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Review

Nuclear diacylglycerol kinases: emerging downstream regulators in cell signaling networks

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Summary. There exists an active lipid metabolism in the nucleus, which is regulated differentially from the lipid metabolism taking place elsewhere in the cell. Evidence has been accumulated that nuclear lipid metabolism is closely involved in a variety of cell responses, including proliferation, differentiation, and apoptosis. A fundamental lipid second messenger which is generated in the nucleus is diacylglycerol, that is mainly known for its role as an activator of some protein kinase C isoforms. Diacylglycerol kinases attenuate diacylglycerol signaling by converting this lipid to phosphatidic acid, which also has signaling functions. Ten mammalian diacylglycerol kinase isoforms have been cloned so far, and some of them are found also in the nucleus, either as resident proteins or after migration from cytoplasm in response to various agonists. Experiments using cultured cells have demonstrated that nuclear diacylglycerol kinases have prominent roles in cell cycle regulation and differentiation. In this review, the emerging roles played by diacylglycerol kinases in the nucleus, such as the control of G1/S phase transition, are discussed.

Key words: Nucleus, Diacylglycerol, Proliferation, Differentiation, Nuclear matrix

Introduction

The existence of inositide- or phosphatidylcholine-based signaling pathways which operate within the nucleus has been demonstrated by several independent groups. It is remarkable that these networks are regulated in a way which is totally different from their plasma membrane counterparts (Martelli et al., 2005a; Bunce et al., 2006; Cocco et al., 2006; Hunt, 2006a; Irvine, 2006; Ye, 2006). Diacylglycerol (DG) is a key second messenger which is generated along these nuclear pathways. It has been shown that nuclear DG could derive from either phosphoinositides or phosphatidylcholine (PC) (Goto et al., 2006). DG derived from phosphoinositide is polyunsaturated, whereas DG originating from PC is mono-unsaturated and saturated (Hunt, 2006b). DG activates numerous proteins, including conventional and novel protein kinase C (PKC) isoforms (Becker and Hannun, 2005), the guanine nucleotide exchange factor vav, and Ras- or Rap guanyl nucleotide-releasing proteins (Topham, 2006). Furthermore, DG recruits a number of proteins to membrane compartments, including chimaerins, protein kinase D, and the Munc 13 proteins (Topham, 2006). Since DG has broad effects, its availability within the cell must be tightly regulated. DG is metabolized in three ways: hydrolysis of a fatty acyl chain by diacylglycerol lipase to generate monoacylglycerol and free fatty acid, addition of either CDP-choline or -ethanolamine to form PC or phosphatidylethanolamine, or phosphorylation of the free hydroxyl group to produce phosphatidic acid (PA) (Topham, 2006). PA is believed to elicit many biological responses by itself. In fact, PA plays a role in cytoskeletal organization by inducing actin polymerization and stress fiber formation. It is also involved in the regulation of enzymes which include inositide kinases, PAK1, PKC-ζ, Ras-GAP, and protein phosphatase 1 (Martelli et al., 2002; Topham, 2006).

Under most circumstances, DG conversion to PA is the major route for signaling DG metabolism. This reaction is catalyzed by a family of enzyme referred to as diacylglycerol kinases (DGKs). Ten mammalian DGK isozymes have been identified to date. They have been organized into five classes. Class I comprises of α, ß, and γ isozymes; class II of δ, η, and the recently identified κ (Imai et al., 2005); class III of ε isoform;

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class IV of ζ and ι; class V of θ (Luo et al., 2004a; Topham, 2006). However, there is additional complexity in the DGK family due to alternative splicing which occurs with DGK-ß, -γ, -δ, -η,-ι, and -ζ (Topham, 2006).

All of the mammalian DGKs share a conserved catalytic domain in the COOH-terminal region and at least a pair of cysteine-rich motifs (DGK-θ has three) similar to the C1A and C1B motifs of PKC but lacking certain consensus residues present in phorbol ester-binding proteins. However, DGK-δ and -η have bipartite catalytic domains. The cysteine-rich domains could bind DG and present it to the catalytic domain, even though this has never been demonstrated in a conclusive manner. Nevertheless, DGK isotypes can be distinguished by the presence of additional domains that conceivably confer to each isozyme specific functions in biological processes, sensitivity to different regulatory mechanisms, and a differential intracellular localization. Indeed, these motifs are likely to play a role in lipid-protein and protein-protein interactions in various signaling pathways. Class I DGKs display Ca^{2+}-binding EF domains in their NH2-terminal half, so that they are more active in the presence of Ca^{2+}. Class II isotypes have a pleckstrin homology (PH)-like domain at their NH2-terminal portion, but no specific function has been ascribed to this domain, even though it could be involved in interactions with lipids. Class III DGK-ε is the only isotype which has no domains with obvious regulatory functions, whereas class IV isozymes display four COOH-terminal ankyrin repeats, a PDZ (postsynaptic density protein-95/discs large/zona occludens-1) domain, and a region homologous to the phosphorylation site of the well-established PKC substrate "myristoylated alanine-rich C-kinase substrate" (MARCKS) (see also below). Class V DAG kinase has a region with a weak homology to a PH domain located in the middle of its sequence. This domain overlaps with a Ras-associating domain (Goto and Kondo, 2004; Luo et al., 2004a; Topham, 2006). With the notable exception of DGK-δ and -ε, all mammalian DGK isozymes are expressed in various central nervous system regions (hippocampus, cerebellum, cerebral hemispheres) at levels equivalent or higher than in other tissues (Topham, 2006). Several DGKs are also expressed in other organs such as lung, heart, skeletal muscles, spleen, and thymus (Topham, 2006). It is noteworthy that several DGK isoforms can be expressed in the same tissue or cell. Furthermore, co-expressed DGK isozymes are usually from different classes, strongly suggesting that each DGK class fulfills a specific role (Topham, 2006).

Nuclear DGK isoforms

There are several reports highlighting that DG was generated within the nucleus independently from extranuclear DG and it could derive from either polyphosphoinositides or PC (Divecha et al., 1991; Baldassare et al., 1997; Neri et al., 1998, 2002a; D'Santos et al., 1999). Nuclear DG signaling is not

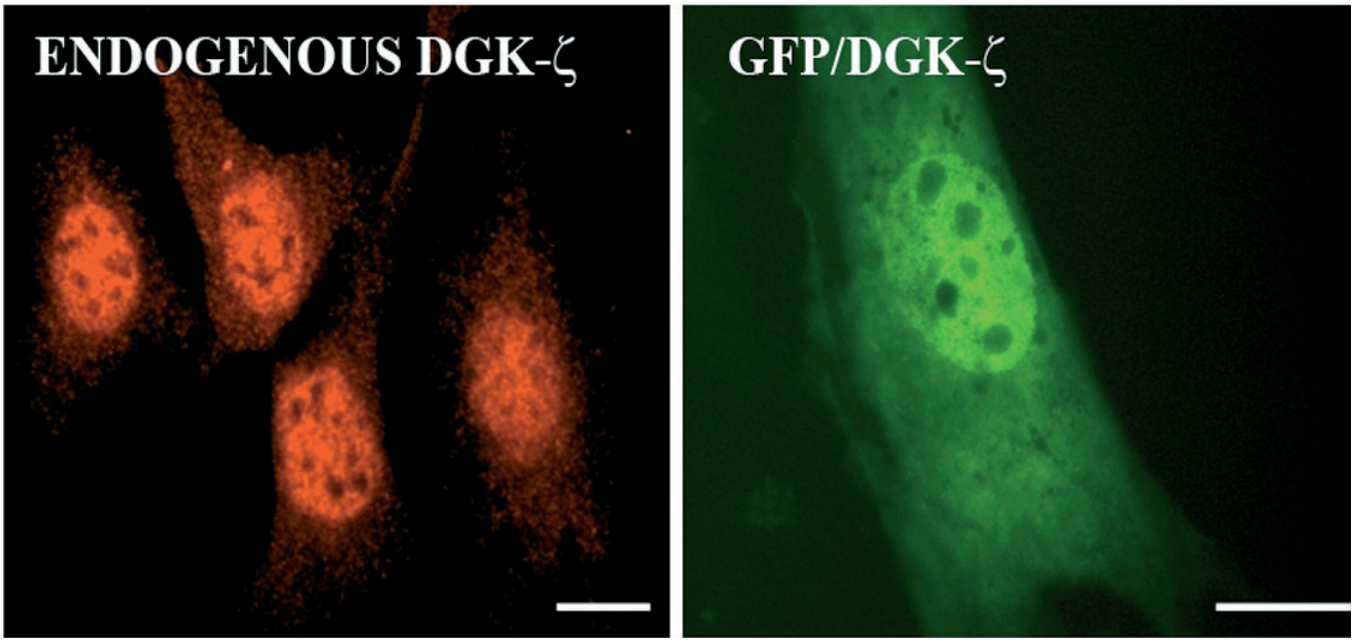

Fig. 1. Fluorescence microscope analysis of the subcellular distribution of endogenous DGK-ζ and of overexpressed GFP/DGK-ζ in C2C12 mouse myoblasts. Endogenous DGK-ζ was detected by means of an affinity purified rabbit antibody followed by Cy3-conjugated anti-rabbit IgG antibody. Note how, in both cases, nucleoli were negative. Scale bars: 5 μm.

confined to the nuclear envelope, as one would expect, but also occurs at discrete location within the nuclear interior (Vann et al., 1997). The involvement of nuclear DG in attracting and/or activating PKC isoforms to the nucleus was first hypothesized in insulin-like growth factor-I -stimulated Swiss 3T3 cells (Divecha et al., 1991) and has been substantiated in other experimental systems (see Neri et al., 2002b; Martelli et al., 2006 for comprehensive reviews on this issue).

Several DGK isoforms have been demonstrated to reside within the nucleus or to translocate to this organelle in response to agonist stimulation, and these include DGK-α, -γ, -δ, -ζ, and -θ (see Table 1). The same cell type could display two different isozymes within the nucleus (Bregoli et al., 2001).

It should be emphasized that, in some cases, endogenous intranuclear DGK isoforms have been identified by means of immunocytochemical staining or immunoblotting carried out on purified nuclear fractions (Flores et al., 1996; Bregoli et al., 2001; Tabellini et al., 2003, 2004), whilst in other studies, DGK isozymes were overexpressed, in most cases as hybrids fused to green fluorescent protein (GFP), then analyzed by immunofluorescence microscopy and/or western blot (Ding et al., 1998; Shirai et al., 2000; Evangelisti et al., 2006; Matsubara et al., 2006). However, both endogenous DGK-ζ and ectopically expressed DGK-ζ/GFP hybrid could be found located within the nucleus of various cell types (Hozumi et al., 2003; Evangelisti et al., 2006) (Fig. 1). DGK-ζ possesses a bipartite nuclear targeting motif located close to the second zinc finger-like sequence in its regulatory domain. Such a motif is similar to the phosphorylation-site domain of the well-known PKC substrate MARCKS protein (Topham et al., 1998). In COS-7 cells overexpressing a DGK-ζ cDNA lacking the sequence homologous to the MARCKS phosphorylation-site domain, the enzyme was almost entirely excluded from the nucleus. This motif contains a serine residue (Ser 265) which is phosphorylated by PKC-α and -γ. In cells overexpressing either of these PKC isozymes, the amount of DGK-ζ present in the nucleus was strongly reduced, whereas treatment with phorbol esters (which downregulated the amount of the two PKC isoforms) resulted in enhanced intranuclear localization of DGK-ζ as well as in an increase in nuclear DGK activity. Thus, DGK-ζ localization in the nucleus seems to be dynamically regulated by phosphorylation of its MARCKS-homology domain by some PKC isoforms, even though it is not clear whether or not these phosphorylative events took place in the nucleus or in the cytoplasm. Nevertheless, this nuclear targeting mechanism for DGK-ζ, does not seem to be universal, because a deletion mutant of DGK-ζ which lacks the COOH-terminal ankyrin repeats but has the NLS, localized to the nucleus of transfected COS-7 cells, whereas the same mutant was cytoplasmic in transfected primary cultured rat neurons (Hozumi et al., 2003). Thus, it could be suggested that both the NLS and the COOH-terminal region are cooperatively engaged in allowing DGK-ζ nuclear localization in neurons. It may be that DGK-ζ NLS is cryptic in the tertiary structure and that molecules interacting with COOH-terminal ankyrin repeats determine whether or not the NLS is exposed to allow nuclear internalization (Goto et al., 2006). Obviously, these molecules could be differently expressed in different cell types, and this would explain the above emphasized findings. In addition to a NLS, DGK-ζ also displays a typical leucine-rich nuclear export sequence (NES) (Goto and Kondo, 1996). However, the role of this NES in shuttling DGK-ζ between the nucleus and the cytoplasm has not been clarified yet.

DGK-ι, which has NLS and ankyrin repeats similar to that of DGK-ζ, has been found by western blot to be nucleus associated when overexpressed in COS-7 cells (Ding et al., 1998). However, a subsequent investigation dealing with expression of three DGK-ι splicing variants in rat brain, showed that none of them was intranuclear when overexpressed as FLAG-tagged constructs in hippocampal neurons (Ito et al., 2004). Also in C2C12 rat myoblasts, overexpressed, GFP-tagged DGK-ι localized to the cytoplasm (Evangelisti et al., 2006).

Table 1. DGK isoforms identified in the nucleus.

ISOFORM	TISSUE OR CELL LINK	REFERENCES
α	rat thymocytes, rat T-lymphocytes, human T-cell line CTLL-2, CHO-K1 cells	Wada et al., 1996; Flores et al., 1996; Shirai et al., 2000
γ	CHO-K1 cells, COS-7 cells, NIH 3T3 cells, SH-SY5Y cells	Shirai et al., 2000; Matsubara et al., 2006
δ	IIC9 cells	Bregoli et al., 2001
ζ	COS-7 cells, A172 cells, Hela cells, rat neurons, rat lung alveolar type II cells, mouse C2C12 myoblasts	Goto and Kondo, 1996; Topham et al., 1998; Hogan et al., 2001; Hozumi et al., 2003; Ali et al., 2004; Evangelisti et al., 2006; Nakano et al., 2006; Sasaki et al., 2006;
ι	COS-7 cells	Ding et al., 1998
θ	rat arterial smooth muscle and endothelial cells, human MelJuso melanoma cells, COS-7 cells, IIC9 cells, HeLa cells, MDA-MB-453 cells, PC12 cells	van Blitterswijk et al., 2000; Bregoli et al., 2001; Walker et al., 2001; Tabellini et al., 2003; Tabellini et al., 2004

From the above results, it could be speculated that different mechanisms regulate the nuclear localization of DGK-ζ and DGK-ι in primary neurons or rat myoblasts when compared to COS-7 cells, despite the fact that both of these isoforms contain similar type of NLS.

Moreover, recent results have highlighted that the two C1-like domains of DGK-γ are important for nuclear localization of this isoform. Indeed, deletion of one of these domains resulted in exclusively cytoplasmic localization of the DGK. Similarly, an overexpressed GFP-tagged C1 domain was specifically localized to the nucleus (Matsubara et al., 2006). Enzymatic activity was not required for DGK-γ nuclear localization, however a kinase dead mutant of DGK-γ showed much more slower nuclear transportation than wild type DGK-γ.

The NLS, or the domain(s), which allow nuclear localization of DGK-α, -δ, and -θ, still await identification.

As to DGK isotype subnuclear localization, evidence suggests that some of them, such as -α, -ζ, and -θ (Wada et al., 1996, Tabellini et al., 2003, 2004; Evangelisti et al., 2006), associate with the nuclear matrix, a highly dynamic structure, mostly composed of nonhistone proteins, which has been proposed to serve as a sort of nuclear skeleton or scaffold (Anachkova et al., 2005). Several of the enzymes involved in nuclear lipid-dependent signaling pathways are associated with the nuclear matrix, which conceivably plays a critical role in the regulation of these complex networks (Gonzales and Anderson, 2006).

DGK-ζ and -θ are also concentrated in dots which correspond to the speckle domains of the nucleus (Tabellini et al., 2003, 2004; Evangelisti et al., 2006). These domains, which are scattered throughout the nucleus (hence their name), are structures enriched in splicing small nuclear ribonuclear particles, and many other transcription- and splicing related proteins plus apparently untranslated poly-adenylated RNA (Handwerger and Gall, 2006). Nuclear speckles also contain various components of nuclear inositol lipid metabolism, including the α and ß isoforms of type II phosphatidylinositol kinase, phospholipase C ß1 (PLC ß1) and its substrate phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P2], and phosphatidylinositol 3-kinase (Manzoli et al., 2005; Martelli et al., 2005b; Bunce et al., 2006; Deleris et al., 2006; Gonzales and Anderson, 2006). Both DGK-ζ and -θ physically interacts with PLC ß1 (Tabellini et al., 2003; Evangelisti et al., 2006), so that it could be hypothesized that their role, within the nuclear speckles, is related to the metabolism of DG which is generated by PLC ß1 through PtdIns(4,5)P2 hydrolysis. We do not know if PA plays any functions in the nucleus. As the nuclear matrix is considered by some investigators to be the equivalent of the cytoskeleton, it might be that nuclear PA is involved in regulating some structural aspects of this nuclear scaffold, in analogy with its role in cytoplasmic actin polymerization. Indeed, actin is rapidly emerging as a key protein for the regulation of many nuclear processes (Pederson and Aebi, 2005; Schoenenberger et al., 2005) and recent evidence has convincingly shown for the first time the existence of polymerized actin in the nucleus (McDonald et al., 2006).

Functions of nuclear DGK isoforms

Cell proliferation and growth

There is general consensus over the fact that nuclear DG is important for cell cycle progression (Divecha et al., 1995; Martelli et al., 2000). Therefore, there is need for a mechanism in the nucleus to turn off DG signal. It has been shown that DGK-ζ is deeply involved in the regulation of the G1/S phase transition because in cells overexpressing an inducible DGK-ζ, the doubling time increased about two-fold over controls and cells accumulated in the G0/G1-phase of the cell cycle (Topham et al., 1998). Moreover, in cells transfected with DGK-ζ cDNA encoding either a kinase-dead mutant or a mutant that did not localize to the nucleus, cell cycle progression was not affected. Thus, these findings convincingly demonstrated that DGK-ζ was important for the regulation of cell proliferation through the control of nuclear DG mass and that both its enzymatic activity and nuclear localization were essential (see also later). Taken together, these findings fit well with the observation that intranuclear DG mass derived from inositide hydrolysis increased as the cells progressed through the cell cycle (D'Santos et al., 1999). Nevertheless, to validate these results, which have been obtained by forced expression of DGK-ζ cDNAs, it would be necessary to identify a physiological model in which an increase in nuclear DGK-ζ activity is linked with a cell cycle arrest.

Very recently, it has been demonstrated that also DGK-γ, when overexpressed in the nucleus, affected cell cycle regulation. Indeed, overexpression of a dominant negative DGK-γ slowed down progression through the S phase. It was also shown that both endogenous and overexpressed DGK-γ preferentially accumulated in the nucleus of serum-starved cells (Matsubara et al., 2006). However, it is at present difficult to reconcile these findings with our existing knowledge of nuclear DG mass changes during the cell cycle, especially considering that nuclear DG levels increased in parallel with DNA synthesis in an *in vivo* model of liver regeneration (Banfic et al., 1993).

Cell differentiation

Changes in nuclear DGK isoforms have been reported also in differentiating models. For example, in response to nerve growth factor (NGF) stimulation of rat PC12 cells, there was a selective increase in nuclear DGK-θ activity (Tabellini et al., 2004) which, presumably, might play an important role in NGF-mediated neural differentiation of these cells. More recently, our group has demonstrated that nuclear DGK-

ζ increased during myogenic differentiation of mouse C2C12 myoblasts and, more importantly, siRNA down-regulation of DGK-ζ severely impaired the differentiation process (Evangelisti et al., 2006). At present, however, we do not know how nuclear DGK-ζ could control myogenic differentiation.

Regulation of nuclear DGK activity

The regulation of DGK cytoplasmic isoforms is complex and only partially understood. It is commonly thought that DGKs become active when DG is generated but activation may require phosphorylative events and interactions with other cofactors (for example Ca^{2+}, which binds the EF hand motifs, or lipids such as phosphatidylserine and spingosine) and/or binding partners (Topham, 2006). DGK-ζ, -δ, and -θ are phosphorylated on serine residues (Luo et al., 2003; Imai et al., 2004; van Baal et al., 2005) and this may influence both their localization in the plasma membrane and their activity. As to binding partners, the syntrophin family of scaffolding proteins regulates DGK-ζ subcellular localization by associating with its COOH-terminal PDZ binding domain to sequester DGK-ζ in the cytoplasm (Hogan et al., 2001). Finally, DGK-θ interacts with, and is inhibited by, active RhoA (Houssa et al., 1999).

As to nuclear DGK isotypes, our knowledge about their regulation is even more limited. Also in the nucleus DGK-θ activity is inhibited by active RhoA (Bregoli et al., 2001). Very recently, it has been shown that the retinoblastoma protein (pRB) and its family members p107 and p130 specifically interacted in vitro and in vivo with the MARCKS domain of nuclear DGK-ζ. DGK-α and -θ interacted with pRB to a much lower extent. The major binding site of pRB with DGK-ζ was mapped at the COOH-terminus of pRB. It is well known that pRB, together with p107 and p130, regulates cell cycle progression by interacting with, and attenuating the activity of, the E2F transcription factor family (Cobrinik, 2005). The DGK-ζ/pRB interaction, which only took place with hypophosphorylated pRB, resulted in a robust enhancement of DGK-ζ activity (Los et al., 2006). However, DGK-ζ did not affect the ability of pRB to regulate E2F-mediated transcription.

Since overexpression of DGK-ζ in the nucleus, was capable of partially reconstituting a cell cycle arrest in a model of γ-irradiated rRB^{null} fibroblasts, it has been hypothesized that nuclear DGK-ζ is either down-stream of pRB or is part of a parallel signaling pathway whose activation results in a cell cycle blockage. Interestingly, the cell cycle arrest was not seen in cells transduced with a kinase dead DGK-ζ, suggesting that DG removal or PA generation are of fundamental importance for cell cycle arrest. In any case, it would be very important to identify the downstream target(s) of nuclear DGK-ζ involved in G1/S phase transition arrest.

However, compartmentalization is conceivably of fundamental importance also at the nuclear level to ensure correct termination of DG signaling by DGKs (Vann et al., 1997), and future investigations should fully address this issue. This hypothesis is strengthened by the observation that DGK-θ activity increased upon its association with the nuclear matrix in NGF-stimulated PC12 cells (Tabellini et al., 2004).

Concluding remarks and future directions

Evidence reviewed here shows that DGKs could be major players in nuclear lipid-dependent signaling pathways. However, numerous questions regarding nuclear DGK isozymes remain unanswered. Indeed, we know very little about the regulation of activity of DGKs operating in the nuclear environment, apart from the -ζ isotype. Also the mechanisms which govern nuclear entry and export of several DGK isozymes are obscure. A further critical step will be the identification of the nuclear interacting partners important for DGK activation/inhibition. Identification of the interacting partners would also be important for a better understanding of the functions of nuclear DGKs, taking into account that at the plasma membrane level, DGKs commonly bind DG-activated proteins to inhibit their activity, as has been reported for PKC-α. Moreover, DGKs also interact with proteins whose activity is regulated by PA. For example, DGK-ζ associates with and activates both phosphatidylinositol 5-kinase type Ia (Luo et al., 2004b) and mammalian target of rapamycin (mTOR) (Avila-Flores et al., 2005). Therefore, experiments aimed at identifying DGK interacting partners in the nucleus might also clarify the role(s) of nuclear PA. Furthermore, the presence of more than one isotype in the nucleus of the same cell suggests that DGKs could operate in distinct signal transduction pathways that need to be identified. Our understanding of nuclear lipid-dependent signal transduction networks is evolving quite rapidly. It is clear that the contributions of these pathways are essential for many critical cell responses. A better knowledge of nuclear DGKs is desirable mostly because, if it is ascertained that they are indeed activated in a manner which is distinct from their cytoplasmic counterparts and regulated in a way peculiar to the nuclear compartment, it should then be possible to design drugs for selective inhibition of the relevant nuclear isotypes while sparing those operating in the plasma membrane.

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