein Kinase C Activity Is Required for Mitosis

Chelerythrine blocks mitotic nuclear lamin disassembly. HL60 cells were obtained from asynchronous cultures (A) or from cultures synchronized with aphidicolin and treated with either nocodazole (B) or chelerythrine (C) as described in the legend to Fig. 1. 15 h after release from aphidicolin, cells were resuspended in lysis buffer containing 2% Nonidet P-40 and fractionated into soluble (S) and insoluble (P) fractions as described under “Experimental Procedures.” Cellular fractions were resolved by SDS-PAGE and subjected to immunoblot analysis using a monoclonal antibody to lamin B1 as described previously (18).

Chelerythrine-treated (G2 phase) and nocodazole-treated (mitotic) HL60 cells (Fig. 3).

Lamin B1 from mitotic cells is highly phosphorylated on three tryptic phosphopeptides (Fig. 5A, labeled 1-3). This pattern is identical to that observed in mitotic lamin B1 from K562 cells and corresponds to phosphorylation within the carboxyterminal domain of lamin B1 (17, 18). Lamin B1 from chelerythrine-treated cells is highly phosphorylated on one peptide, phosphopeptide 1, whereas phosphorylation on phosphopeptides 2 and 3 is drastically reduced (Fig. 5B). Recombinant b11 PKC phosphorylates lamin B1 on two phosphopeptides (Fig. 5C), corresponding to phosphorylation of the consensus PKC phosphorylation sites Ser395 (peptide 4) and Ser405 (peptide 2) (17, 18). The identity of phosphopeptide 2 was confirmed by mixing lamin B1 phosphopeptides from mitotic cells with those from either chelerythrine-treated cells (Fig. 5D) or lamin B1 phosphorylated by recombiant b11 PKC in vitro (Fig. 5E). Inhibition of Ser405 phosphorylation (peptide 2) is indicative of direct inhibition of b11 PKC-mediated phosphorylation of lamin B1 in the presence of chelerythrine. Inhibition is selective, since phosphorylation of phosphopeptide 1, which is not attributable to PKC, is unaffected by chelerythrine treatment. Phosphorylation of peptide 3 is also inhibited in the presence of chelerythrine. However, phosphorylation of this peptide cannot be attributed to the direct action of b11 PKC, since it is not phosphorylated by recombinant b11 PKC in vitro (Fig. 5C; Refs. 17 and 18). It is possible that chelerythrine directly inhibits the kinase responsible for phosphorylation of peptide 3. Alternatively, peptide 3 may be phosphorylated by a kinase whose activity is modulated by or is dependent upon b11 PKC activation in vivo.

Chelerythrine Results in Selective Degradation of the b11 PKC Isoform at G2 Phase—Attempts at rescuing chelerythrine-treated cells by extensive washing and/or treatment with phorbol myristate acetate or bryostatin, both potent activators of PKC, failed to stimulate entry into mitosis. The irreversibility of the chelerythrine-induced G2 phase blockade was not due to cytotoxicity since chelerythrine-treated cells maintain high viability (as measured by trypan blue exclusion) in the G2-arrested state for at least 48 h after treatment. Our inability to reverse the effects of chelerythrine led us to examine the levels of PKC isotypes in chelerythrine-treated cells (Fig. 6).

Total cell lysates from synchronized and chelerythrine-treated cells were obtained during the time period when synchronized cells traverse mitosis (11-15 h after release from aphidicolin; see Fig. 1). Total cell extracts were subjected to immunoblot analysis using a panel of isotype-specific antibodies against a, b11, and c PKC. Immunoblot analysis reveals that b11 PKC is selectively degraded in chelerythrine-treated cells, while a and c PKC levels remain unchanged (Fig. 6A). The degradation of b11 PKC is not a consequence of the cell synchronization procedure or the transit of cells through mitosis, since...
**Fig. 5.** Chelerythrine inhibits mitotic phosphorylation of lamin B. HL60 cells were synchronized with aphidicolin blockade, cells were lysed, and lamin B was isolated and subjected to tryptic phosphopeptide analysis as described under “Experimental Procedures.” Alternatively, lamin B was phosphorylated by purified PKC in vitro. A, phosphopeptide map of mitotic lamin B from nocodazole-treated cells. B, phosphopeptide map of lamin B from G2 phase cells blocked with chelerythrine. C, phosphopeptide map of lamin B phosphorylated by PKC in vitro. D, mixture of A and B. E, mixture of A and C.

**Fig. 6.** Chelerythrine induces selective degradation of \( \beta_h \) PKC during G2 phase in synchronized cells. A, HL60 cells were synchronized and treated with either chelerythrine (+) or diluent (−) as described above. Cells were harvested at the indicated times and total cell lysates were subjected to immunoblot analysis for \( \alpha \), \( \beta_h \), and \( \zeta \) PKC as described under “Experimental Procedures.” B, temporal relationship between \( \beta_h \) PKC degradation and progression through G2/M phase. HL60 cells were synchronized with aphidicolin and treated with either chelerythrine or diluent as described above. Cellular \( \beta_h \) PKC levels were determined, radiolabeled in the presence of [\( { }^{32} \)P]orthophosphoric acid and treated with either nocodazole or chelerythrine as described under “Experimental Procedures.” At 15 h after release from aphidicolin, cell lysates were subjected to immunoblot analysis for \( \alpha \), \( \beta_h \), and \( \zeta \) PKC as described under “Experimental Procedures.” Alternatively, lamin B was phosphorylated by purified \( \beta_h \) PKC in vitro. A, phosphopeptide map of mitotic lamin B from nocodazole-treated cells. B, phosphopeptide map of lamin B from G2 phase cells blocked with chelerythrine. C, phosphopeptide map of lamin B phosphorylated by \( \beta_h \) PKC in vitro. D, mixture of A and B. E, mixture of A and C.

levels in synchronized, chelerythrine-treated cells were assessed along with cell cycle progression through G2/M phase in parallel cultures of synchronized control cells (Fig. 6B). As can be seen, chelerythrine-induced degradation coincides with cell cycle progression through G2/M phase. Degradation is not dependent upon the length of exposure to chelerythrine, since it is observed specifically at G2/M phase whether chelerythrine is added from 1 to 5 h before mitosis, yet it is not observed when chelerythrine is added after mitosis has occurred (data not shown). These results indicate that \( \beta_h \) PKC becomes highly sensitive to chelerythrine-mediated degradation specifically during G2 phase prior to mitosis, a finding that is interesting in light of our recent demonstration that \( \beta_h \) PKC is selectively translocated and activated at the nucleus during G2 phase (18). The selective degradation of \( \beta_h \) PKC may account for the irreversibility of the chelerythrine-induced G2 blockade.

Chelerythrine Induces Activation-dependent Degradation of PKC Isotypes in Vivo—Fig. 6 demonstrates that chelerythrine induces rapid and selective degradation of \( \beta_h \) PKC in G2 phase. Our previous studies showed that \( \beta_h \) PKC, but not \( \alpha \) or \( \zeta \) PKC, is selectively activated at the nucleus during G2 phase (18). These results suggested that chelerythrine may induce activation-dependent degradation of PKC. In order to test this hypothesis, we assessed the effect of chelerythrine on the stability of the \( \alpha \), \( \beta_h \), and \( \zeta \) PKC isotypes in unsynchronized cells in the presence and absence of PKC activators (Fig. 7). Incubation of unsynchronized cells with chelerythrine leads to little or no change in stability of the \( \alpha \) and \( \zeta \) PKC isotypes and to a slow loss of \( \beta_h \) PKC over a 9-h period (Fig. 7). Activation of PKC by addition of diC8 has little effect on the stability of \( \alpha \), \( \beta_h \), and \( \zeta \) PKC, however, when cells are exposed to both chelerythrine and diC8, rapid degradative loss of the \( \alpha \) and \( \beta_h \) PKC isotypes is observed (Fig. 7). Interestingly, \( \zeta \) PKC is not degraded under these conditions, consistent with the observation that \( \zeta \) PKC does not bind, and is not activated by, conventional PKC activators (26). Similar results were obtained using phorbol myristate acetate and bryostatin to activate \( \alpha \) and \( \beta_h \) PKC, indicating that this effect is not specific for diC8-mediated activation (data not shown).

The rates of degradation of \( \alpha \) and \( \beta_h \) PKC in the presence of chelerythrine and PKC activators are similar. However, some degradation of \( \beta_h \) PKC is observed even in the absence of exogenous activator. It is not apparent whether this reflects a
difference in the activation status of the α and βII PKC isotypes in unsynchronized cells or an intrinsic difference in the susceptibility of these PKC isotypes to chelerythrine-induced degradation. The degradation of α and βII PKC differs in another respect. Immunoreactive βII PKC disappears completely, whereas α PKC undergoes "laddering" as the full-length protein is degraded into lower molecular weight fragments prior to complete loss of immunoreactive α PKC.

Fig. 7 demonstrates that chelerythrine is capable of activation-dependent degradation of both α and βII PKC. However, in synchronized cells a selective loss of the βII PKC isotype is observed during G2 phase. These results are consistent with our previous finding that βII PKC, but not α PKC, is selectively activated during G2 phase in synchronized cells (18).

**DISCUSSION**

The protein kinase C family has been implicated in the regulation of differentiation and proliferation in human leukemia cells (20, 22, 27–30). We previously demonstrated that the α and βII PKC isotypes are involved in cellular differentiation, whereas the βII PKC isotype is required for proliferation (22). βII PKC levels correlate directly with proliferative capacity, and inhibition of βII PKC expression using antisense oligonucleotides leads to profound inhibition of cellular proliferation (22). The current study provides a plausible mechanism by which βII PKC participates in the proliferative pathway.

Earlier fractionation studies demonstrated that βII PKC is selectively translocated and activated at the nucleus of human leukemia cells in response to proliferative stimuli (20, 30). At the nucleus, βII PKC directly phosphorylates a major nuclear envelope component, lamin B1 (17, 18, 20, 21, 27–30). Phosphorylation site mapping identified the βII PKC phosphorylation sites on lamin B as Ser395 and Ser405 (17, 18). Furthermore, phosphorylation of these two sites by purified βII PKC leads to nuclear lamina disassembly in vitro (17). Finally, combined cellular fractionation and phosphorylation studies demonstrated that βII PKC is selectively translocated and activated at the nucleus of synchronized cells at the time of the G2/M phase transition and that the βII PKC phosphorylation site on lamin B, Ser405, becomes prominently phosphorylated during the G2/M phase transition (18). We have not detected phosphorylation of Ser395 in synchronized cells during any phase of cell cycle, suggesting that this site may not be of physiologic relevance. Our previous data suggested a role for βII PKC activation and lamin B phosphorylation in cellular events during G2/M phase of cell cycle. In the present report, we provide compelling evidence that βII PKC activation is a requisite event in the G2/M phase transition.

Treatment of synchronized cell populations with the highly selective PKC inhibitor chelerythrine leads to profound cell cycle arrest in G2 phase. This blockage is characterized by the presence of a fully replicated genome, decondensed nuclear chromatin morphology including dense nucleolar structures, and the presence of an intact, highly polymerized nuclear lamina. Inhibition studies demonstrate that chelerythrine does not inhibit the prominent mitotic protein kinase, p34cdc2/cyclin B, either in vitro or in whole cells. Phosphopeptide mapping demonstrates that chelerythrine inhibits βII PKC-mediated phosphorylation of lamin B, at Ser405, a site previously implicated in mitotic nuclear lamina disassembly (17, 18). In addition, a second, unidentified mitotic phosphorylation site on lamin B1 was also inhibited by chelerythrine treatment. Phosphorylation of this site does not appear to be directly mediated by either βII PKC or p34cdc2/cyclin B kinase, since neither kinase phosphorylates this site in vitro (18). It is possible that chelerythrine directly inhibits the kinase that phosphorylates this site or that phosphorylation at this site requires βII PKC activity. Current studies are ongoing to identify this site, the kinase responsible for its phosphorylation, and the effect of βII PKC activation on phosphorylation.

The cell cycle effects of chelerythrine are not readily reversible. However, this effect does not appear to be due to cytotoxicity, but rather to the selective degradation of βII PKC at G2 phase in chelerythrine-treated cells. Chelerythrine-induced degradation of PKC is not peculiar to βII PKC, since chelerythrine leads to degradation of both α and βII PKC in unsynchronized cells in the presence of PKC activators. Several observations suggest that chelerythrine-induced PKC degradation is activation-dependent. First, both α and βII PKC are rapidly degraded when HL60 cells are treated with both chelerythrine and a PKC activator. Second, PKC is not degraded in these cells in the presence of chelerythrine and either diC8, phorbol myristate acetate, or bryostatin. It is well established that PKC, unlike α and βII PKC, is not activated by these conventional PKC activators (31). The lack of degradation of PKC is not due to an intrinsic insensitivity to chelerythrine inhibition, since chelerythrine inhibits PKC activity in vitro. Third, βII PKC is selectively degraded during G2 phase in synchronized cells treated with chelerythrine, consistent with our previous observation that βII PKC, but not α or ε PKC, is selectively activated during this phase of cell cycle (18).

Our data are interesting in light of the proposed mechanism of action of chelerythrine (23). Kinetic studies have determined that chelerythrine inhibition is substrate-competitive and that chelerythrine binding likely involves the active site of PKC (23). According to the prevailing model of PKC regulation, inactive, cytosolic PKC assumes a conformation in which an amino-terminal pseudosubstrate domain occupies the active site of the enzyme, making it inaccessible to substrates (32). Binding of co-factors and activators to the regulatory domain leads to a conformational change that displaces the pseudosubstrate from the active site, thereby allowing substrates to bind and leading to enzyme activation (33). Since chelerythrine binding to PKC involves the active site and is competitive with

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2 L. J. Thompson and A. P. Fields, unpublished results.
substrate, PKC activation may lead to enhanced binding of chelerythrine. It is well established that the active conformation of PKC is highly susceptible to proteolytic degradation, which appears to be the molecular basis underlying phorbol ester-induced PKC degradation and down-regulation (33, 34). Phorbol esters activate PKC by binding to diacylglycerol binding sites within the regulatory domain. The high affinity of phorbol esters for PKC causes the enzyme to remain in its active conformation for extended periods of time, leading to chronic activation of the enzyme and proteolytic degradation (33, 34). Our data suggest that chelerythrine may act in a manner similar to the phorbol esters. In the case of chelerythrine, binding of endogenous activators induces the active conformation. Release of the pseudosubstrate domain from the active site allows subsequent binding of chelerythrine at or near the active site. High affinity binding of chelerythrine may hold PKC in its active conformation, leading to increased susceptibility to proteolytic degradation. Further studies will be required to determine whether this proposed mechanism is responsible for the activation-dependent degradation of PKC by chelerythrine.

The present study provides compelling evidence that $\beta_1$, PKC activation is a requisite step for entry into mitosis. $\beta_1$, PKC activation appears to be necessary, either directly or indirectly, for a number of mitotic events including chromosome condensation and mitotic nuclear lamina disassembly. Our results indicate that $\beta_1$, PKC is a cell cycle regulator distinct from p34$^{cdk2}$/cyclin B kinase. Many cell cycle regulatory proteins, including p34$^{cdk2}$/cyclin B kinase and PKC, are highly conserved evolutionarily. The yeast PKC homolog, PKC1, is required for the G2/M phase transition in yeast (13). Ablation of PKC1 leads to recessive lethality and arrest of cells in G2 phase prior to mitosis (13). Functionally, PKC1 appears to regulate aspects of the osmotic stability of the yeast cell wall necessary for completion of the cell division cycle (35) and is a mitotic checkpoint independent from the yeast homolog of p34$^{cdk2}$/cyclin B kinase. Our data are consistent with those obtained in yeast, since the activity of p34$^{cdk2}$/cyclin B kinase is unaffected by chelerythrine either in vitro or in whole cells, suggesting that $\beta_1$, PKC may lie in a distinct pathway from that of p34$^{cdk2}$/cyclin B kinase. Furthermore, our data indicate that mitotic activation of p34$^{cdk2}$/cyclin B kinase is not sufficient for entry into mitosis, consistent with the data of others (7).

Given the critical role that PKC plays in the regulation of mitosis in species as diverse as yeast and man, it is tempting to speculate that the pathways in which PKC participates are likewise conserved. PKC1 has been shown to regulate the activity of a number of downstream protein kinases including the yeast homologs of the mammalian dual specificity kinase, mitogen-activated protein kinase kinase, and the serinethreonine kinase, mitogen-activated protein kinase (36, 37). Current studies are under way to determine whether these kinases lie downstream of $\beta_1$, PKC in mammalian cells during the G2/M phase transition.

The present data, coupled with our previous studies, indicate that lamin B1 is a relevant and perhaps critical target for direct, $\beta_1$, PKC-mediated phosphorylation during the G2/M phase transition. Phosphorylation of lamin B1 by purified $\beta_1$, PKC leads to nuclear lamina disassembly in vitro and the $\beta_1$, PKC phosphorylation site on lamin B1, Ser$^{405}$, is prominently phosphorylated in mitotic cells (17, 18), suggesting that phosphorylation of Ser$^{405}$ plays a key role in mitotic nuclear lamina disassembly in vivo. Indeed, chelerythrine inhibits phosphorylation of Ser$^{405}$ (and a second unidentified site) on lamin B1 and inhibits mitotic nuclear lamina disassembly. These data suggest that one mechanism by which chelerythrine blocks entry into mitosis is by preventing phosphorylation of a site or sites on lamin B1, required for mitotic nuclear lamina disassembly. Clearly, chelerythrine inhibits other aspects of mitosis including chromosome condensation and mitotic spindle formation. Therefore, $\beta_1$, PKC represents an important G2/M phase cell cycle regulator that is required for multiple aspects of mitosis. Although the mechanism by which $\beta_1$, PKC is translocated and activated at the nucleus during G2 phase is not known, it has recently been shown that the nucleus has a distinct phosphoinositide cycle that is responsive to both mitogenic stimuli and intrinsic cell cycle regulation (38–41). In regenerating rat hepatocytes, nuclear phosphoinositide turnover increases during G2 phase, leading to an increase in nuclear diacylglycerol levels and the translocation of PKC to the nucleus (41). In addition, a nuclear membrane lipid factor, termed NMAF, has been identified from human leukemia cells that selectively activates $\beta_1$, PKC at the nuclear membrane (21). Therefore, it is likely that nuclear phospholipid metabolism can generate signals for the nuclear activation of $\beta_1$, PKC during G2 phase of cell cycle. In this regard, it has recently been reported that in Saccharomyces cerevisiae the yeast homolog of phosphatidylinositol 4-kinase, STT4, lies upstream of PKC1 (42). The existence of cell cycle-regulated pathways involving PKC in both yeast and mammals suggests that PKC is a phylogenetically conserved regulator of cell cycle events, particularly those associated with the G2/M phase transition.

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Protein Kinase C Activity Is Required for Mitosis

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