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Lamin B is rapidly phosphorylated in lymphocytes after activation of protein kinase C

(signal transduction/nuclear lamina/protein kinase M/two-dimensional chromatography)

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ABSTRACT **Lamin B was shown to be a major substrate of cellular phosphorylation in the response of lymphocytes to phorbol esters. Lamins A and C, which were not observed in lymphocytes, were also substrates of phorbol-stimulated phosphorylation in those cell types that express them. Lamin B phosphopeptides labeled with ^{32}P in intact cells treated with phorbol 12-myristate 13-acetate were compared to those produced by *in vitro* phosphorylation with protein kinase M, cAMP-dependent protein kinase, and Ca^{2+}/calmodulin-dependent protein kinase II. The phosphopeptides labeled by *in vivo* stimulation with phorbol esters are very similar to those phosphorylated *in vitro* by protein kinase M, a catalytic domain of protein kinase C. Phorbol treatment of interphase cells significantly reduces the amount of detergent-insoluble lamin B, suggesting that phosphorylation of lamin may alter the architecture of the nuclear lamina. In addition, we have shown that treatment of a B-cell line with antibodies to IgM induces a modest increase in lamin B phosphorylation. These results strongly suggest that ligands that are known to activate protein kinase C at the cell surface or in the cytosol also lead to the activation of a nuclear kinase activity with a protein kinase C-type specificity.**

Protein kinase C (PKC) plays a crucial role in signal transduction in many cell types (1). Physiologically, PKC is activated by diacylglycerol, a lipophilic second messenger generated by receptor-coupled phosphodiesterase (2). PKC has also been identified as the major cellular receptor for tumor-promoting phorbol esters, which activate PKC in a manner similar to diacylglycerol (3). Phorbol esters, when added to intact cells, rapidly partition into hydrophobic environments throughout the plasma membrane and cytoplasm (4), where they specifically bind and activate PKC (5, 6). This cytoplasmic activation of PKC influences nuclear events, including the rapid transcription of specific sets of genes (7–10). The mechanisms through which these nuclear events are achieved are unknown. Because neither PKC nor phorbol esters apparently enter the nucleus (1, 4), it has been postulated that the influence of phorbol esters upon transcription may result from indirect events, such as the phosphorylation of cytoplasmic proteins (4). Alternatively, protein kinase M (PKM), a proteolytic fragment of PKC that is known to be generated after phorbol treatment, may be translocated into nuclei to cause phosphorylation of nuclear proteins. In this study, we show that a nuclear protein, lamin B, is one of the principal lymphocytic proteins to be rapidly phosphorylated after treatment of intact cells with phorbol 12-myristate 13-acetate (PMA). Lamin B, one of the three lamins that constitute the nuclear lamina in most adult mammalian cells (11, 12), is the only lamin we observe in lymphocytes. The *in vitro* pattern of phosphorylation of purified lamin B by PKM, which has the same specificity as intact PKC (13), is similar to the *in vivo* pattern of phosphorylation of lamin B in cells treated with PMA. In addition, we show that cross-linking of the antigen receptor of a B-lymphoma line also leads to increased phosphorylation of lamin B. These observations suggest that ligands that activate PKC in the cytosol or plasma membrane also induce a PKC-like activity in the nucleus. Furthermore, these results may identify a critical pathway for regulation of nuclear events by PKC.

MATERIALS AND METHODS

Cells and Cell Culture. Mouse lines include the B-cell lymphoma WEHI-231, the T-cell lymphoma EL-4, the mastocytoma MC-1, and the fibroblast line DAP.3; other lines include the rat glioma CG-17, the human promyelocytic leukemia line HL-60 (provided by L. Hall, National Institute of Allergy and Infectious Diseases), and the CHO line 10001 and its cAMP-resistant derivative 10248 (14). Dense splenocytes or splenic B cells were obtained by Percoll gradient centrifugation (15). Cells were cultured in RPMI 1640 medium/10% heat-inactivated fetal calf serum/50 μM 2-mercaptoethanol/pyruvate/penicillin/streptomycin at 37°C. Cycloheximide was included in some cultures at 0.5 mM.

Antibodies. Guinea pig antisera and purified antibodies to lamin B and to lamins A/C were generously provided by Larry Gerace (The Johns Hopkins University School of Medicine). Goat anti-IgM antibodies were coupled to dextran by J. Inman (National Institute of Allergy and Infectious Diseases).

Proteins. Lamin B was prepared by following published procedures (16) from the splenocytes of 120 mice. PKM, the catalytic fragment of rat PKC, was purified to homogeneity as described (17). Calcium/calmodulin-dependent protein kinase II (CaM-PK-II) was kindly provided by Claude Klee (National Cancer Institute). The catalytic subunit of cAMP-dependent protein kinase (PKA) was from Sigma (P2645).

Phosphorylation. Intact cells were labeled with $^{32}P_i$ as described (18). Briefly, cells were incubated with 0.5 mCi of $^{32}P_i$ (1 Ci = 37 GBq) for 2–4, followed by treatment with 0.32 μM PMA for the times indicated. *In vitro* phosphorylation assays were performed at 30°C. Assay mixtures of 75 μl contained 2–20 μg of substrate protein, 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 5 μM ATP, and 25 μCi of [γ-^{32}P]ATP. Calmodulin (2 μg) was included in the CaM-PK-II reaction mixture. At time 0, PKA (1.5 μg), CaM-PK-II (1.5 μg), or PKM (≈0.2 μg) was added. Reactions were

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Abbreviations: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PKM, Ca^{2+}/phospholipid-independent catalytic domain of protein kinase C; CaM-PK-II, Ca^{2+}/calmodulin-dependent protein kinase II; PKA, catalytic subunit of cAMP-dependent protein kinase.

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stopped at 25 min by the addition of $NaDodSO_4$ to 0.5% and heating in a 90°C water bath.

Cell Lysis. Cells, at a density of 4×10^6 ml^{-1}, were lysed at 95°C for 2 min in $NaDodSO_4$ lysis buffer [0.4% $NaDodSO_4$/0.05 M Tris, pH 7.4/10 μM vanadate/10 mM NaF/2 mM iodoacetamide/1 mM phenylmethylsulfonyl fluoride/leupeptin (5 μg/ml)/aprotinin (100 units/ml)], placed on ice, and treated with 100 μg of DNase and 50 μg of RNase for 10 min. For lamin solubility studies, cells were lysed on ice for 5 min in 0.5% Nonidet P-40/50 mM Tris·HCl, pH 7.5/2 mM EDTA/2 mM iodoacetamide/1 mM phenylmethylsulfonyl fluoride/leupeptin (5 μg/ml)/aprotinin (100 units/ml)/10 μM vanadate/10 mM NaF. Insoluble material was separated by spinning for 5 min in an Eppendorf tabletop centrifuge.

Electrophoresis. Two dimensional electrophoresis and $NaDodSO_4$/PAGE were performed by standard protocols (18, 19).

Immunoprecipitation of Lamin B. ^{32}P-labeled lamin was immunoprecipitated with guinea pig anti-lamin B and electrophoresed as described (12). The amount of ^{32}P-labeled lamin B was quantified by scintillation counting.

Immunoblotting. After $NaDodSO_4$/PAGE, proteins were electroblotted onto nitrocellulose paper in 0.05% $NaDodSO_4$/25 mM Tris·HCl/20% MeOH/192 mM glycine, pH 8.6, for 4 hr at 250 mA and 4°C. Blots were quenched with 2% bovine serum albumin in phosphate-buffered saline for 30 min followed by incubation with guinea pig antisera to lamins diluted 1:200 in rinse buffer (2% bovine serum albumin/0.4% Nonidet P-40/0.1% $NaDodSO_4$/0.1% Tween 20/0.1 M NaCl/0.05 M borate, pH 8.6) for 4 hr at room temperature. After rinsing, blots were incubated with ^{125}I-labeled protein A (Amersham; 1.85×10^{-3} MBq·ml^{-1}) for 2 hr, rinsed, and autoradiographed. Relative amounts of lamin were estimated by densitometry of autoradiograms of duplicate experimental samples compared to standard dilution curves.

Phosphopeptide Mapping. Phosphorylated lamin B was excised from two-dimensional gels, electroeluted (20), and precipitated in 90% EtOH. Tryptic digestion and two-dimensional peptide chromatography on cellulose plates was as described (12). In brief, the first dimension was electrophoresed toward the cathode in acetic acid/formic acid/H_2O (195:65:1040), and the second dimension was chromatographed in 1-butanol/pyridine/acetic acid/H_2O (75:50:15:60). ^{32}P-labeled peptides were visualized with autoradiography.

RESULTS

Splenic lymphocytes were labeled with $^{32}P_i$, stimulated with PMA, and lysed. Two-dimensional gel analysis demonstrated that a protein previously designated as protein 6 (18) was the most heavily labeled of the phosphoproteins induced by PMA (Fig. 1 *A* and *B*). We have shown that protein 6 is greatly enriched in the Nonidet P-40-insoluble fraction, suggesting that it is either nuclear or cytoskeletal. The molecular mass, solubility, and isoelectric characteristics of protein 6 suggested that it might be the nuclear intermediate filament protein lamin B.

Two-dimensional gel electrophoresis was used to study the relationship of protein 6 to lamin B. Splenic B cells were prelabeled with $^{32}P_i$, treated for 20 min with or without PMA, lysed, electrophoresed in two dimensions, and electroblotted onto nitrocellulose. ^{32}P-labeled protein 6 was visualized by autoradiography (Fig. 1*D*). These blots were immunoblotted with antibodies to lamin B. The position of lamin B directly overlays that of protein 6 (Fig. 1 *F* vs. *D*). In contrast, in cells not exposed to PMA, ^{32}P-labeled forms of protein 6 were not apparent (Fig. 1*C*), but immunoreactive lamin B was observed (Fig. 1*E*). Treatment with PMA induced an acidic shift of lamin B (Fig. 1 *F* vs. *E*) consistent with *de novo* phosphorylation; virtually all lamin B shows this acidic shift, suggesting that most lamin B molecules are phosphorylated in response to PMA. We have previously shown a similar acidic shift in ^{35}S-labeled protein 6 upon PMA treatment (18).

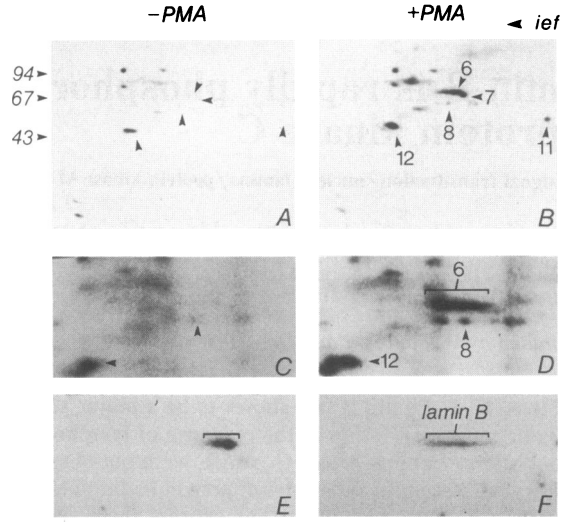

FIG. 1. Lamin B, formerly termed protein 6 (18), is a major target of phorbol ester-stimulated phosphorylation. Dense splenocytes were prelabeled with $^{32}P_i$ for 4 hr, followed by PMA for 30 min (*B*, *D*, and *F*), or nothing (*A*, *C*, and *E*). Cells were lysed and resolved by isoelectric focusing (ief) in the first dimension and $NaDodSO_4$/PAGE in the second dimension. (*A* and *B*) Autoradiograms of two-dimensional gels of total cell lysates. Molecular mass (kDa) is indicated at left; pI range is 4.2–7.0 from left to right. (*C* and *D*) ^{32}P-labeled proteins from two-dimensional gels were electroblotted onto nitrocellulose paper and visualized by autoradiography for 48 hr. The regions shown are 2-fold enlargements of a portion of the total pattern shown in *A* and *B*. (*E* and *F*) The blots pictured in *C* and *D* were treated with alkaline phosphatase overnight (Sigma type III), aged for 30 days, and immunoblotted with antiserum to lamin B. The location of lamin B was visualized after autoradiography for 4 hr.

Further evidence that PMA induces phosphorylation of lamin B is shown in Fig. 2*a*. WEHI-231 cells were prelabeled with $^{32}P_i$ followed either by PMA or nothing. After 20 min, the cells were lysed and lamin B was immunoprecipitated and analyzed by $NaDodSO_4$/PAGE. While PMA treatment did not alter the amount of lamin B that was immunoprecipitated (lanes 2 and 3), it did greatly increase the amount of ^{32}P incorporated into lamin B (lanes 4 and 5).

Immunoblotting of lysates from the B-lymphoma line WEHI-231 with antisera to lamins A, B, and C revealed the presence of lamin B, but not A and C, in these cells (Fig. 2*b*). We have not observed lamins A and C in any of the murine T- or B-lymphocytic cell lines examined; each of the lines expressed lamin B. As expected, cells of the fibroblast line DAP.3 express lamins A, B, and C.

As shown in Fig. 2*c*, PMA induces phosphorylation of lamin B in cells of diverse lineage and species including dense splenic B cells, a T cell lymphoma (EL-4), and a mast cell line (MC-1), all of mouse origin; a rat glioma line (CG-17); Chinese hamster ovary cells (CHO) and a line derived from them that is deficient in cAMP-dependent protein kinase (10001 and 10248, respectively); and a human promyelocytic leukemia line (HL-60). It should be noted that the two additional phosphoproteins that coprecipitate with lamin B from the CG-17 and CHO lysates comigrate with lamins A and C (data not shown) and that phorbol esters also induce the phosphorylation of lamins A and C. From these results, it is clear that the phosphorylation of nuclear lamins in the response to phorbol esters is a widespread phenome-

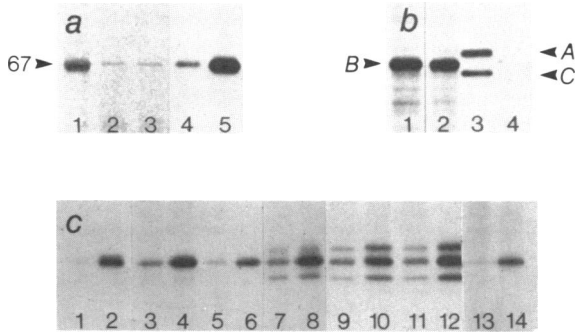

FIG. 2. Expression and phosphorylation of nuclear lamins. (*a*) A comparison of stained (lanes 2 and 3) vs. autoradiographed (lanes 4 and 5) immunoprecipitates of lamin B from ^{32}P-labeled WEHI-231 cells that had been treated with PMA for 30 min (lanes 3 and 5) or nothing (lanes 2 and 4). Samples were analyzed by NaDodSO$_4$/PAGE; lane 1 contained bovine serum albumin. (*b*) Expression of lamins A, B, and C in B cells compared to fibroblasts. Lysates of WEHI-231 (lanes 2 and 4) or DAP.3 (lanes 1 and 3) cells were electrophoresed in NaDodSO$_4$/polyacrylamide gels and immunoblotted with antisera to lamin B (lanes 1 and 2) or to lamins A and C (lanes 3 and 4). (*c*) The effects of PMA on lamin phosphorylation in cells of different origin. Immunoprecipitates of lamin B from ^{32}P-labeled cells treated with PMA (even-numbered lanes) or nothing (odd-numbered lanes) were analyzed by NaDodSO$_4$/PAGE. Cells include dense splenic B lymphocytes (lanes 1 and 2), EL-4 (lanes 3 and 4), MC-1 (lanes 5 and 6), CG-17 (lanes 7 and 8), 10001 (lanes 9 and 10), 10248 (lanes 11 and 12), and HL-60 (lanes 13 and 14).

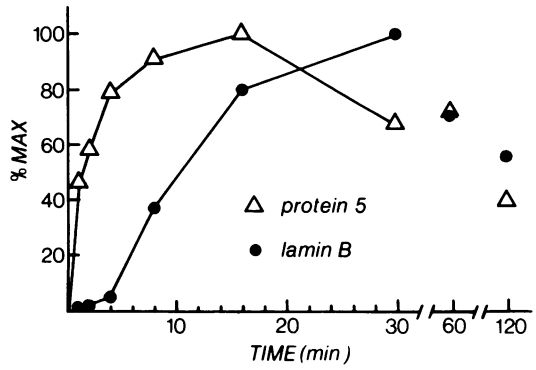

FIG. 3. Comparison of the kinetics of PMA-induced phosphorylation of protein 5 with lamin B in WEHI-231 cells. ●, Lamin B; △, protein 5. WEHI-231 cells were labeled with ^{32}P$_i$ and treated with either PMA or nothing at time 0. Cells were lysed at various times in hot NaDodSO$_4$. Lamin B was isolated by immunoprecipitation, NaDodSO$_4$/PAGE, and excision from the dried gel. Protein 5 was isolated by two-dimensional electrophoresis, autoradiography, and excision of the acidic, 65- to 70-kDa phosphoprotein from the dried gel. The amount of ^{32}P incorporated into the excised proteins was determined by scintillation counting. Maximum PMA-induced phosphorylation of lamin B occurred at 30 min, with 3250 cpm in the immunoprecipitates from PMA-treated cells compared to 350 cpm from untreated cells. Maximum PMA-induced phosphorylation of protein 5 occurred at 16 min, with 8270 cpm in the treated vs. 3250 cpm in the untreated cells. The percentage of maximum PMA-induced phosphorylation was determined by the equation: % max phosphorylation = (cpm treated − cpm untreated)/(cpm maximum − cpm untreated) × 100.

non. Furthermore, it is noteworthy that at least one of these lines (HL-60) is induced to undergo both differentiation and growth arrest in response to phorbol esters (21).

Since nuclear laminar proteins disperse throughout the cell during mitosis (11), it is possible that phosphorylation of lamin may occur not only in the nucleus but also in extranuclear compartments, depending on the mitotic status of the cell. The observation that PMA induces phosphorylation of virtually all lamin B in normal resting lymphocytes (Fig. 1), which are in G_0 and require >24 hr of mitogenic stimulation before entering S phase, argues strongly that the rapid phosphorylation of lamin B is occurring principally in the nucleus. This conclusion is strengthened by the observations that lamins appear to be restricted to the nucleoplasmic face of the nuclear membrane during interphase (22) and that the bulk of the lamin B that is phosphorylated in response to PMA is insoluble in nonionic detergents (18), a hallmark of its nuclear location (12).

The kinetics of the phorbol ester-induced phosphorylation of lamin B was compared to that of an apparent plasma membrane-associated protein (protein 5 in ref. 18). As shown in Fig. 3, the induced phosphorylation of protein 5 begins without apparent delay after the addition of PMA, while that of lamin B begins only after a delay of 4–5 min.

The ability of PKM (17, 23) to phosphorylate lamin B was investigated. A lamin-enriched extract (16) from murine splenocytes (Fig. 4, lane a) was phosphorylated *in vitro* by PKM. Antibodies to lamin B immunoprecipitated a ^{32}P-labeled protein with the molecular weight of lamin B (Fig. 4, lane c), demonstrating that lamin B is a substrate of PKM.

The fine specificity of lamin B phosphorylation induced in intact cells by PMA was compared to the *in vitro* pattern induced by PKM using two-dimensional phosphopeptide mapping. As shown in Fig. 5*B*, a total of six tryptic phosphopeptides were resolved when both PMA-induced and PKM-induced phosphopeptides were cochromatographed. Of these, four phosphopeptides were shared between the phorbol- and PKM-induced patterns. Previous experiments have shown that, while phosphopeptides 1, 2, 4, 5, and 6 are constitutively phosphorylated in intact interphase cells, PMA treatment greatly enhances the phosphorylation of peptides 4–6 (data not shown). Notably, the major phosphopeptides induced by PKM were also peptides 4–6.

The *in vitro* pattern of phosphorylation of lamin B by PKM was compared to that of the PKA, and CaM-PK-II. As shown in Fig. 5 *D–F*, there are major differences between the phosphopeptides produced by the three enzymes. Notably, while all three enzymes phosphorylate peptide 5, only PKM phosphorylates peptides 4–6, which are the major phosphopeptides induced by PMA in intact cells. In addition, the pattern of lamin B phosphorylation stimulated by PKM is identical to that stimulated by PKC (data not shown), confirming similar observations for other proteins (13). Collectively, this evidence strongly suggests that the nuclear kinase that is activated in the cellular response to phorbol esters possesses a PKC-like specificity.

Since it has previously been suggested that PKC is activated by cross-linking the antigen receptor of B cells (18, 24), the ability of antibodies to surface immunoglobulin to induce lamin phosphorylation was investigated (Table 1). Treatment with anti-IgM and anti-IgM dextran increased the

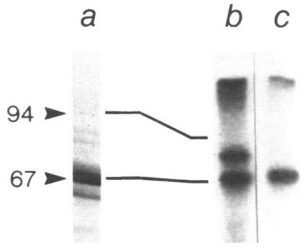

FIG. 4. Lamin B is a substrate of PKM. Lane a, Coomassie blue-stained fraction of splenocyte proteins highly enriched for lamin B and analyzed by NaDodSO$_4$/PAGE. Molecular mass markers (kDa) are on the left. Lane b, proteins from the fraction shown in lane a that are labeled with ^{32}P by *in vitro* phosphorylation with PKM. Lane c, phosphoproteins immunoprecipitated with antibodies to lamin B from the phosphorylated mixture shown in lane b.

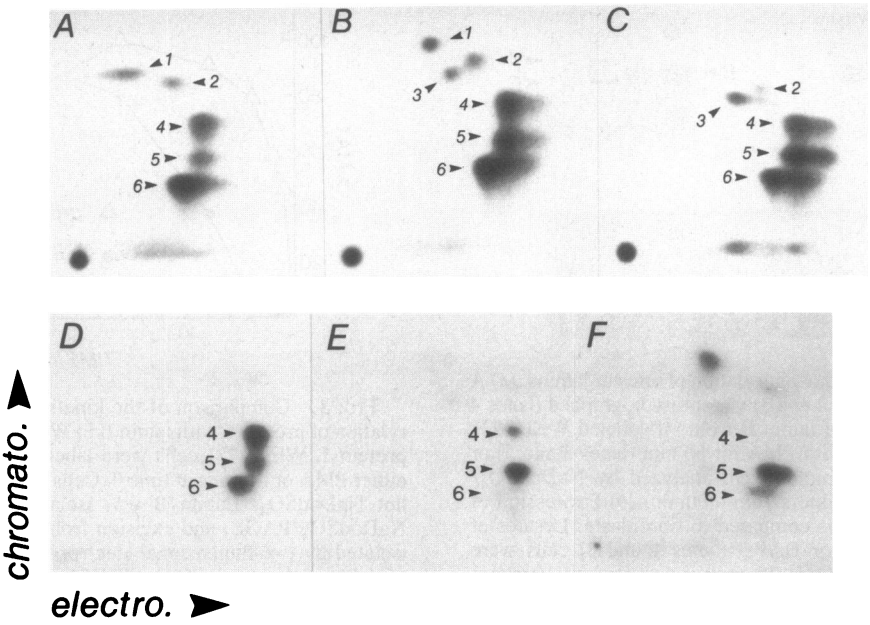

FIG. 5. Comparison of the phosphopeptides of lamin B induced by PMA in intact cells with those induced by PKM, PKA, and CaM-PK-II. (*A–C* and *D–F*) represent two different experiments. (*A*) *In vivo* phosphorylation after PMA treatment of ^{32}P-labeled WEHI-231 cells; (*C*) *in vitro* phosphorylation with PKM; (*B*) mixture of PMA (*A*) and PKM (*C*)-induced phosphorylation; (*D*) *in vitro* phosphorylation with PKM; (*E*) *in vitro* phosphorylation with PKA; (*F*) *in vitro* phosphorylation with CaM-PK-II.

^{32}P incorporated into lamin B 1.6- and 2.0-fold, respectively. In contrast, antibodies to IA/E had no effect on lamin phosphorylation. These data are consistent with other reports that PKC activated by diacylglycerol, which presumably occurs upon cross-linking of the antigen receptor, provokes far less cellular protein phosphorylation than PKC, which is activated by PMA (18).

It has been reported that mitotic lamin is hyperphosphorylated relative to interphase lamin, leading to the speculation that the increased phosphorylation of mitotic lamin leads to its dissolution (26). We therefore examined the effect of phorbol treatment upon lamin solubility in dense splenocytes that were cocultured with cycloheximide. Cells were treated with PMA and at various times thereafter were lysed in Nonidet P-40. Soluble and insoluble proteins were separated by centrifugation. As shown in Table 2, PMA induced a 50% decrease in the amount of detergent-insoluble lamin and a concomitant 33% increase in the amount of soluble lamin. Although there was an unaccountable overall loss of 30–40% of the total lamin, perhaps due to proteolysis, it appears from these and similar experiments with WEHI-231 cells (data not shown) that the phosphorylation of lamin B caused by PMA in intact interphase cells correlates with its partial loss from the nuclear matrix.

Table 1. Anti-IgM stimulates lamin B phosphorylation in WEHI-231

Group	Treatment	Relative phosphorylation Exp. 1	Exp. 2
1	—	1.0	1.0
2	Anti-IgM	1.6	—
3	Anti-IgM dextran	2.1	1.8
4	Anti-Ia	0.9	—
5	PMA	19.7	—

WEHI-231 cells were loaded with $^{32}P_i$ and then treated with goat anti-IgM (5 μg/ml), anti-IgM dextran (5 μg/ml), anti-Ia (M5/114 at 10 μg/ml) (25), or PMA. After 30 min, cells were lysed and lamin B was immunoprecipitated. Relative phosphorylation was determined densitometrically.

DISCUSSION

The present study demonstrates that lamin B can be hyperphosphorylated in response to ligands that activate PKC. The difference in the kinetics of phosphorylation between the nuclear lamina and the plasma-membrane proteins in the PMA response may reflect different mechanisms of phosphorylation in these two cellular compartments. PKC might directly phosphorylate both plasma membrane and nuclear proteins. Membrane-bound activators (PMA or diacylglycerol) are thought to induce the translocation of PKC from the cytosol to the membranes. During this phase of PKC activation, cytosolic and plasma membrane-associated proteins are phosphorylated. Subsequently, membrane-bound PKC may be cleaved to a soluble low molecular weight form of PKC (PKM) that no longer requires Ca^{2+} or phosphatidylserine for activation (23). PKM could then move into the nucleus and phosphorylate lamin B and other nuclear targets. The 4- to 5-min delay in the nuclear phosphorylation events relative to those at the plasma membrane might therefore reflect the time necessary for the activation, cleavage, and transport of PKM from the cell periphery to the nucleus. Alternatively, the activating ligand (PMA or diacylglycerol) could move into the nucleus via membrane fusion events, diffusion, or by specific association with complexes that might move between plasma membrane and nucleus. PKC could then be bound and activated in the nucleus. Here, the delay in the nuclear phosphorylation would reflect the time necessary for PMA to reach the nucleus. The lack of phorbol ester localization in the nucleus

Table 2. PMA alters solubility of lamin B in splenocytes

	Time, min 0	20	40	450
Insoluble	88	53	41	47
Soluble	12	16	15	16

Percentage of lamin B in Nonidet P-40-insoluble and -soluble forms at various times after addition of PMA to cycloheximide-treated dense splenocytes. Lamin B content was estimated from densitometry of immunoblots.

(3, 4) argues against this mechanism. A third possibility is that PKC activated in the periphery initiates a cascade of events leading to the activation of a nuclear protein kinase with a substrate specificity similar to PKC. In this case, the delay in the nuclear phosphorylation reflects the time necessary for the secondary signals to affect their nuclear targets. Previous reports that the *in vitro* phosphorylation of nuclear enzymes including DNA polymerase α (27) and topoisomerase II (28) affects their catalytic properties, coupled with the present demonstration that phorbol ester stimulates PKC-type phosphorylation of nuclear proteins *in vivo*, suggest that PKC-like phosphorylation of nuclear enzymes and structural proteins may play an important role in ligand-induced regulatory pathways. The finding that phorbol ester stimulates the phosphorylation of nuclear lamin, together with the observations that it induces the rapid transcription of specific sets of genes (7–10) and that the nuclear lamina may interact with interphase chromatin to regulate higher-order chromatin structure (11), suggests that the regulated phosphorylation of lamin B may influence interphase transcriptional activity. In addition, it is likely that the hyperphosphorylation of lamin that occurs during signal transduction in interphase cells plays a substantially different role than that which precedes nuclear dissolution during mitosis.

We would like to thank Dr. Larry Gerace (The Johns Hopkins University School of Medicine) for graciously providing antisera and purified antibodies to lamins A, B, and C; Dr. John Inman (National Institute of Allergy and Infectious Diseases, Laboratory of Immunology) for preparing conjugates of anti-IgM and dextran T-2000; Dr. Claude Klee (National Cancer Institute) for providing CaM-PK-II; Dr. Rose Mage (National Institute of Allergy and Infectious Diseases, Laboratory of Immunology) for her critical reading of the manuscript; and Ms. Cynthia Watson for excellent assistance.

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