Identification of Protein Kinase C (PKC) Phosphorylation Sites on Human Lamin B

POTENTIAL ROLE OF PKC IN NUCLEAR LAMINA STRUCTURAL DYNAMICS

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Protein kinase C (PKC) is activated at the nuclear membrane in response to a variety of mitogenic stimuli. In human leukemic cells, the δ subtype of PKC is selectively translocated and activated at the nucleus. We recently identified the nuclear envelope component lamin B as a major substrate for nuclear PKC both in whole cells and in vitro. Using highly purified human δ PKC and isolated nuclear envelopes from the human promyelocytic (HL60) leukemia cell line, we have now determined the major sites for δ PKC-mediated lamin B phosphorylation. Using a combination of cyanogen bromide cleavage, direct microsequencing, tryptic phosphopeptide, and phosphate release analyses, two major sites of PKC-mediated phosphorylation, Ser385 and Ser406, have been identified. These sites lie within the carboxyl-terminal domain of lamin B immediately adjacent to the central α-helical rod domain. Functionally, δ PKC-mediated phosphorylation of these sites leads to the time-dependent solubilization of lamin B indicative of mitotic nuclear envelope breakdown in vitro. δ PKC-mediated lamin B phosphorylation is inhibited by 1) a monoclonal antibody directed against the active site of PKC, 2) a PKC pseudosubstrate inhibitor peptide, and 3) a PKC peptide substrate. Two observations indicate that PKC-mediated lamin B phosphorylation and solubilization is due to direct phosphorylation of lamin B by PKC rather than indirect activation of a cdc2 kinase. Neither immunodepletion with p139-Sepharose beads nor the presence of a p34cdc2 kinase peptide substrate had any effect on PKC-mediated lamin B phosphorylation. Therefore, we conclude that δ PKC represents a physiologically relevant lamin kinase that can directly modulate nuclear lamina structure in vitro. Nuclear δ PKC, like p34cdc2 kinase, may function to regulate nuclear lamina structural stability during cell cycle.

The nuclear lamina is a proteinaceous fibrillar network underlying the inner nuclear membrane that gives the nucleus structural integrity, segregates the nuclear interior from the surrounding cytoplasm and provides attachment sites for chromatin (1-3). The major constituents of this structure, the nuclear lamins, are members of the intermediate filament protein family (1). The nuclear lamina is a dynamic structure which undergoes growth during cell cycle progression and is reversibly depolymerized at the G2/M phase transition of cell cycle (2). Many of the structural dynamics of the lamina are thought to be regulated by the reversible phosphorylation of the nuclear lamins. Mitotic depolymerization is dependent upon the stoichiometric hyperphosphorylation of the lamins, an event which contributes to the increased solubility of mitotic lamins (4). Following mitosis, the lamins become increasingly insoluble and are progressively dephosphorylated as they reassociate to form the polymeric lamina (2, 4).

Several lamin kinases have been identified to date including p34cdc2 kinase (5-8), S6 kinase II (9, 10), and PKC (11-16). Recent attention has focused on p34cdc2 kinase, the active component of M-phase promoting factor, since it is required for G2/M phase progression, and it can elicit a mitotic-like nuclear envelope breakdown (NEBD) in vitro. However, biochemical evidence suggests the involvement of multiple lamin kinases in NEBD in vitro (7, 10).

Recent work from this laboratory demonstrated that treatment of human leukemia cell lines with the mitogenic PKC activator bryostatin 1 leads to the rapid translocation and activation of δ PKC at the nuclear where it induces time- and dose-dependent phosphorylation of lamin B, the sole lamin species expressed in these cells (14, 15). From these data we suggested that nuclear δ PKC activation and subsequent lamin B phosphorylation was involved in mitogenic stimuli, including the proliferative effects of bryostatin (14, 15). Although PKC appears to be a physiologically relevant lamin kinase, its physiologic role remains to be elucidated.

In the present study, we report the identification of the major sites of phosphorylation on human lamin B mediated by δ PKC. These sites are restricted to the carboxyl-terminal tail region immediately adjacent to the central α-helical coil domain. This region of the lamins has been found to be phosphorylated during mitosis and is thought to be involved in the regulation of lamin assembly and disassembly. In addition, these results indicate that PKC, like p34cdc2 kinase, may play a role in regulating nuclear lamina structural dynamics during the cell cycle.

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The abbreviations used are: PKC, protein kinase C; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid; NEBD, nuclear envelope breakdown; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylaminopropanesulfonic acid; PVDF, polyvinylidene difluoride; TLC, thin layer chromatography.
EXPERIMENTAL PROCEDURES

Cell Culture, Radioisotopic Labeling, and Isolation of Nuclear Envelopes—HL60 cells were maintained in logarithmic growth in Iscove's modified Dulbecco's medium containing 5 mM HEPES (Sigma) supplemented with 10% (v/v) fetal bovine serum and 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO) in a 37°C/5% CO2 incubator. For radioisotopic labeling, cells were washed in phosphate-free RPMI 1640 medium (GIBCO) and incubated with 150 µCi/ml [32P]orthophosphoric acid (Du Pont-New England Nuclear, 850-900 Ci/mmol; 100,000 Ci at 37°C prior to treatment with 100 µg/ml bryostatin 1 for 30 min to stimulate lamin B phosphorylation (11).

Nuclear envelopes were isolated from HL60 cells as previously described (11) except that the cells were lysed by vortexing for 30 s in lysis buffer containing 50 mM NaCl, 10 mM MgSO4, 10 mM EGTA, and 1 mM dithiothreitol with 0.1% Nonidet P-40 containing 1% phenylmethylsulfonyl fluoride and 20 µg/ml leupeptin.

Expression, Isolation, and Characterization of Human β2 PKC Produced in the Baculovirus-Insect Cell Expression System—Two independent clones for human β2 PKC were isolated from a human temporal cortex library (Stratagene). Clone 1 contained a deletion between base pairs 1189 and 1261 based on the previously published sequence. Clone 2 was a partial clone with sequence beginning at approximately base pair 400 of the previously published sequence. The two clones were spliced together to generate a full-length human β2 PKC construct as follows: a 1.3-kilobase BamHI/SacI fragment from clone 1 (representing the 5′ end of β2 PKC) was ligated to a 0.3-kilobase SacI/BglII fragment from clone 2 (3′ end of β2). The resulting 2.0-kilobase BamHI/BglII fragment (full-length human β2 PKC) was gel isolated and then ligated into the BamHI site of the baculovirus transfer vector, pVL941.

The generation of recombinant β2 PKC baculovirus was accomplished as previously described (18). Recombinant human β2 PKC was produced in Spodoptera frugiperda cells (Sf9) infected with $\phi$1437 baculovirus. Sf9 cells were routinely harvested and characterized by infecting 5 × 10^6 cells in 10 ml of serum-free medium with 0.5 ml of infected cell culture supernatant. Infected cells were fed twice weekly with fresh medium to maintain a doubling time of 10–12 h. Sf9 cells were infected with recombinant baculovirus at a multiplicity of 10. Infected cells were harvested 72 h after infection and stored at −20°C.

The resulting 2.0-kilobase BamHI/BglII fragment was excised from the baculovirus transfer vector, pVL941, and ligated into the BamHI site of the baculovirus transfer vector, pVL941. The two clones were spliced together to generate a full-length human β2 PKC construct as follows: a 1.3-kilobase BamHI/SacI fragment from clone 1 (representing the 5′ end of β2 PKC) was ligated to a 0.3-kilobase SacI/BglII fragment from clone 2 (3′ end of β2). The resulting 2.0-kilobase BamHI/BglII fragment (full-length human β2 PKC) was gel isolated and then ligated into the BamHI site of the baculovirus transfer vector, pVL941.

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final concentration of 1% (w/v) followed by a 10-min incubation on ice. The reaction mix was microfuged at 12,000 x g for 10 min at 4°C, and supernatants and pellets were subjected to SDS-PAGE analysis in 8% acrylamide gels followed by transfer to nitrocellulose as previously described (11). Autoradiography and immunoblot analysis was performed using a specific anti-lamin antibody as previously described (12). Lamin B phosphorylation and content was evaluated by laser densitometric scanning.

RESULTS

Expression and Characterization of Human βII PKC in the Baculovirus-Insect Cell System—Our previous studies in human leukemic cells demonstrated the translocation and activation of a PKC activity at the nucleus following bryostatin treatment where it phosphorylated nuclear lamin B (11, 14, 15). Moreover, we recently identified this nuclear-activated PKC as the βII isoform (14, 15). We therefore wished to use purified human βII PKC to determine the sites of phosphorylation on human lamin B. To obtain a source of human βII PKC that was not cross-contaminated with other PKC isoforms, we utilized the baculovirus-insect cell system to express recombinant human βII PKC. This system has been used successfully for the production of bovine α, rat βII, and γ-PKC (22). Since uninfected Sf9 insect cells contain very little detectable PKC activity and no immunoreactive PKC, infection with recombinant baculovirus yields a convenient source for isotype pure βII PKC (22).

Infection of Sf9 insect cells with recombinant baculovirus containing the human βII PKC cDNA led to the efficient expression of human βII PKC. To characterize the βII PKC expressed in these cells, immunoblot analysis and cofactor studies were conducted (Fig. 1). Partially purified βII PKC preparations consisted of a prominent band with a relative molecular mass (Mr) of 85,000 (Fig. 1A) consistent with the migration of native βII PKC. The isotype purity of this preparation was confirmed by immunoblot analysis using our previously characterized isotype-specific antibodies recognizing the α, βI, βII and γ isoforms of PKC (14). Recombinant βII PKC was immunoreactive with the βII-specific antibody but not the other isotype-specific antibodies indicating the absence of multiple isoforms in the preparation (Fig. 1B).

To assess that the recombinant βII PKC behaved like native PKC, the Ca2+ and phospholipid dependencies of the enzyme were determined as described under “Experimental Procedures” (Fig. 1, C and D). Recombinant βII PKC exhibited Ca2+-phosphatidylserine-dependent histone kinase activity with maximal activity observed at ~150 μM Ca2+ and 10 mol % phosphatidylserine, similar to values reported previously for βII PKC from other sources (22). Therefore, our recombinant human βII PKC preparation is isotype pure and exhibits enzymatic properties consistent with those of the native enzyme.

Human βII Protein Kinase C-mediated Phosphorylation Is Confined to the Carboxyl-terminal Domain of Lamin B—Nuclear lamin B belongs to the family of intermediate filament proteins and as such possesses several structural motifs common to all family members. The most prominent of these is the presence of a large, highly conserved α-helical domain which makes up the central portion of the molecule. This region is thought to be responsible for the formation of a highly stable coiled-coil dimer between two lamin molecules (3). Flanking the central α-helical region are non-helical NH2- and COOH-terminal domains (3). Previous analysis has identified the NH2- and COOH-terminal domains immediately adjacent to the α-helical coil domain as a region of multiple-site phosphorylation (5, 8, 10, 23). It appears clear that phosphorylation of these sites is involved in the regulation of higher order lamin filament assembly/disassembly (8, 10, 24). Given the physiologic importance of lamin phosphorylation within the NH2- and COOH-terminal regions, we sought to determine where βII PKC-mediated phosphorylation sites lie in relation to these regions. For this purpose, CNBr was used to generate relatively large peptide fragments which could easily be resolved by SDS-PAGE. Sequence analysis predicted that CNBr would generate fragments of characteristic size corresponding to the NH2-terminal domain (predicted Mr, 22,400) and the COOH-terminal domain including the region immediately adjacent to the central α-helical domain (predicted Mr, 10,400).

CNBr digestion and SDS-PAGE analysis of in vivo and in

FIG. 1. Characterization of purified human βII PKC expressed in the baculovirus-insect cell system. Human βII PKC was expressed in the baculovirus-insect cell system as described under “Experimental Procedures.” Panel A, Coomassie Blue-staining pattern of βII PKC preparation. Panel B, Immunoblot analysis of βII PKC preparation as in A using isotype-specific antibodies to α, βII, βI, and γ-PKC isotypes as previously described (14). Panels C and D, Ca2+ and phosphatidylserine-dependent histone kinase activity of βII PKC preparation assayed as described under “Experimental Procedures.”
In vitro phosphorylated lamin B revealed that the major phosphorylation sites mediated by βII PKC reside in a fragment which migrates as a doublet of ~10-12 kDa consistent with phosphorylation of the COOH-terminal domain adjacent to the central α-helical domain (Fig. 2). To confirm the identity of this fragment, the lower band of the doublet was excised and subjected to direct microsequencing analysis (Fig. 3). The sequence obtained identified this fragment as corresponding to the predicted CNBr cleavage product Glu737 to Met666. This fragment corresponds to the carboxyl end of the central α-helical domain and the flanking region of the carboxyl-terminal domain. Sequence analysis of the upper band in the doublet was inconclusive; however, two-dimensional tryptic phosphopeptide maps of the two bands were identical to each other and to those generated by tryptic digestion of intact lamin B suggesting identity of the actual phosphorylation sites within these fragments (data not shown). The most likely explanations of the doublet are that the bands are related fragments generated by incomplete CNBr cleavage, or alternatively that the upper band may represent a more highly phosphorylated form of the lower fragment. In this regard, phosphorylation of intact lamin B causes the protein to migrate more slowly in SDS-PAGE gels (4; see also Fig. 6). Interestingly, phosphorylated lamin B isolated from whole cells contains a phosphorylated fragment of ~24 kDa in addition to the 10-12-kDa doublet (Fig. 2A). Based upon its molecular mass, this 24-kDa band may correspond to the predicted NH2-terminal domain CNBr fragment. However, this fragment is not highly phosphorylated in vivo and is not detected after in vitro phosphorylation (Fig. 2B), where phosphorylation can be directly attributed to βII PKC. These results indicate that the major phosphorylation sites mediated by βII PKC are contained within the carboxyl-terminal domain of lamin B immediately adjacent to the central α-helical domain.

βII PKC Phosphorylates Lamin B Predominantly at Ser390 and Ser405—Although microsequencing of the phosphorylated CNBr fragment localized βII PKC-mediated phosphorylation sites to the COOH-terminal domain of lamin B, specifically between Glu737 and Met666, the large size of the fragment precluded direct sequencing through potential PKC phosphorylation sites. Previous phosphoamino acid analysis of human lamin B phosphorylated either in whole HL60 cells or in vitro with PKC indicated that phosphorylation was confined to serine residues within the molecule (11). Furthermore, analysis of the deduced amino acid sequence of human lamin B revealed the presence of only three potential PKC phosphorylation sites, Ser390, Ser405, and Ser466, within the identified CNBr fragment (no potential Thr sites exist in this fragment). All three sites conform to the determined consensus PKC phosphorylation motif of S*/T*-X-K/R. As indicated in Fig. 3, each of these potential sites localize to separate tryptic fragments. Therefore, tryptic digestion of intact lamin B employed to generate small peptides that would contain only one potential PKC phosphorylation site within this region.

Tryptic digests of in vivo and in vitro phosphorylated lamin B were subjected to two-dimensional thin layer chromatography and autoradiography. Lamin B phosphorylated in vitro by βII PKC generates two predominant 32P-labeled tryptic fragments labeled spots 1 and 2 (Fig. 4, right panel). In vivo labeled lamin B (Fig. 4, left panel) generates these two major spots and an additional spot not seen in in vitro labeled lamin B (spot 3).

Attempts to directly microsequence in vitro phosphorylated tryptic fragments after HPLC or two-dimensional TLC separation proved unsuccessful, so alternative methods were employed to identify the phosphorylated residues, including phosphate release analysis and comparative chromatographic migration analysis. [32P]Phosphate released after each cycle of manual Edman degradation was employed to detect a phosphorylated residue at that cycle. For this analysis, tryptic residues were separated by two-dimensional TLC, eluted from the cellulose plate, and subjected to manual Edman degradation as described (21). Analysis of an aliquot removed after each cycle revealed [32P]Phosphate release after cycle 3 in peptide 1 and after cycle 6 in peptide 2, with approximately

(E) ISAYRKLL
E77I S A Y R K L L E G E E R L K L S P S P S*SRV T V SRASS*SR
S*V R T TRGK K R K V D V E E E A S S S V S I S H S A SATGN
V C I E I D V D G K F I R L K N T S E Q D O P M 666

Fig. 3. PKC-mediated phosphorylation of lamin B is restricted to the carboxyl-terminal domain. Complete sequence of the phosphorylated CNBr fragment. Direct sequence obtained from microsequencing is indicated in boldface. Residues in parentheses indicate some ambiguity in sequence determination in that cycle. * denotes a consensus serine PKC phosphorylation site. Tryptic fragments containing consensus phosphorylation sites are underlined.
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Fig. 4. Two-dimensional tryptic phosphopeptide analysis of PKC-phosphorylated lamin B. In vivo and βII PKC in vitro phosphorylated lamin B was subjected to tryptic digestion as described (21). The resulting tryptic digest was subjected to two-dimensional TLC analysis as described: first dimension electrophoresis was performed in pH 1.9 buffer (formic acid/acetic acid/dH2O 50:156:1794) at 1000 V for 20 min followed by second dimension ascending chromatography in phosphochromatography buffer (n-butanol/pyridine/acetic acid/dH2O 75:50:15:60) for 3 h. Phosphopeptides were visualized by autoradiography.

Fig. 5. Phosphate release analysis of in vitro phosphorylated lamin B. Tryptic phosphopeptides 1 (panel A) and 2 (panel B) from lamin B phosphorylated with βII PKC in vitro (Fig. 4) were eluted from cellulose TLC plates in pH 1.9 buffer and subjected to manual Edman degradation as described (21). Aliquots from each cycle were evaluated for phosphate release as described (21). Total recovery of input counts was greater than 90% as monitored by Cerenkov counting. Results are displayed graphically as counts released/cycle as a percentage of total recovered counts.

To further confirm the identity of the tryptic fragments, comparative chromatographic migration analysis was performed in two different solvent systems. A migration or \( R_f \) value was calculated from the known amino acid composition of each peptide for each solvent system as described (21) and compared to the migration of the tryptic fragments following two-dimensional TLC separation. Table I compares the actual migration of peptides 1 and 2 in the two chromatographic solvents along with predicted relative mobilities for each peptide. As shown, the experimentally determined mobilities correlate well with predicted values, again corroborating the proposed assignment of peptides 1 and 2. In addition, synthetic peptides corresponding to the sequences of peptides 1 and 2 show nearly identical migration with the respective peptide following one-dimensional chromatography (\( R_f \) values in bold, Table I). Based on the above results, the major sites of βII PKC-mediated phosphorylation of lamin B are identified as Ser395 and Ser603.

βII PKC Phosphorylation Leads to Solubilization of Nuclear Envelope-associated Lamin B—A hallmark of the G2/M phase transition in eukaryotes is NEBD, a process requiring hyperphosphorylation of the lamins, the lamins become soluble in the reaction mixture. For lamin B, which remains membrane bound in the cell during mitosis, non-ionic detergent is added to obtain freely soluble lamin B in the reaction supernatant.

As can be seen in Fig. 6A, incubation of nuclear envelopes with βII PKC leads to solubilization of lamin B as evidenced by loss of lamin B from the nuclear envelope pellet fraction (P) and recovery of the protein in the Nonidet P-40 supernatant (S). This solubilization was accompanied by PKC-mediated phosphorylation of lamin B. Lamin B solubilization was rapid and time-dependent, being apparent by 5 min and becoming maximal by 120 min (Fig. 6B). This time course is similar to that observed for lamin solubilization using extracts from mitotic cells (26). These data demonstrate that βII PKC can elicit nuclear lamina disassembly in vitro.

Since the p34cdc2 kinase has also been shown to elicit nuclear lamina disassembly in vitro, we assessed whether a cdc2-like activity was present in our βII PKC or nuclear envelope preparations. This did not seem likely since our nuclear envelopes contain no intrinsic kinase activity (11, 14), and a possible site of phosphorylation.

Table I

| Peptide Composition | Phosphochromatography buffer | Isobutyric buffer |
|---------------------|------------------------------|-----------------|
| 1 403ASSSR          | 0.29 (0.32)                  | 0.53, 0.48 (0.52) |
| 2 390LSPSPSSR       | 0.43 (0.41)                  | 0.78, 0.76 (0.61) |

*Composition based on consensus PKC sites generated from tryptic digestion of labeled CNBr fragment.

\( R_f \) determined relative to indicator dye e-DNP-lysine, experimentally determined values are listed first; synthetic peptide migration is listed in bold, and values shown in parentheses are predicted mobilities calculated based on amino acid composition (21).

n-Butanol/pyridine/acetic acid/dH2O (75:50:15:60).

Isobutyric acid/n-butanol/pyridine/acetic acid/dH2O (65:2:5:3:29).
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**Fig. 6.** PKC-mediated phosphorylation of nuclear-envelope associated lamin B leads to time-dependent lamin solubilization in vitro. Panel A, HL60 nuclear envelopes were incubated with human \( \beta_{1} \) PKC for the indicated times, followed by assessment of lamin B phosphorylation and solubilization as described under “Experimental Procedures.” S designates the supernatant fraction after Nonidet P-40 extraction and P designates the insoluble pellet remaining after reactions were terminated. Panel B, time course of lamin B solubilization as assessed by laser densitometric scanning of anti-lamin B immunoblots.

the recombinant \( \beta_{1} \) PKC preparation exhibited only Ca\(^{2+} \) and phospholipid-dependent kinase activity (Fig. 1). Likewise, the identified phosphorylation sites, Ser\(^{395} \) and Ser\(^{405} \), do not conform to the CDC2 kinase consensus motif (27). However, further evidence that the observed NEBD was elicited directly by PKC comes from the observation that \( \beta_{1} \) PKC-mediated lamin B phosphorylation is inhibited by: 1) a monoclonal antibody against the PKC active site which specifically inhibits PKC activity, 2) a PKC substrate peptide, and 3) a PKC pseudosubstrate inhibitor peptide. In contrast, preincubation of the reaction mix with p13\(^{395} \) beads, which specifically bind cdc2 kinase, failed to remove the lamin kinase activity (Fig. 7A). Human cdc2 kinase prepared from mitotically arrested HL60 cells, as described under “Experimental Procedures” also phosphorylates lamin B (Fig. 7B). Lamin B phosphorylation mediated by cdc2 kinase was inhibited by a cdc2 substrate peptide, but not by the PKC pseudosubstrate inhibitor peptide (Fig. 7B). Histone phosphorylation is included to demonstrate isolation of the active mitotic form of cdc2 kinase (20). cdc2 phosphorylation of lamin B also was not inhibited by the PKC monoclonal antibody nor PKC substrate peptide (data not shown). These observations indicate that although both kinases can phosphorylate lamin B, the lamin solubilization observed in the NEBD assays shown in Fig. 6A can be attributed to direct phosphorylation by \( \beta_{1} \) PKC.

**Discussion**

Existence of Multiple Lamin Kinases—The intermediate filament nuclear lamins are integral components of the nuclear envelope which provide structural integrity to the nucleus during interphase (1–3). Disassembly of the lamin network is thought to be required for nuclear envelope breakdown at the time of mitosis (3). Previously it was shown that phosphorylation of the lamins precedes disassembly while dephosphorylation accompanies reassembly of the lamin network following chromosome segregation, leading to the hypothesis that phosphorylation is the key trigger for lamina disassembly (2, 4). Thus, the search for “mitosis-specific” lamin kinases was initiated.

Previously, we have shown in HL60 and K562 cells that lamin B is a major substrate for PKC both in vivo and in vitro (11, 14, 15). In the present report we have identified the major sites of PKC-mediated lamin B phosphorylation as Ser\(^{395} \) and Ser\(^{405} \). In addition, phosphorylation of the lamins by PKC has been demonstrated by other groups both in vivo (16) and in vitro (10, 23). Clearly PKC has been identified as a physiologically relevant lamin kinase but the functional consequence of PKC-mediated lamin phosphorylation remained to be elucidated.

Recently, several other kinases also have been identified as physiologically relevant lamin kinases, most notably, p34\(^{cdc2} \) kinase (5–8), and S6 kinase II (9, 10). Interestingly, in a detailed study of phosphorylation sites on human lamin C by Ward and Kirschner it was found that sequences surrounding the identified phosphorylation sites did not match the consensus phosphorylation site motifs of any single kinase (10). Therefore, they postulated that several lamin kinases act on the lamins to modulate lamina structure during the cell cycle. Specifically, they proposed that phosphorylation of sites within the carboxyl-terminal domain by one kinase may facilitate subsequent phosphorylation of adjacent sites by other lamin kinases. Indeed, the existence of multiple lamin kinases is corroborated by our study, since treatment of intact HL60 cells with bryostatin generates an additional lamin B phosphorylation site which cannot be directly attributed to phosphorylation by PKC (Fig. 2A), suggesting the possible involvement of another lamin kinase. Such cooperative or hierarchical phosphorylation involving multiple lamin kinases could explain the phosphorylation of sites on mitotic lamins which are consensus sites for different kinases. In this regard, we have found that \( \beta_{1} \) PKC phosphorylates human lamin B in vitro at more than 50 times the rate of mitotic cdc2 kinase suggesting that PKC is a better intrinsic lamin kinase than is cdc2 kinase.\(^{2} \) It is intriguing to speculate on the existence of a hierarchical phosphorylation scheme involving PKC and cdc2 kinase. If such a scheme exists, our data would suggest that PKC first phosphorylates lamin B at Ser\(^{395} \) and Ser\(^{405} \), thereby facilitating subsequent cdc2 kinase-mediated phosphorylation at the adjacent site, Ser\(^{402} \). This possibility remains to be tested.

Role of Lamin Phosphorylation in Regulating Nuclear Lamina Structure—Although it has been demonstrated that phosphorylation of the lamins is important in regulating lamin disassembly preceding mitosis, conflicting data exists in the literature regarding mitotic phosphorylation sites as well as sites which are key for disassembly of the lamins. Fig. 8 summarizes the identified phosphorylation sites on the lamins attributed to the known lamin kinases. Peter et al. (5) demonstrated that phosphorylation of Ser\(^{16} \) on chicken lamin B\(_{2} \) by cdc2 kinase resulted in lamina disassembly in vitro. In

\(^{2} \) B. A. Hocevar and A. P. Fields, unpublished observation.
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FIG. 7. Lamin B solubilization is due to PKC-mediated phosphorylation not to phosphorylation by p34\(^{cd2}\) kinase. Panel A, recombinant human \(\beta\)\(\text{II}\) PKC was incubated with purified lamin B as described under "Experimental Procedures" with the following substrates/inhibitors as indicated: PKC monoclonal antibody, 6 \(\mu\)g of IgG; PKC substrate peptide, 5, 10, and 50 \(\mu\)M; p13\(^{met}\)-agarose beads; p34\(^{cd2}\) kinase substrate peptide, 50 \(\mu\)M; PKC pseudosubstrate inhibitor peptide, 50 \(\mu\)M. Panel B, p34\(^{cd2}\) kinase was incubated with purified lamin B and histone H1 as described under "Experimental Procedures" with the following substrates/inhibitors as indicated: PKC pseudosubstrate inhibitor peptide, 50 \(\mu\)M. Histone phosphorylation is included to indicate isolation of active mitotic form of cdc2 kinase.

FIG. 8. Schematic diagram of known phosphorylation sites on the nuclear lamins. Identified phosphorylation sites on the nuclear lamins, human lamin B\(_1\) (HLB1), human lamins A and C (HLA/C), and chicken lamin B\(_2\) (CLB2). Denotes a PKC phosphorylation site (solid arrow) (this report and Ref. 10), a p34\(^{cd2}\) kinase phosphorylation site (thin arrow) (8, 10), and a S6 kinase II phosphorylation site (open arrow) (10).

Further studies, this group demonstrated that phosphorylation at this site was sufficient to cause head-to-tail depolymerization of \(\text{in vitro}\) synthesized chicken lamin B\(_2\) polymers (8). Although this site conforms to the consensus phosphorylation sequence requirements of cdc2 kinase, this is not a consensus phosphorylation site for PKC and indeed is not detected after \(\text{in vitro}\) phosphorylation by PKC in our study (Fig. 2B). In contrast, Ward and Kirschner (10) identified Ser\(^{392}\) as the only mitosis-specific phosphorylation site on human lamin C, while phosphorylation at Ser\(^{22}\) (the human lamin C homologue of Ser\(^{16}\) on chicken lamin B\(_2\)) and Ser\(^{404}\) increased in phosphorylation over interphase levels. It is important to note that while Ser\(^{404}\) has been identified as a S6 kinase II phosphorylation site on lamin C, sequence differences renders the analogous site (Ser\(^{465}\) on human lamin B\(_1\)) a consensus PKC phosphorylation site (see Fig. 8). Indeed in this report, we identify Ser\(^{405}\) as a major site of PKC-mediated phosphorylation. Interestingly, in a study by Heald and McKeon (24) it was found that a double mutation of Ser\(^{22}\) and Ser\(^{292}\) on human lamin A was required to partially block lamina disassembly \(\text{in vivo}\), while single mutations of these sites were insufficient to cause this blockade indicating the importance of carboxyl-terminal phosphorylation in lamina disassembly \(\text{in vivo}\). In the present study, we have demonstrated that \(\beta\)\(\text{II}\) PKC-mediated phosphorylation of Ser\(^{396}\) and Ser\(^{405}\) on human lamin B\(_1\) is sufficient to cause nuclear lamina disassembly \(\text{in vivo}\).

The common theme that emerges from these \(\text{in vivo}\) and \(\text{in vitro}\) phosphorylation studies is that phosphorylation of both the NH\(_2\)- and COOH-terminal domains immediately adjacent to the \(\alpha\)-helical coil region is important in lamina disassembly \(\text{in vivo}\). The lamins are known to dimerize, presumably through interaction of the \(\alpha\)-helical regions, and phosphorylation of residues close to these regions could cause disruption of the hydrophobic interactions holding the network together. In addition, phosphorylation of these sites may be involved in other processes proposed to be mediated by the lamins, such as chromatin attachment (28) and DNA replication (29).

\(\beta\)\(\text{II}\) PKC Is a Potential Mitotic Lamin Kinase—Several lines of evidence exist to implicate the involvement of PKC in G\(_2/\)M phase progression, and specifically in the process of nuclear envelope breakdown. First, global intracellular Ca\(^{2+}\) tran-
sients have been observed to precede nuclear envelope break-
down in amphibian eggs as well as mammalian Swiss 3T3 cells (30, 31). In fact, microinjection of the calcium chelator
BAPTA as well as severe Ca\textsuperscript{2+} deprivation by treatment with
tonomycin in EGTA-containing media blocks nuclear enve-
lope breakdown, suggesting the involvement of a Ca\textsuperscript{2+}-sensi-
tive kinase (31). Moreover, rat diploid fibroblasts treated with
staurosporine analogs, selective inhibitors of PKC, fail to enter mitosis and instead undergo DNA re-replication (32).
Finally, a functional homologue of PKC identified in Saccha-
romyces cerevisiae, termed PKC1, was shown to be essential
for cell viability. Cells depleted of the PKC1 gene product
arrested at a point in cell cycle following S phase but prior to
mitosis, while deletion of the gene resulted in recessive le-
thality (33).

Although PKC has been implicated in the process of NEBD,
no aspect of NEBD has previously been shown to be directly
modulated by PKC. In this report, we identify the major sites
of phosphorylation mediated by PKC as Ser\textsuperscript{395} and Ser\textsuperscript{405}
in vitro. In particular, phosphorylation sites is sufficient to cause NEBD
in vivo. Identification of the relative roles of individual phosphoryla-
tion sites and their proposed kinases in modulating nuclear
lamina structural dynamics in vivo remain to be determined.

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