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Characterization of Cellular DGK- θ

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Introduction

Diacylglycerol kinases (DGKs, EC 2.7.1.107) regulate a number of signaling pathways by modulating two important signaling lipids: diacylglycerol (DAG) and phosphatidic acid (PtdOH). These enzymes transfer the γ phosphate of ATP to DAG to generate PtdOH, simultaneously reducing DAG levels and increasing PtdOH levels. Reduction of the DGK substrate, DAG, attenuates pathways that involve DAG-dependent components such as PKC (Nishizuka, Y., 1992), a nuclear PIP kinase (EC 2.7.1.68), Ras (ras), and (Munc13) (Kazanietz, M. G., 2000). Similarly, the DGK product, PtdOH, is an activator of other signaling enzymes such as Raf-1 kinase, PKC- ζ , human protein phosphatase-1 (EC 3.1.3.48), and the protein tyrosine-phosphatase PTP1C (EC 3.1.3.48) (Zhao, Z., Shen, S. H., and Fischer, E. H., 1993; Limatola, C., Schaap, D., Moolenaar, W. H., and van Blitterswijk, W. J., 1994; Tomic, S., Greiser, U., Lammers, R., Kharitonov, A., Imyanov, E., Ullrich, A. et al., 1995; Ghosh, S., Strum, J. C., Sciorra, V. A., Daniel, L., and Bell, R. M., 1996; Rizzo, M. A., Shome, K., Watkins, S. C., and Romero, G., 2000; Jones, J. A. and Hannun, Y. A., 2002). It is becoming increasingly clear that by their ability to reciprocally regulate the cellular levels of two critical lipids, these enzymes are exquisitely poised to coordinately regulate a variety of signaling pathways.

While the realization that DGKs play important signaling roles has grown, there is little data pertaining to their regulation. We have focused some studies on understanding the regulation of one DGK, DGK- θ . Like many other DGKs, this enzyme is regulated in part by sub-cellular redistribution (reviewed in (Wattenberg, B. W., Pitson, S. M., and Raben, D. M., 2006). When quiescent embryonic fibroblasts are treated with α -thrombin, a known mitogen for these cells and other fibroblasts (Wright, T. M., Rangan, L. A., Shin, H. S., and Raben, D. M., 1988; Coughlin, S. R., 2000), DGK- θ redistributes to the nucleus (Bregoli, L., Baldassare, J. J., and Raben, D. M., 2001; Bregoli, L., Tu-Sekine, B., and Raben, D. M., 2002). Similar results have been observed with neuronal cells (Tabellini, G., Bortul, R., Santi, S., Riccio, M., Baldini, G., Cappellini, A. et al., 2003; Tabellini, G., Billi, A. M., Fala, F., Cappellini, A., Evagelisti, C., Manzoli, L. et al., 2004). DGK- θ , like many other DGKs, is also regulated by two phospholipids: phosphatidylserine (PtdSer) and phosphatidic acid (PtdOH) (Tu-Sekine, B., Ostroski, M., and Raben, D. M., 2006; Tu-Sekine, B., Ostroski, M., and Raben, D. M., 2007; Tu-Sekine, B. and Raben, D. M., 2009). There is also evidence this isoform is regulated by products of a PI 3-kinase (EC 2.7.1.153) (Walker, A. J., Draeger, A., Houssa, B., van Blitterswijk, W. J., Ohanian, V., and Ohanian, J., 2001). In addition to

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regulation by these lipids, the small GTPase RhoA has been shown to bind to and inhibit this enzyme (Houssa, B., de Widt, J., Kranenburg, O., Moolenaar, W. H., and van Blitterswijk, W. J., 1999). Here we summarize the data on the regulation of DGK- θ by PtdSer and PtdOH, and introduce data on an apparent perturbation of PtdSer-based lipid biosynthesis resulting from DGK- θ expression in IIC9 fibroblasts. We also report that a Class I PI 3-kinase activity is involved in regulating nuclear DGK- θ activity in embryonic fibroblasts and that this regulation appears to be indirect through a PI 3-kinase-mediate regulation of nuclear RhoA localization.

Materials and methods

Materials

Reagents—Silica gel 60 TLC plates (aluminum sheets) were purchased from EM Science (Germany). Cytoscient scintillation-counting fluid was obtained from ICN (Costa Mesa CA). Tissue culture media components were purchased from MediaTech, Inc. (Herndon VA). Plastic culture dishes were purchased from Falcon Labware. Highly purified human thrombin (~4000 NIH units/ml) and BSA (RIA grade, fraction V) were purchased from Sigma (St. Louis, MO). β -octylglucoside was from Calbiochem (Santa Cruz, CA). All lipids were purchased from Avanti. Novafector was purchased from Venn Nova (Pompano Beach, FL). Other chemicals were of reagent grade.

Embryonic Fibroblasts and Cell Culture—IIC9 embryonic fibroblasts, a subclone of Chinese hamster embryo fibroblasts (CHEF19, ATCC), were grown, maintained and serum starved as previously described (Wright, T. M., Rangan, L. A., Shin, H. S., and Raben, D. M., 1988). Cultures were grown and maintained in Minimal Essential Medium- α (α MEM)/Ham's F12 medium (1:1, v/v) containing 7.5% (v/v) fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM-L-glutamine. Subconfluent (60-70% confluent) cultures were serum-deprived by washing with low-glucose Dulbecco's modified Eagle's medium (D-MEM) and incubated in fresh D-MEM containing 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM-L-glutamine for 48 hrs. The cultures were then incubated in fresh low-glucose D-MEM in the presence or absence of α -thrombin (1.5 NIH units/ml) for the indicated times.

Transient transfections of DGK- θ —IIC9 embryonic fibroblasts were grown in 175 cm² flasks until 50-60% confluent. Cultures were washed once with PBS and once with OPTIMEM I (reduced serum medium of Eagles's MEM, Life Technologies), then incubated with 10 ml OPTIMEM I containing 20 μ g of the cDNA-containing vector in 100 μ l of Novafector. Following an overnight incubation, 10 ml of 15% FCS-containing complete medium with 2X penicillin, streptomycin and L-glutamine was added, and cells were grown for an additional day before being subjected to serum deprivation. Transfection efficiency was evaluated by parallel transfections with 20 μ g pEGFP-N3 vector (Clontech).

DGK- θ Construct—hDGK θ in pcDNA3 was kindly provided by Dr. W. J. van Blitterswijk (Division of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam).

Preparation of Cellular Fractions—Total homogenates, cytoplasmic fractions and nuclear samples were prepared as follows: Cells were washed with ice-cold fractionation buffer (10 mM Hepes, pH 7.5, 1mM EDTA, 0.5mM EGTA; FB), and incubated in cold FB supplemented with 10mM NaF, 20uM quinacrine, 200uM NDC and 1X Roche Complete Protease Inhibitor cocktail, and allowed to swell on ice for 5-10 minutes. Cells were removed from flasks by scraping, and the resulting suspension was dounced 10-15 times in a

glass homogenizer (Kontes, Vineland NJ) to release the nuclei. Nuclear suspensions were centrifuged at $700 \times g$ in a RT6000B refrigerated Sorvall centrifuge for 15 min to pellet nuclei and unbroken cells. The cytosol was poured off and saved, and pellets were resuspended in 0.5 ml FB plus inhibitors. The nuclear pellet was homogenized on ice in a 2-ml Dounce homogenizer with a type B pestle for 7 passes. The nuclei-containing suspension from 1-2 150 mm plates was layered onto a 3 ml 30% (w/v) sucrose cushion and centrifuged at $8,000 \times g$ at 4°C for 30 min. The purified nuclei were resuspended in FB and used immediately or stored at -80°C . Protein concentrations of all samples were determined using the BCA Protein Assay kit (Pierce) or the BioRad Protein Assay (Bio-Rad). The purity of nuclear preparations was assessed by immunoblotting for α -tubulin with a monoclonal antibody (Zymed). Membranes were prepared by ultracentrifugation of the post-nuclear supernatant at $100,000 \times g$ for 1 hour. The membrane pellet was resuspended in 0.1% T $\times$ 100 by pipetting and brief sonication and stored at -80°C . The remaining cytosol was supplemented to 0.1% T $\times$ 100 to stabilize the protein and stored at -80°C .

Nuclear Envelope Removal—Purified nuclei were resuspended in 0.5 ml FB containing 0.05% T $\times$ 100 and incubated for 1 minute on ice. Nuclei were passaged 5X through a 25G syringe. The insoluble fraction was pelleted at 6,000 rpm in a benchtop microfuge for approximately 2-3 minutes. The resulting supernatant was designated the nuclear envelope. The pellet was washed 3X 1ml FB to remove residual detergent.

Immunofluorescence—Cells were grown on glass coverslips in 60 mm plates to 50-60% confluence prior to transfection with cDNAs. Transfected cells were serum starved for 2 days in DMEM as described above, then stimulated with thrombin for the times indicated. Cells were placed on ice, the medium was aspirated, and cells were washed with 10 ml ice cold PBS. Cells were immediately fixed in 5 ml MeOH:Acetone (1:1, v:v) for 20-30 min at -20°C . Fixation solution was removed and cells were washed with 5 ml PBS 2×5 min with gently agitation. Following washes cells were blocked 1 hour in 5% non-fat dry milk (NFDM) containing 1% FBS in PBS. Blocking reagent was removed and cells were incubated with primary antibody in a 100ul droplet of PBS containing 5% NFDM, 1% serum. Slides were covered gently with a square of parafilm to prevent evaporation, and incubated o/n at 4°C (PKC α M4 mAb, 1:50; PI3K pAb 1:25). Antibody was removed with 2×5 ml washes for 5 min each with gentle agitation. Secondary antibody was applied in the same manner as primary antibody. Plates were protected from light and incubated at 37°C for 30 min. Secondary was removed with 3×5 ml washes in PBS for 5 min. Residual PBS was carefully removed with a Kimwipe prior to mounting slides using Vectashield (Vector Laboratories, Burlingame CA).

Western Blot Analysis—Samples were resuspended in Laemmli sample buffer and separated by SDS-PAGE. Proteins were transferred to a PVDF membrane (Invitrogen) and blocked for 1 hr in Tris-buffered saline, pH 7.4, containing 0.1% (v/v) Tween 20 (TBST), supplemented with 3% BSA, RIA grade V (Sigma). Membranes were incubated with the indicated antibodies under the conditions described in the figures. The immunoreactive bands were visualized using peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (Zymed) and developed using SuperSignal West Pico detection reagent (Pierce).

DGK Enzymatic Assays—DGK activity was assayed in vitro using octylglucoside/diacylglycerol (OG/DG) mixed micelles. Lipids were dried under N_2 and micelles were formed using 50 mM Tris, 5.5 mM MgCl_2 , 10 mM NaF pH 8.2 (or as indicated in figure legends) by vortexing and sonicating until the suspension appeared clear. AT ^{32}P and DTT were added to the micelle mixture to a final concentration of 1mM ATP, specific activity 10uCi ^{32}P /100ul reaction, 1mM DTT. Protein (10 μl ; approx 10-30 μg) was added to the

micelle-containing reaction solution, and the reaction was initiated by brief shaking (for soluble fractions) or by vortexing and sonicating for 10-15 sec (for nuclear preps). Reactions were incubated at 25° C for 30 min, or at 37° C for 15 minutes, and terminated by the addition of chloroform/methanol/1% perchloric acid (1:2:0.75) (v:v). The organic phase was washed twice with 1% perchloric acid, dried under nitrogen gas, and spotted onto a silica gel 60 TLC plate. Phosphatidic acid (PA) was separated from other lipids using a solvent system containing chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1) (v:v). The amount of [γ ³²P]PA was measured by liquid scintillation spectrophotometry.

Phosphatidylinositol 3-Kinase Enzymatic Assay—Nuclei were extracted from quiescent cells or from cells stimulated with α -thrombin and purified as described above. 200 μ g purified nuclei were suspended in 80 μ l buffer C (20mM TrisHCl pH7.5, 0.25 M sucrose, 1mM EDTA, 1 $\times$ Roche complete protease inhibitors, 10mM NaF, 5 uM U73122, 0.5 mM dithiothreitol) and sonicated 3 $\times$ 3s in a bath sonicator. Samples were incubated with 20U DNaseI/200 μ g nuclei for 30 min at room temperature. The reaction was started by addition of 10 μ l reaction mix (10 mM ATP, 50 mM MgCl₂, 10uCi AT³²P in 50 mM Tris, 150 mM NaCl, pH 7.5). Reactions were incubated at 37° C for 90 min and terminated by the addition of chloroform/methanol/1% perchloric acid (1:2:0.75) (v:v). The organic phase was washed twice with 1% perchloric acid, the lower phase was removed, dried under nitrogen gas, and spotted onto a silica gel 60 TLC plate impregnated with 1% oxalic acid and allowed to run overnight using a solvent system containing MeOH:H₂O 2:3 (v:v), 1% potassium oxalate. The amount of [³²P]PIP₃ was measured by densitometry using a MultiImage Light Cabinet with AlphaImager software v5.5 (Alpha Innotech Corp, San Leandro CA) or phosphoimager, and by scintillation count.

Lipid Extraction—Lipids were extracted by the method of Bligh and Dyer with the following modifications: chloroform was supplemented with 50ug/ml β -hydroxy butyrate (BHT) to prevent oxidation of double bonds; the aqueous phase was 1M NaCl. Samples were incubated for at least 30 minutes or o/n at -20C before phase separation to facilitate complete solubilization of lipids. The organic phase was washed 3X with 1M NaCl before drying under N₂. Samples were stored for 1-3 days at -20C prior to shipment for MS analysis

Mass Spectroscopy—Electrospray ionization tandem mass spectroscopy (ESI MS/MS) was performed by the Kansas Lipidomics Research Center Analytical Laboratory.

Results and Discussion

Regulation of DGK- θ by Phosphatidylserine and Phosphatidic Acid

Phosphatidylserine (PtdSer) is known to modulate the activity of various DGKs. In a previous study (Tu-Sekine, B., Ostroski, M., and Raben, D. M., 2007), we examined the effects of various phospholipids on DGK- θ activity and substrate affinity. While our study confirmed the activating effect of PS on this enzyme, it also revealed that phosphatidic acid (PtdOH), the product of the DGK reaction, is a more effective activator than PtdSer. Further kinetic analysis showed that PtdOH decreased the apparent surface K_M ($K_{M(surf)}^{app}$) of DGK- θ for its DAG substrate (dioleoylglycerol (DOG)) and promoted binding to vesicles in a dose-dependent manner. A similar analysis of the PtdSer-mediated activation of DGK- θ indicated that PtdSer also decreases the $K_{M(surf)}^{app}$ of DGK- θ , but higher concentrations of PtdSer were required to achieve the same effect observed with PtdOH. In contrast to PtdOH, PtdSer promoted binding to vesicles only at levels that exceeded those required to saturate enzyme activity ((Tu-Sekine, B., Ostroski, M., and Raben, D. M., 2007) and see Figure 1). This suggests that PtdSer and PtdOH modulate DGK- θ activity via different mechanisms.

If PtdOH and PtdSer are physiologically relevant regulators of DGK- π , then these lipids would be expected to reside in the sub-cellular compartment in which DGK- π is localized in response to agonists. Previous studies demonstrated the nuclear translocation of DGK- θ in neuronal cells and embryonic fibroblasts (Bregoli, L., Baldassare, J. J., and Raben, D. M., 2001; Bregoli, L., Tu-Sekine, B., and Raben, D. M., 2002; Tabellini, G., Bortul, R., Santi, S., Riccio, M., Baldini, G., Cappellini, A. et al., 2003; Tabellini, G., Billi, A. M., Fala, F., Cappellini, A., Evagelisti, C., Manzoli, L. et al., 2004). We also showed that DGK θ is localized to the nuclear matrix of IIC9 fibroblasts (Bregoli, L., Tu-Sekine, B., and Raben, D. M., 2002). We therefore examined the levels of phospholipids present in the nuclear envelope, the nuclear matrix (defined here as the pellet remaining after detergent treatment of sucrose purified nuclei sufficient to remove the nuclear membrane as observed by electron microscopy), and non-nuclear membranes from quiescent embryonic fibroblasts. This analysis allowed us to determine the percent composition of the major phospholipids in these compartments. As shown in the overall distribution of phospholipids is similar in all three compartments. The most notable difference is a nearly 2-fold increase in PtdSer levels in the nuclear envelope relative to the nuclear matrix and the non-nuclear membranes. This may explain, in part, why DGK- θ is often found associated with the nucleus. Levels of PtdOH were not significantly impacted by DGK- θ expression, and no major differences in this lipid were detected between the various compartments tested. However, DGK- θ over-expression did have a striking effect on the PtdSer levels of the nuclear envelope, decreasing them by approximately 50% over the control sample. This decrease was accompanied by a drop in PtdEth levels by almost 30% in the same compartment. This is interesting in light of the fact that a majority of the PtdEth in many cultured cells is produced by decarboxylation of PtdSer (Vance, J. E., 2008). The precise significance of these differences in nuclear lipids, and their alterations in response to stimuli, remains to be determined and will require a better understanding of the sub-nuclear distribution of DGK- θ .

Consequences of Nuclear DGK- θ Activity: Regulation of Nuclear PKC- α

One of the challenges in DGK research is to identify the physiological roles of these enzymes. Indeed, the precise physiological role of DGK- θ has remained a mystery. One possibility is that nuclear DGK- θ may be involved in regulating the localization of DAG-responsive protein kinase Cs (PKCs) (EC 2.7.11.13) to the nucleus. Such PKCs have been shown to translocate to nuclei in response to agonists (Leach, K. L., Powers, E. A., Ruff, V. A., Jaken, S., and Kaufmann, S., 1989; Leach, K. L., Ruff, V. A., Wright, T. M., Pessin, M. S., and Raben, D. M., 1991; Leach, K. L., Ruff, V. A., Jarpe, M. B., Adams, L. D., Fabbro, D., and Raben, D. M., 1992; Leach, K. L. and Raben, D. M., 1993a; Leach, K. L. and Raben, D. M., 1993b; Murray, N. R., Burns, D. J., and Fields, A. P., 1994; Goss, V. L., Hocesvar, B. A., Thompson, L. J., Stratton, C. A., Burns, D. J., and Fields, A. P., 1994). DGK- θ , therefore, could regulate the localization of PKC- α by depleting nuclei of DAG.

To test this hypothesis, we examined the effect of suppressing nuclear DGK- θ on the levels of nuclear PKC- α . Previous data from our laboratory has shown that stimulation of quiescent embryonic fibroblasts leads to a transient increase in nuclear PKC- α (Leach, K. L., Ruff, V. A., Jarpe, M. B., Adams, L. D., Fabbro, D., and Raben, D. M., 1992; Leach, K. L. and Raben, D. M., 1993a; Leach, K. L. and Raben, D. M., 1993b). If DGK- θ modulates DAG levels to regulate nuclear PKC- α , then suppression of DGK- θ should lead to an increase or sustained increase in nuclear PKC- α . For these studies, embryonic fibroblasts were transfected with either RhoA or a DGK- θ that was rendered catalytically inactive by mutation of glycine 648 in the presumed ATP-binding region to alanine (G648A). RhoA was used as this small GTPase has been shown to bind to and inhibit DGK- θ activity (Houssa, B., de Widt, J., Kranenburg, O., Moolenaar, W. H., and van Blitterswijk, W. J., 1999; Bregoli, L., Baldassare, J. J., and Raben, D. M., 2001). As shown in Figure 2, while

treatment of the quiescent embryonic fibroblasts with α -thrombin induces a transient localization of PKC- α to the nucleus, this translocation is sustained in cells expressing either constitutively active RhoA, which inhibits DGK- θ (Houssa, B., de Widt, J., Kranenburg, O., Moolenaar, W. H., and van Blitterswijk, W. J., 1999), or a catalytically inactive form of DGK- θ (KD-DGK- θ). These data support the notion that nuclear DGK- θ is involved in regulating the level of nuclear PKC- α .

PI 3-Kinase and RhoA Regulation of DGK- θ

One of the goals of our research is to understand the mechanism involved in regulating nuclear DGK- θ . Previously, we have shown that DGK- θ translocates to the nucleus approximately 5 minutes after thrombin stimulation and remains resident for up to 30 minutes. However, increases in nuclear DGK activity are transient ((Bregoli, L., Baldassare, J. J., and Raben, D. M., 2001)). While it has not been definitively defined, there are some hints in the literature that PI3-kinase is involved in regulating this enzyme. For example, Walker et al. have presented data suggesting that activation, but not membrane translocation, of DGK- θ induced in small arteries by noradrenalin was dependent on a PI3-kinase activity (Walker, A. J., Draeger, A., Houssa, B., van Blitterswijk, W. J., Ohanian, V., and Ohanian, J., 2001). Consistent with this, stimulation of quiescent embryonic fibroblasts leads to an increase in nuclear PI3-kinase activity (Figure 3). Furthermore, we have detected both a p110 β , as well as p110 γ , catalytic subunit in the nucleus of the embryonic fibroblasts (Figure 4), and additional isoforms may be present. Activation of the p110 β subunit is dependent on recruitment to membranes, and this function is regulated by another protein, the p85 protein (Yu, J., Zhang, Y., McIlroy, J., Rordorf-Nikolic, T., Orr, G. A., and Backer, J. M., 1998), which is also present in the nucleus of this cell line (Figure 4). Class I PI3-K isoforms α , β and δ all utilize the p85 protein for membrane association, and a mutant form of p85 (Δ p85) which lacks the region responsible for membrane association is known to act in a dominant negative manner (Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G. et al., 1994). To determine whether Class I (p85-associated) PI 3-kinase contributed to the induced PI 3-kinase activity in these cells we examined the effect of thrombin stimulation on IIC9 fibroblasts expressing this mutant p85. As shown in Figure 5, expression of Δ p85 prevented α -thrombin-induced nuclear PI 3-kinase activity in the embryonic fibroblasts. These data indicate that a p85-associated PI 3-kinase is the major PI 3-kinase activated in response to acute stimulation with α -thrombin.

Suppression of p85-Dependent PI 3-Kinase Inhibition Does Not Prevent Induced Nuclear DGK- θ Activation

As indicated, PI 3-kinase has been shown to activate DGK- θ activity in small arteries in response to noradrenaline (Walker, A. J., Draeger, A., Houssa, B., van Blitterswijk, W. J., Ohanian, V., and Ohanian, J., 2001) and our data indicates a p85-dependent PI 3-kinase is activated in the nuclei of the embryonic fibroblasts. Given this, we fully expected that inhibition of PI 3-kinase would prevent the induced activation of nuclear DGK- θ in these cells. Surprisingly, while LY294002 does not affect DGK- θ activity (Figure 6), neither inhibition of PI 3-kinase by treatment with LY294002 (Figure 7A) nor expression of Δ p85 (Figure 7B) prevented the induced increase in this activity. In fact, all of these treatments led to a more sustained increase in nuclear DGK- θ activity (Figure 7).

PI 3-Kinase Inhibition Suppresses α -Thrombin-induced Nuclear RhoA

Translocation—The data above suggests that PI3-K suppresses nuclear DGK- θ activity, and that suppression of PI3-K activity stimulates DGK- θ activity. One potential mechanism for suppressing DGK- θ involves RhoA, a protein which is known to inhibit DGK- θ activity (Houssa, B., de Widt, J., Kranenburg, O., Moolenaar, W. H., and van Blitterswijk, W. J., 1999; Bregoli, L., Baldassare, J. J., and Raben, D. M., 2001). Interestingly, there are data

indicating that lipid products of PI 3-kinase may modulate RhoA translocation as well as activation (Karnam, P., Standaert, M. L., Galloway, L., and Farese, R. V., 1997; Missy, K., Van, Poucke, V, Raynal, P., Viala, C., Mauco, G., Plantavid, M. et al., 1998; Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D. et al., 1998; Mamoon, A. M., Baker, R. C., and Farley, J. M., 2001). We therefore examined the possibility that PI 3-kinase may be involved in modulating the nuclear translocation of RhoA. Strikingly, inhibition of PI 3-kinase by LY294002 or transient expression of $\Delta p85$ suppressed the induced nuclear translocation of RhoA in the embryonic fibroblasts (Figure 7C). Taken together, these data suggest that activation of a p85-associated PI 3-kinase is involved in RhoA translocation to the nucleus resulting in the inhibition of DGK- θ although involvement of another isoform of PI 3-kinase, such as the Class II p110 γ , cannot be completely ruled out.

Inhibitors and DGK- θ

Future studies to identify the pathway(s) involved in activating nuclear DGK- θ will likely involve the use of pharmacological inhibitors. It is important therefore, to ensure that these inhibitors do not affect the enzymatic activity of the enzyme. We therefore examined a number of inhibitors for their effect on DGK- θ . As noted above, 10 μ M LY294002 does not affect DGK- θ activity (Figure 6). In contrast, inclusion of 1-2 mM sodium pyrophosphate (NaPPi), often used to inhibit phosphatase activity, results in a substantial reduction of DGK activity (Figure 8A), while 1 mM 3- β -glycerophosphate has no effect on enzyme activity (Figure 8B). The effect of NaPPi does not appear to be through chelation of Mg $^{2+}$, as 5 mM EDTA has very little effect on enzyme activity under our assay conditions (Figure 7). The other inhibitors examined had little to no effect on DGK- θ activity with the exception of the general inhibitor of PI-PLC termed U73122 (Figures 8 and 9), while the inactive analog U73343 has only a minor effect on DGK- θ activity (Figure 10). The inhibition was the same whether the DGK- θ was present in cytosol from quiescent embryonic fibroblasts, or from quiescent fibroblasts that had been stimulated with α -thrombin for 5 minutes (data not shown), and appears to be competitive with respect to substrate (dioleoylglycerol) concentration (surface concentration) (Figure 10). Although the K_i of U73122 for DGK- θ is about 10-fold higher ($\sim 20 \mu$ M) than the K_i for PI-PLC (approximately 2 μ M), this information should be considered when using this inhibitor in various studies.

Summary

In this report we have summarized the data regarding the regulation of DGK- θ by two phospholipids: PtdSer and PtdOH. Our previous data has shown that stimulation of quiescent fibroblasts with a potent mitogen (α -thrombin) leads to an increase in nuclear localized DGK- θ (Bregoli, L., Baldassare, J. J., and Raben, D. M., 2001; Bregoli, L., Tu-Sekine, B., and Raben, D. M., 2002), as has been seen in neuronal cells (Tabellini, G., Bortul, R., Santi, S., Riccio, M., Baldini, G., Cappellini, A. et al., 2003; Tabellini, G., Billi, A. M., Fala, F., Cappellini, A., Evagelisti, C., Manzoli, L. et al., 2004). Furthermore, these previous studies demonstrated that DGK- θ is actually localized to the nuclear matrix (Bregoli, L., Tu-Sekine, B., and Raben, D. M., 2002; Tabellini, G., Bortul, R., Santi, S., Riccio, M., Baldini, G., Cappellini, A. et al., 2003). As we have also previously shown that PtdSer and PtdOH modulate DGK- θ activity (Tu-Sekine, B., Ostroski, M., and Raben, D. M., 2006; Tu-Sekine, B., Ostroski, M., and Raben, D. M., 2007), we examined the phospholipid composition of the nuclear matrix as well as the phospholipid composition of the intact nuclei and non-nuclear membranes. This analysis has revealed that there are phospholipids in the “matrix” of nuclei and the composition of these lipids largely resembles those of the other membranes. The physical form and localization of the matrix-associated lipids has not been established, though the nearly identical percent composition of the non-nuclear membranes and the nuclear matrix lend credence to the hypothesis that at least some of the internal nuclear lipid is derived from invaginations of the cellular membranes through

the nuclear interior known as nuclear tubules (Fricker, M., Hollinshead, M., White, N., and Vaux, D., 1997; Lee, R. K., Lui, P. P., Ngan, E. K., Lui, J. C., Suen, Y. K., Chan, F. et al., 2006).

An important finding resulting from this analysis is that the nuclear envelope is enriched in PtdSer relative to the nuclear matrix and to cellular membranes, and that over-expression of DGK- θ reduces both the PtdSer and PtdEth levels of the nuclear envelope. While the mechanism behind these fluctuations is unknown, the data are consistent with a DGK-regulated phosphatidylcholine-dependent phosphatidylserine synthase (PSS-1) (EC 2.7.8.8) activity at the nuclear envelope. PtdSer synthesis in mammalian cells proceeds by headgroup exchange with PtdCho or PtdEth, catalyzed by PSS-1 or PSS-2, respectively. Interestingly, the decrease in both PtdSer and PtdEth at the nuclear membrane mirrors effects observed in the CHO-K1 mutant cell lines M.6.1.1 and PSA-3, which have been shown to be deficient in PSS-1 activity (Vance, J. E. and Steenbergen, R., 2005). While there is very little information on the regulation of mammalian PSS enzymes, phosphorylation has been shown to regulate serine exchange activities in rat brain (Kanfer, J. N., McCartney, D., and Hattori, H., 1988), and we have shown that nuclear DGK- θ regulates the exit of PKC- α from the nucleus (see Regulation of PKC- α). To our knowledge, there is currently no reported study of nuclear phosphatidylserine synthases.

One other notable fluctuation in cellular lipids from DGK- θ expression is an increase in plasmalogen PtdEth in both the NNM and nuclear matrix. While the majority of studies conducted on plasmalogen synthesis agree in that there is an almost complete dependence on the classical CDP-ethanolamine pathway to provide the headgroup for precursor plasmalogen synthesized in the peroxisome, there are several studies utilizing radiolabeled serine that report approximately 20-30% of cellular plasmalogen-PtdEth can be derived from PtdSer in at least some cell models (Yorek, M. A., Rosario, R. T., Dudley, D. T., and Spector, A. A., 1985; Xu, Z. L., Byers, D. M., Palmer, F. B., Spence, M. W., and Cook, H. W., 1991). Whether nuclear PtdSer-derived PtdEth a precursor for the membrane plasmalogen-PtdEth present in DGK- θ expressing cells is not evident from the data presented here, though one potential mechanism for an increase in flux through this pathway could be an increase in PtdSer-derived PtdEth. At this juncture we can only speculate on the cause of the lipid perturbations, and further work is required to support our hypothesis that DGK- θ impacts PtdSer and plasmalogen metabolism.

The physiological role for nuclear DGK- θ has remained a mystery. It has long been recognized that an obvious role for DGKs is to modulate cellular levels of its DAG substrate thereby modulating DAG-sensitive proteins like such as many PKCs (Merida, I., Avila-Flores, A., and Merino, E., 2008). Our data support this notion as suppression of DGK- θ , either by expression of RhoA or a dominant-negative construct of DGK- θ , leads to a sustained increase in nuclear PKC- α (Figure 2).

In addition to the regulation by PtdSer and PtdOH, we report here that the regulation of nuclear DGK- θ by RhoA is modulated by a Class I PI 3-kinase. Furthermore, we have examined the effect of various inhibitors on the activity of DGK- θ in lysates over-expressing this isoform. This is important as many studies often use pharmacologic inhibitors to determine the role of selected enzymes in signaling pathways. While this approach is useful for comparative analyses, there are no data pertaining to the effect of these inhibitors on DGK- θ . We have found that one well-established PI-PLC inhibitor, U73122, but not its inactive analog U73343, competitively inhibits this isoform with respect to its diacylglycerol (DAG) substrate. These data indicate that studies designed to examine the role of PI-PLC in modulating DGK- θ activity using this inhibitor should be interpreted with caution.

Major Topics

1. Diacylglycerol kinase
2. DGKtheta
3. Lipids
4. Enzymology
5. Diacylglycerol
6. Phosphatidylserin
7. Phosphatidic acid
8. Signal Transduction
9. Kinases
10. Enzymes
11. Mixed Micelles
12. Competitive Inhibition
13. PI 3-Kinase
14. DGK-θ
15. RhoA

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Figure 1. PA and PS Exert Differential Effects on Binding of DGK θ to Vesicles

Membrane-free cytosol from thrombin-stimulated cells was subjected to activity and binding assays in small unilamellar vesicles (SLUVs) over a range of (A) PA and (B) PS concentrations. Fold change in specific activity is depicted as a line graph, and fold change in binding is presented as a bar graph. Typical error values ranged from 3-10%. Data is representative of two experiments conducted in triplicate (activity) and quadruplicate (binding).

Figure 2. Inhibition of DGK- θ Results in Sustained Nuclear Localization of PKC- α

Embryonic IIC9 fibroblasts were either mock transfected or transfected with constitutively active (V14RhoA) or kinase-dead DGK- θ (G648A, KD-DGK- θ) to inhibit DGK- θ . Cells were brought to quiescence by serum-deprivation and stimulated with α -thrombin for the indicated times. PKC- α localization was examined using immunohistochemistry.

Figure 3. Nuclear PI3K is Stimulated by α -Thrombin

(A) Nuclear extracts from quiescent and thrombin-stimulated IIC9 cells were examined for PI3K activity as outlined in materials and methods (B) The fold increase in stimulated total PI3K activity over quiescent controls was determined using densitometry. Values represent the mean and SD of three individual experiments. +/+ = positive control for PI3K activity (lysates from SF9 cells overexpressing PI3K p110 β and p85)

Figure 4. PI3K p110 β , p110 γ and the p85 regulatory subunit are present in the nuclei of embryonic fibroblasts

A. Cells were brought to quiescence and stimulated with thrombin for the times indicated. Localization of p110 β and p110 γ of PI3-kinase was determined by immunofluorescence of fixed cells incubated with p110 β or p110 γ polyclonal antibodies, and (B) nuclear localization was confirmed by immunoprecipitation of nuclear lysates followed by western blot analysis using the same antibody. C. Nuclei(N) were isolated from α -thrombin-induced (5 min) IIC9 embryonic fibroblasts and the presence of p85-associated PI3-kinase in 50 μ g of protein was examined by western blot analysis. C= no antibody IP control.

Figure 5. α -Thrombin Stimulates a p85-associated PI 3-kinase

Quiescent cells were either mock transfected (Control), or transfected with vector alone (Vector) or a construct expressing a dominant negative p85 (Δ p85=deltap85). Cells were incubated in the presence or absence of α -thrombin (1 NIH unit/ml) for 5 minutes and p85-associated PI 3-kinase activity was quantified.

Figure 6. LY294002 does not inhibit DGK- θ

HEK293T nuclear-free lysates from mock-transfected (control) or FlagDGK θ transfected cells (FlagDGK θ) were assayed for DGK activity at pH 8.2 using OG mixed micelles containing 6 mol% DOG and 9 mol % PS as described in Methods. Samples were preincubated in 10 μ M LY294002 for 30 min at 25C prior to assay. LY294002 was included in the final assay mixture at 10 μ M. PNS = post nuclear supernatant

Figure 7. Inhibition of PI3-kinase leads to constitutive activation of nuclear DGK- θ

A. Embryonic IIC9 fibroblasts were brought to quiescence and treated with 10 μ M LY294002 or DMSO (carrier) for 30 minutes prior to stimulation by thrombin for the times indicated. DGK activity was measured in 10–40 μ g nuclear protein for 30 minutes at 25C in mixed micelles containing 2.5 mM DAG (DOG) and 5 mM PtdSer. The data are the mean from single experiments performed in duplicate. B. Embryonic IIC9 fibroblasts or fibroblasts that had been transfected with $\Delta p85$ prior to stimulation for the times indicated. (C) Cell lysates from quiescent and thrombin-stimulated cells were fractionated as described in methods and purified nuclei were immunoblotted to detect RhoA. Control = mock transfected cells. *Error bars* represent standard error.

A

B

Figure 8. DGK θ is inhibited by the phosphatase inhibitor sodium pyrophosphate

(A) Quiescent cytosol from IIC9 cells over-expressing DGK θ was assayed in the presence of the common phosphatase inhibitors sodium pyrophosphate (NaPPi) and β -glycerol phosphate using OG mixed micelles containing 6 mol% DOG and 8 mol% PS at pH 8.2 as outlined in Methods.

(B) Quiescent cytosol from IIC9 cells over-expressing DGK θ was assayed over a range of NaPPi concentrations to evaluate dose dependency of inhibition. *Error bars* represent standard error.

Figure 9. DGKθ is inhibited by U73122 but not wortmannin, Akt inhibitor or GTPγS

(A) Quiescent cytosol from IIC9 cells over-expressing DGKθ was assayed in the presence of vehicle, protein inhibitors, or GTPγS, to identify potential sensitivity to reagents used to examine DGKθ signaling pathways. Assays were conducted using OG mixed micelles at pH 8.2 containing 3 mol% DOG and 6 mol% PS as described in Methods. U73122 = PI-PLC inhibitor (25 μM), wortmannin = PI3K inhibitor (50 nM), Akt inhibitor = 1L-6-hydroxymethyl-chiro-inositol 2(R)-2-O-methyl-3-O-octadecylcarbonate (Calbiochem) (50 μM), and GTPγS = RhoA and general GTPase activator (100 μM). *Error bars* represent standard error.

Figure 10. U73122 is a competitive inhibitor of DGK-θ

(A) Quiescent cytosol from IIC9 cells over-expressing DGKθ was assayed at 3 concentrations of U73122 over a range of DOG concentrations (0.5, 1.0, 3.0, 6.0, 9.0 mol %) in OG mixed micelles containing 6 mol% PS at pH 8.2 to evaluate the inhibition kinetics. Results are presented in the form of a Lineweaver-Burke plot. K_i values were calculated using the equation $K_i = [\text{inhibitor}] / ((K_{M\text{obs}}/K_M) - 1)$ (B) Identical samples were assayed using OG micelles containing 3 mol% DOG and 6 mol% PS at pH 8.2 in the presence of increasing concentrations of the inactive compound U73343 to determine the specificity of the interaction. *Error bars* indicate standard error. (C) Chemical structures of U73122 and U73343.

Phospholipid composition of non-nuclear membranes, nuclear envelope and nuclear matrix of embryonic fibroblasts

TABLE 1

Quiescent embryonic fibroblasts (HIC9s) were fractionated as described and lipids were extracted from non-nuclear membranes, nuclear envelope and nuclear interior. The total lipid composition of each fraction was determined using ESI MS/MS (Kansas Lipidomics Facility). Data are expressed as mol % (mean $\pm$ SD). NNM n=3; NE n=6, NI n=5. Variations in lipid composition between fractions are noted. Statistical significance was determined using an unpaired, 2-tailed Student's t-test. (*) indicates $p < 0.05$ (lysoPtdCho, lysophosphatidylcholine; PtdCho, phosphatidylcholine; SM sphingomyelin; ePtdCho, ether-linked phosphatidylcholine; lysoPtdEth, lysophosphatidylethanolamine, PtdEth, phosphatidylethanolamine; ePtdEth, ether-linked phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdOH, phosphatidic acid).

Lipid Class	Non-nuclear membranes (NNM)		Nuclear Envelope (NE)		Nuclear Matrix (NM)	
	wt	DGK θ	wt	DGK θ	wt	DGK θ
lysoPtdCho	0.5% $\pm$ 0.0%	0.4% $\pm$ 0.1%	0.2% $\pm$ 0.1%	0.1% $\pm$ 0.0%	0.2% $\pm$ 0.1%	1.3% $\pm$ 1.3%
PtdCho	42.0% $\pm$ 1.1%	41.0% $\pm$ 1.4%	44.7% $\pm$ 5.9%	47.8% $\pm$ 4.1%	48.9% $\pm$ 6.2%	40.7% $\pm$ 2.0%
SM	9.6% $\pm$ 2.1%	8.8% $\pm$ 0.5%	11.7% $\pm$ 0.9%	8.6% $\pm$ 1.1%	5.8% $\pm$ 3.8%	6.6% $\pm$ 1.4%
ePtdCho	2.2% $\pm$ 2.9%	5.1% $\pm$ 0.2%	5.5% $\pm$ 1.4%	6.4% $\pm$ 0.6%	6.3% $\pm$ 0.7%	9.5% $\pm$ 0.6%***
lysoPtdEth	0.4% $\pm$ 0.2%	0.2% $\pm$ 0.0%	0.6% $\pm$ 0.5%	0.2% $\pm$ 0.0%	0.7% $\pm$ 0.6%	0.5% $\pm$ 0.1%
PtdEth	15.4% $\pm$ 0.5%	12.6% $\pm$ 1.0%	12.0% $\pm$ 1.6%	8.6% $\pm$ 0.8%*	12.4% $\pm$ 2.0%	13.3% $\pm$ 1.2%
ePtdEth	10.4% $\pm$ 0.3%	13.5% $\pm$.5%*	9.2% $\pm$ 0.9%	10.8% $\pm$ 1.1%	9.0% $\pm$ 1.0%	15.2% $\pm$ 1.7%***
PtdIns	5.0% $\pm$ 0.9%	8.2% $\pm$ 0.8%	5.1% $\pm$ 1.6%	8.9% $\pm$ 8.1%	4.6% $\pm$ 2.7%	4.7% $\pm$ 0.5%
PtdSer	9.1% $\pm$ 0.5%	9.0% $\pm$ 1.2%	15.4% $\pm$ 1.3%*	7.6% $\pm$ 1.3%*	6.7% $\pm$ 3.3%	6.5% $\pm$ 1.4%
PtdOH	0.5% $\pm$ 0.1%	0.3% $\pm$ 0.1%	0.6% $\pm$ 0.5%	0.1% $\pm$ 0.1%	0.7% $\pm$ 0.3%	0.7% $\pm$ 0.3%