A major mechanism by which protein kinase C (PKC) function is regulated is through the selective targeting and activation of individual PKC isoforms at distinct subcellular locations. PKC $\beta_{II}$ is selectively activated at the nucleus during G2 phase of cell cycle where it is required for entry into mitosis. Selective nuclear activation of PKC $\beta_{II}$ is conferred by molecular determinants within the carboxyl-terminal catalytic domain of the kinase (Walker, S. D., Murray, N. R., Burns, D. J., and Fields, A. P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 9156–9160). We previously described a lipid-like PKC activator in nuclear membranes, termed nuclear membrane activation factor (NMAF), that potently stimulates PKC $\beta_{II}$ activity through interactions involving this domain (Murray, N. R., Burns, D. J., and Fields, A. P. (1994) *J. Biol. Chem.* 269, 21385–21390). We have now identified NMAF as phosphatidylglycerol (PG), based on several lines of evidence. First, NMAF cofractionates with PG as a single peak of activity through multiple chromatographic separations and exhibits phospholipase sensitivity identical to that of PG. Second, purified PG, but not other phospholipids, exhibits dose-dependent NMAF activity. Third, defined molecular species of PG exhibit different abilities to stimulate PKC $\beta_{II}$ activity. 1,2-Dioleoyl-PG possesses significantly higher activity than other PG species, suggesting that both fatty acid side chain composition and the glycerol head group are important determinants for activity. Fourth, in vitro binding studies demonstrate that PG binds to the carboxyl-terminal region of PKC $\beta_{II}$, the same region we previously implicated in NMAF-mediated activation of PKC $\beta_{II}$. Taken together, our results indicate that specific molecular species of nuclear PG function to physiologically regulate PKC $\beta_{II}$ activity at the nucleus.

Protein kinase C (PKC) is a family of serine/threonine kinases involved in the transmission of a wide variety of extracellular signals (1, 2). The individual PKC family members are classified according to their cofactor requirements (3, 4). Classical, or calcium-dependent PKC isoforms require calcium, diacylglycerol (DAG), and phosphatidylserine (PS) for activation. The novel PKCs require DAG and PS, but not calcium, whereas the atypical PKCs do not require DAG or calcium, but appear to require PS for activation. PKC isoforms exhibit tissue- and cell-type-specific patterns of expression, suggesting specialization of function. Indeed, accumulating evidence indicates that PKC isoforms serve distinct, nonoverlapping functions in cellular physiology (reviewed in Refs. 1, 3, and 5).

An important mechanism by which PKC function is regulated is through the targeting of PKC isoforms to distinct subcellular locations (1, 5). In human leukemia cells, which express PKC $\alpha$, $\beta_{II}$, and $\xi$, we have found that PKC $\beta_{II}$ is selectively activated at the nucleus during the G2 phase of cell cycle (6, 7). At the nucleus, PKC $\beta_{II}$ directly phosphorylates the nuclear envelope poly peptide lamin B at sites involved in mitotic nuclear lamina disassembly (8–10). Inhibition of nuclear PKC $\beta_{II}$ activity leads to cell cycle arrest in G2 phase, demonstrating the importance of nuclear PKC $\beta_{II}$ activation in the entry of cells into mitosis (7). In contrast, PKC $\alpha$ and PKC $\xi$ are not observed at the nucleus, and we have demonstrated that they play key roles in leukemia cell differentiation and survival/apoptosis, respectively (11, 12).

Given its involvement in cell cycle progression, we investigated the mechanisms underlying the selective nuclear translocation and activation of PKC $\beta_{II}$. Using chimeric PKC molecules, produced by exchanging the regulatory and catalytic domains of PKC $\alpha$ and $\beta_{II}$, we determined that the catalytic domain of PKC $\beta_{II}$ contains molecular determinants that are important for selective nuclear targeting of the enzyme (13). In related biochemical studies, we examined the mechanism by which PKC $\beta_{II}$ is activated at the nucleus (14). We found that component(s) within the nuclear membrane selectively stimulate PKC $\beta_{II}$ activity 3–6-fold above the level achieved in the presence of optimal concentrations of calcium, DAG, and PS (14). This nuclear membrane activation factor (NMAF) was shown to be soluble in nonionic detergents and organic solvents, and to be insensitive to protease treatment, suggesting that it is a lipid (14). In the present study, we identify NMAF as phosphatidylglycerol (PG) based on the fractionation profile of NMAF and the ability of purified PG to activate PKC $\beta_{II}$. Interestingly, individual purified PG species vary in their ability to activate PKC $\beta_{II}$, suggesting that the nuclear membrane contains specific PG species that serve to potently stimulate PKC $\beta_{II}$ activity. Finally, we demonstrate that PG binds to the carboxyl-terminal region of PKC $\beta_{II}$, consistent with the role of this region in nuclear activation of PKC $\beta_{II}$ (14). Our data indicate that nuclear membrane PG is an important physiologic regulator of PKC activity at the nucleus.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Isolation of Nuclear Membrane Extracts—Human promyelocytic (HL60) leukemia cells were maintained in suspension...*
Lipid Vesicle Binding Assay—A fusion protein between glutathione S-transferase (GST) and the carboxyl terminus of PKC \( \beta_t \) (amino acids 576–673; GST-PKC \( \beta_t \) CT) was generated by polymerase chain reaction of the carboxyl-terminal fragment of the human PKC \( \beta_t \) cDNA using the following primers: forward 5'-CGGGATCCCACTGATGAC-3' and reverse 5'-CCCTCGAGGATTAGCTCTTGACT-3'. These primers allowed introduction of a 5'-BanHI and a 3'-XhoI restriction site to facilitate directional cloning of the polymerase chain reaction product into the pGEX-5X-3 expression vector (Amersham Pharmacia Biotech). GST fusion protein was expressed in E. coli. Specificity of the assay was confirmed using other purified phospholipids. All assays were carried out for 15 min at room temperature, conditions under which [\( ^{32}P \)]phosphate incorporation is linear (14). Unless otherwise stated, nuclear membrane extracts from 10^7 cell equivalents were assayed in the standard vesicle assay and a mixed micelle assay (22), indicating that NMAF activity is not the result of an artifact inherent to the assay system (data not shown). These data, along with our previous observation that NMAF activity is resistant to exhaustive protease treatment (14), provide convincing evidence that NMAF is lipid and not protein.

Thin Layer Chromatographic Fractionation of NMAF—In order to characterize the lipid component(s) of nuclear membrane extract responsible for NMAF activity, nuclear membrane extracts were resolved into major lipid classes by TLC. TLC plates were spotted with nuclear membrane extract in chloroform and developed using a solvent system that allows resolution of the major phospholipid classes. The developed TLC plates were then divided into regions based on the migration of lipid standards, scraped, and eluted as described under “Experimental Procedures.” Individual TLC fractions were then assayed for NMAF by measuring the ability to stimulate PKC \( \beta_t \) activity in both our standard vesicle assay and a mixed micelle assay (22), indicating that NMAF activity is not the result of an artifact inherent to the assay system (data not shown). These data, along with our previous observation that NMAF activity is resistant to exhaustive protease treatment (14), provide convincing evidence that NMAF is lipid and not protein.

RESULTS

Initial Characterization of Nuclear Membrane Activation Factor—Based on our initial observation that NMAF activity is solubilized from nuclear envelopes by either nonionic detergent or organic solvent extraction, we hypothesized that NMAF may be a lipid (14). Further characterization demonstrated that NMAF activity fractionated completely into the organic phase of a two-phase (Folch) extraction and that NMAF activity is largely resistant to heat inactivation by boiling for 5 min, supporting the hypothesis that NMAF is lipid and not protein. In addition, NMAF exhibits the same stimulatory effect on PKC \( \beta_t \) activity by both our standard vesicle assay and a mixed micelle assay (22), indicating that NMAF activity is not the result of an artifact inherent to the assay system (data not shown). These data, along with our previous observation that NMAF activity is resistant to exhaustive protease treatment (14), provide convincing evidence that NMAF is lipid and not protein.
Phosphatidylglycerol Activates Nuclear PKC

NMAF activates PKC \( \beta_1 \) in the presence of maximal concentrations of these cofactors (14). Substantiating this finding, PS and DAG are clearly resolved from NMAF activity after TLC separation (Fig. 1). The migration of NMAF on TLC plates suggested that NMAF might correspond to one of the three phospholipids PG, PC, or PE. Therefore, we directly determined the ability of these phospholipids to activate PKC \( \beta_1 \) (Fig. 2). As can be seen, PG was able to stimulate PKC \( \beta_1 \) activity in a dose-dependent fashion above the level induced by the conventional PKC activators, DAG, PS, and calcium. In contrast, PC and PE showed little or no stimulatory activity at concentrations up to 250 \( \mu \)g/ml, demonstrating that the NMAF-like activity exhibited by phospholipid addition is specific for PG.

If PG were NMAF, one would predict that NMAF and purified PG would exhibit the same sensitivity to phospholipases. Therefore, we assessed the effect of PI-PLC, PC-PLC, and PLA2 on the ability of NMAF and purified PG to stimulate PKC \( \beta_1 \) activity (Fig. 3). Treatment of NMAF or purified PG with either PI-PLC or PC-PLC had little effect on their PKC stimulatory activity. In contrast, treatment with PLA2 led to substantial inhibition of both NMAF and PG activity. These data demonstrate that NMAF and PG exhibit a similar pattern of sensitivity to phospholipases, consistent with the suggestion that NMAF is PG. These data also provide direct confirmation that NMAF is distinct from PC and PI and that it is not a lysophospholipid.

**PG Is Present in Nuclear Membrane Extracts and Cofractionates with NMAF Activity**—Given the ability of PG to stimulate PKC activity, we next assessed whether PG is present in nuclear membrane extracts and whether endogenous PG cofractionates with NMAF activity. For this purpose, we subjected nuclear membrane extracts to fractionation by silica column chromatography (Fig. 4A). Samples were loaded onto silica columns in chloroform, and lipid constituents were eluted into solvents of increasing polarity. Individual fractions were collected and assayed for the ability to stimulate PKC activity (Fig. 4A). NMAF activity was retained on the column and eluted as a single peak in 1:1 chloroform/methanol (CHCl3/CH3OH). Purified PG standard also eluted as a single peak in 1:1 CHCl3/CH3OH, further suggesting that PG corresponds to NMAF (data not shown). Therefore, we directly assessed the levels of PG in the silica column fractions from nuclear membrane extracts using a specific enzymatic assay for PG mass (20). Nuclear membrane PG elutes specifically in 1:1 CHCl3/CH3OH along with NMAF activity (Fig. 4B). From the quantitative PG assay, we calculate that nuclear membranes contain \( -0.39 \) \( \mu \)g of PG/10\(^7\) cells. This level of PG corresponds to a concentration of \( -10 \) \( \mu \)g/ml in our standard assay. These data confirm the presence of PG in the nuclear membrane and demonstrate that nuclear PG comigrates with NMAF activity.

**Individual Species of PG Differ in Ability to Activate PKC \( \beta_2 \)**—Having identified NMAF as PG, we wished to determine whether the fatty acid side chain composition of individual PG

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**Fig. 1**. Fractionation of NMAF by thin layer chromatography. Nuclear membrane extracts were prepared from HL60 cell nuclear envelopes as described previously (14). Extracts in chloroform were spotted onto Silica Gel 60 plates and resolved by one-dimensional TLC in chloroform/methanol/water (65:25:4, v/v/v). The migration of purified phospholipid standards was determined as described under "Experimental Procedures," and the positions of standards are depicted in the schematic diagram. The TLC plate was divided into eight fractions as indicated, based on the migration of the lipid standards and the silica scraped from each fraction and recovered. Lipids in each fraction were eluted with chloroform/methanol (3:1), dried under N2 gas, resuspended in aqueous buffer, and assayed for activation of PKC in the standard histone kinase assay under conditions supporting maximal PKC activity (100 \( \mu \)M Ca\(^{2+}\), 20 \( \mu \)M diacylglycerol, 40 \( \mu \)g/ml phosphatidylserine). Results are plotted as -fold activation relative to control (no lipid extract added) for each fraction and compared with the -fold activation obtained with unfractionated NMAF (top bar). Results represent the mean of three independent determinations ± SD. LPC, lysophosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol.

**Fig. 2**. Purified phosphatidylglycerol exhibits PKC activation properties similar to NMAF. The ability of purified phospholipids to activate PKC \( \beta_1 \) was assessed. Kinase assays were conducted under the conditions described in the legend to Fig. 1 in either the absence or presence of the indicated amount (0.25–250 \( \mu \)g/ml) of purified phospholipid. Results are expressed as -fold activation of kinase activity in the presence of purified phospholipid. PG, ○; PC, ×; and PE, □. Results represent the mean of three independent determinations ± S.D.

**Fig. 3**. NMAF and PG exhibit similar sensitivities to phospholipases. Purified PG (open bars) and nuclear membrane extract (hatched bars) were either assayed directly (control) or treated with either PI-PLC (+ PI-PLC), PC-PLC (+ PC-PLC) or PLA2 (+ PLA2) for 30 min. Phospholipids were then extracted and assayed for NMAF activity as described under "Experimental Procedures." Results are plotted as percent activity remaining after treatment and represent the mean of three independent determinations ± S.D.

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species is important for PKC stimulatory activity. Therefore, we assessed the ability of individual purified molecular species of PG to activate PKC βII (Fig. 5). The ability of various species of PG to activate PKC βII was assessed. Kinase assays were conducted under the conditions described in the legend to Fig. 1 in the presence of purified PG species. Results are expressed as fold activation of kinase activity in the presence of purified PG species. Results represent the mean of three independent determinations ± S.D. The major PG species in the original PG mixture, oleic acid and palmitic acid, were compared for NMAF activity. C, PG mixture; ●, 1,2-dioleoyl-PG; ○, 1,2-dipalmitoyl-PG; and □, 1-palmitoyl-2-oleoyl-PG. Activity is stereospecific for dioleoyl (C18:1 Δ9 cis)-PG. C18 PG species were compared for NMAF activity. ○, original PG mixture; ●, 1,2-dioleoyl (C18:1 Δ9 cis)-PG; □, 1,2-dielaidoyl (C18:1 Δ9 trans)-PG; and ×, 1,2-distearoyl (C18:0)-PG.

We next wished to directly compare the ability of nuclear membrane-derived PG and purified PG to stimulate PKC βII activity (Fig. 6). Nuclear PG was isolated by silica column chromatography as described above and assayed for activity along with purified 1,2-dioleoyl-PG and a mixture of PG species. Nuclear membrane-derived PG and purified 1,2-dioleoyl-PG exhibit comparable activity that is more potent than that exhibited by the PG mixture. From these data, an apparent

**FIG. 5. Different molecular species of PG differ in ability to activate PKC βII.** The activity of various species of PG to activate PKC βII was assessed. Kinase assays were conducted under the conditions described in the legend to Fig. 1 in the absence or presence of the indicated amount (2.5–250 μg/ml) of purified PG species. Results are expressed as fold activation of kinase activity in the presence of purified PG species. Results represent the mean of three independent determinations ± S.D. A, the major PG species in the original PG mixture differ in activity. The activity of PG species containing the major fatty acid side chain constituents represented in the original mixture, oleic acid and palmitic acid, were compared for NMAF activity. C, PG mixture; ●, 1,2-dioleoyl-PG; ○, 1,2-dipalmitoyl-PG; and □, 1-palmitoyl-2-oleoyl-PG.
EC₅₀ was estimated for each PG source. The apparent EC₅₀ for the purified PG mixture was ~90 μg/ml. In contrast, both 1,2-dioleoyl-PG and nuclear membrane PG were more potent than the PG mixture, with apparent EC₅₀ values of ~10 μg/ml. Therefore PG, possibly 1,2-dioleoyl-PG, exhibits sufficient activity to account for the activity we previously identified as NMAF.

The Carboxyl-terminal Region of PKC βII Selectively Binds PG—Our previous results demonstrated that NMAF-mediated activation of PKC βII is isozyme selective and suggested that the carboxyl-terminal region of PKC βII is important for this activation (14). Therefore we devised a lipid vesicle binding assay to directly assess whether the carboxyl-terminal region of PKC βII binds to PG. In this assay, PG vesicles were incubated with a GST fusion protein containing the carboxyl terminus of PKC βII (GST-PKC βII CT). Vesicle-bound protein was separated from unbound protein by centrifugation through a 100-kDa cutoff filter and the bound fraction analyzed for the presence of the GST fusion protein (Fig. 7). Under these conditions, GST-βII CT bound to PG-containing vesicles (Fig. 7A). Binding was selective for GST-PKC βII CT, since little or no binding of GST to PG-containing vesicles was detected (Fig. 7B). Likewise, binding of GST-PKC βII CT was selective for PG, since PC vesicles did not bind GST-PKC βII CT (Fig. 7A, lane 3). Binding to PG vesicles was dependent on the PG content of mixed vesicles containing different proportions of PG and PC, indicating that binding is both concentration-dependent and saturable (Fig. 7C). The presence of PS and DAG did not influence binding to PG (data not shown), consistent with the fact that PS and DAG bind to the C2 region within the regulatory domain of PKC βII, which is not present in the GST-PKC βII CT fusion protein. In conclusion, our data demonstrate that PG binds selectively to the carboxyl-terminal region of PKC βII. These data are consistent with our finding that this region of PKC βII contains important determinants involved in the nuclear translocation and activation of PKC βII (13, 14).

DISCUSSION

Protein kinase C function is regulated by multiple mechanisms, including the tissue- and cell type-specific expression of individual PKC isotypes. In addition, PKC isotypes exhibit intrinsic differences in cofactor requirements and sensitivities to lipid second messengers. Appropriate intracellular targeting of PKC also appears to be critical for proper PKC isotype function in vivo. Intracellular targeting can be achieved through specific interactions of PKC with a growing family of docking proteins. For instance, members of the protein kinase A anchoring protein family appear to serve a role in PKC-mediated signaling at the postsynaptic density (23). Protein kinase A anchoring proteins target PKC and other signaling molecules to the same intracellular compartment through simultaneous binding to distinct binding sites on the protein kinase A anchoring protein (23). Still other PKC-binding proteins serve as receptors for activated protein kinase C and play functional roles in PKC targeting and translocation events (24, 25). It will be of interest to determine whether similar mechanisms aid in the targeting of PKC βII to the nucleus of human leukemia cells. In this paper, we provide direct evidence that specific lipid components within the nuclear membrane play a key role in PKC signaling by stimulating PKC activity at the nucleus.

In previous studies, we demonstrated that PKC βII is selectively translocated to the nucleus of human leukemia cells in response to proliferative stimuli (1, 7–9). Nuclear PKC βII translocation and activation is cell cycle-regulated, occurring during the G₂ phase of cell cycle (6, 7). At the nucleus, PKC βII phosphorylates sites on the nuclear envelope polypeptide lamin B that are involved in the process of mitotic nuclear lamina disassembly (6, 7, 9). Inhibition of nuclear PKC activity leads to cell cycle arrest in G₂ phase prior to mitosis, demonstrating...
that nuclear PKC activity is required for cell cycle progression through the G2/M phase transition (7). Using PKC chimera, we demonstrated that nuclear translocation of PKC \( \beta_{II} \) is dependent upon the carboxyl-terminal catalytic domain of the enzyme (13). At the nucleus, PKC \( \beta_{II} \) activity is stimulated by a component within the nuclear membrane, termed NMAF, that serves to potently activate the enzyme (14). An active component of NMAF has now been identified.

Identification of NMAF as Phosphatidylglycerol—Based on several lines of evidence, we have shown that NMAF corresponds to PG. First, NMAF is a heat-stable, lipophilic activity that comigrates with PG on thin layer and silica column chromatographies. Second, purified PG, but not other phospholipids, exhibits NMAF-like activity. Third, NMAF and PG exhibit similar sensitivities to phospholipases. Fourth, PG is present in the nuclear membrane in sufficient quantities to stimulate PKC \( \beta_{II} \) activity. Fifth, specific PG species exhibit different activities indicating that the selectivity of NMAF activity lies not only in the glycerol head group, but also in the fatty acid side chain constituents. 1,2-Dioleoyl PG was found to be significantly more potent at stimulating PKC \( \beta_{II} \) activity than the other PG species tested. The selectivity for oleic acid is stereospecific since 1,2-dioleoyl-16:0-PC, which is identical to 1,2-dioleoyl PG except for the orientation of the \( \Delta 9 \) double bond in the C18:1 fatty acid chain, exhibits activity that is about one log lower than 1,2-dioleoyl-PG. These results argue against the possibility that PG causes a nonspecific membrane effect, such as a change in membrane charge density, leading to PKC activation. Whereas it is possible that the nuclear membrane contains other lipid components that contribute to NMAF activity, we provide convincing evidence that PG has sufficient activity and is present in appropriate quantities to account for NMAF activity.

Previous studies indicated that PG-mediated activation of PKC \( \beta_{II} \) might involve the carboxyl-terminal region of the catalytic domain of PKC \( \beta_{II} \) (14). Our lipid vesicle binding studies provide direct evidence in support of this conclusion. Specifically, we demonstrate that the carboxyl terminus of PKC \( \beta_{II} \) binds selectively to PG-containing vesicles. These results are interesting in light of our previous demonstration that the carboxyl terminus of PKC \( \beta_{II} \) is also important for the nuclear translocation and activation of PKC \( \beta_{II} \) (13). Taken together, these results suggest that nuclear PG functions to modulate nuclear PKC \( \beta_{II} \) translocation and activation. In a recent study, we determined that the cell cycle-dependent activation of PKC \( \beta_{II} \) in the nucleus during G2 phase is coupled to the generation of nuclear DAG through the action of a nuclear PI-PLC activity (26). We have determined nuclear PG levels during cell cycle progression and find that they do not change appreciably during cell cycle.2 It is possible therefore that nuclear PG functions primarily to facilitate or enhance the selectivity of NMAF activity during G2 phase. Further studies will be aimed at elucidating the mechanism by which nuclear PG stimulates PKC \( \beta_{II} \) activity, the fatty acid side chain composition and activation has been investigated. In the presence of calcium and DAG, PKC exhibits an identical sigmoidal dependence on PS for membrane binding and activation (27). DAG increases the affinity of PKC for PS, but not for other acidic phospholipids (28). Though PE and PG can reduce the amount of PS required for maximal binding and activity, they cannot replace the requirement for PS (28). Based on these studies, it has been suggested that PKC exhibits a dual requirement for acidic phospholipid, a specific requirement for PS binding and nonspecific electrostatic interactions with other acidic phospholipids (28). Our data also indicate that PG, in addition to PS, can influence PKC activity. However, our data suggest that, like the specific requirement for PS, the interactions involving PG are specific. Furthermore, our binding studies demonstrate that PG binds to the carboxyl-terminal region of PKC \( \beta_{II} \), a site distinct from the binding site for PS in the regulatory domain. In addition, our data indicate that specific PG species can differentially influence PKC activity, indicating that specific lipid-PKC interactions underlie the stimulatory effects of PG.

Phosphatidylglycerol Is a Physiologic Activator of Nuclear PKC \( \beta_{II} \)—The effects of phospholipid composition and membrane fluidity on PKC activity have been assessed by selective enrichment of rat liver membranes with various phospholipids (29). Addition of PG, PS, PE, or dioleoyl-PC to membrane can lead to enhanced activation of PKC, with PG being the most effective activator (29). Our data are consistent with these findings and indicate that nuclear membrane PG is a physiologically relevant regulator of PKC activity. However, the exact mechanism by which PG stimulates PKC \( \beta_{II} \) activity remains to be fully elucidated. One potential mechanism stems from the observation that PKC interacts well with acidic phospholipids (28, 30). Bazzi and Nelsestuen (30) demonstrated that calcium-dependent binding of PKC to phospholipid vesicles induces clustering of acidic phospholipids including PG. They suggested that PKC may induce certain acidic phospholipids to form microdomains within physiologic membranes. Therefore, the presence of specific phospholipids, such as certain PG species, may influence PKC activity by forming clustered subdomains that serve to enhance the membrane binding and catalytic activity of PKC. Likewise this clustering may influence substrate selection through enhanced interactions of PKC and substrate at the membrane surface. Future studies will focus on determining the mechanisms by which nuclear PG stimulates PKC \( \beta_{II} \) activity, the fatty acid side chain composition and PKC stimulatory activity of individual nuclear PG species, and the potential involvement of nuclear PG in the temporal and spatial regulation of nuclear PKC \( \beta_{II} \) activity.

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