Nuclear expression of diacylglycerol kinases: possible involvement in DNA replication

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The existence of intranuclear lipid-dependent signal transduction systems has been demonstrated by several independent groups. Remarkably, intranuclear lipid-dependent signal transduction pathways are regulated independently from their membrane/cytosolic counterparts. A sizable body of evidence suggests that nuclear lipid signaling controls critical biological functions such as cell proliferation, differentiation, and apoptosis. Diacylglycerol (DG) is a fundamental lipid second messenger which is produced in the nucleus. Since the levels of nuclear DG fluctuate during the cell cycle progression, it has been suggested that this lipid second messenger has important regulatory roles. Most likely, nuclear DG serves as a chemoattractant for some isoforms of protein kinase C that migrate to the nucleus in response to a variety of agonists. The nucleus also contains diacylglycerol kinases (DGKs), i.e. the enzymes that, by converting DG into phosphatidic acid (PA), terminate DG-dependent events. This review aims at highlighting the different isozymes of DGKs present within the nucleus as well as at discussing their potential functions with particular emphasis placed on DNA replication.

Key words: nucleus, signal transduction pathways, lipids, diacylglycerol, proliferation.

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There is now solid evidence that lipid-dependent signaling pathways operate within the nucleus (D'Santos *et al.,* 1998; D'Santos *et al.,* 2000; Cocco *et al.,* 2001; Martelli *et al.,* 2004). A key second messenger which is generated at the nuclear level along these pathways is DG. Data reported by independent laboratories have shown that nuclear DG can derive from either phosphoinositides or phosphatidylcholine (D'Santos *et al.,* 1999; Divecha *et al.,* 2000). DGKs catalyze phosphorylation of DG to yield PA. By lowering DG levels, DGKs may downregulate membrane localization of protein kinase C (PKC) isoforms and/or may terminate transient receptor-induced PKC activation. DG-dependent PKC isozymes include conventional PKC-α, -β_I, -β_{II}, -γ, and novel PKC -δ, -ε, -η,-θ (Martelli *et al.,* 2003).

Several reports indicate that also PA has signaling proprieties. For example, studies have indicated that PA is involved in vescicle trafficking (Siddhanta and Shields, 1998), and can bind and regulate the activity of numerous enzymes, including the Ras-GAP (Yu *et al.,* 1988), PKC-ζ (Limatola *et al.,* 1994). Furthermore, PA has a potential role in progression of cells to S phase of the cell cycle (Flores *et al.,* 1996) and regulate actin stress fiber function (Jamney and Lindberg, 2004).

Several DGK isozymes are present in the nucleus where they may be involved in regulating the amount of intranuclear DG. In some cases, the activity of nuclear DGKs has been demonstrated to be very critical for the control of cell proliferation (Topham *et al.,* 1998; Divecha, 1998). In this review, we shall concentrate on the subnuclear localization and functions of the various types of DGKs that have been described to be associated with the nucleus.

DGK isoforms

DGKs have been identified in a wide variety of species including yeast, *Drosophila melanogaster*, *Caenorhabditis elegans*, plants, and mammals. Nine mammalian isoforms of DGK, organized into five classes, have thus far been cloned: class I comprises the α, β, and γ isozymes; class II the δ and η; class III the ε isoform; class IV the ζ and ι; class V the θ Van Blitterswijk and Houssa, 2000). All of the mammalian DGK isotypes share a conserved catalytic domain in the COOH-terminal region which has an ATP-binding site similar to protein kinase catalytic domains with the sequence Gly-X-Gly-X-X-Gly. All DGKs have 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. It is likely that these cysteine-rich domains bind DG and present it to the catalytic domain. However, 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 selective 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 contain a pair of EF hand-like domains (that bind Ca^{2+}) in their NH_2-terminal half. Class II isotypes have a pleckstrin homology-like domain at their NH2-terminal portion, whereas class IV isozymes are characterized by a nuclear localization signal (NSL), by a nuclear export signal (NES), and by ankyrin repeats. The NLS sequence of DGK-ζ overlaps with a motif similar to the phosphorylation site domain of the myristoylated alanine-rich C-kinase substrate (MARCKS) protein (Bunting *et al.,* 1996: Topham *et al.,* 1998). Phosphorylation of this site by conventional PKC isoforms results in nuclear exclusion of DGK-ζ (Topham *et al.,* 1998). However, nuclear localization of DGK-ζ also depends on a cooperative interaction between the NLS and the COOH-terminal region including ankyrin repeats in a manner which suggests that the NLS is a cryptic site whose exposure is regulated by the COOH-terminal region (Hozumi *et al.,* 2003). The ankyrin repeat motif, which is present in a number of proteins, is thought to be involved in protein-protein interactions (Blank *et al.,* 1992). The NES is a leucine-rich region spanning from amino acids 362 to 370 of DGK-ζ (Leu-Ser-Thr-Leu-Asp-Gln-Leu-Arg-Leu). It seems plausible that rapid disappearance of DGK-ζ from the nucleus of hippocampal neurons in response to hypoxic conditions (Ali *et al.,* 2004) may be due at least in part to active transport via NES and the nuclear exporter Crm1/exporter 1 system (Fornerod *et al.,* 1997). Class V DGK has a pleckstrin homology domain located in the middle of its sequence. This domain overlaps with a Ras-associating domain. Class III DGK is the only isotype wich has no domains with obvious regulatory functions.

Nuclear DGK isoforms

The first report dealing with nuclear DGK showed that intranuclear DGK activity was enriched in the nuclear matrix fraction prepared from NIH 3T3 fibroblasts and rat liver cells (Payrastre *et al.,* 1992). It is worth recalling here that the nuclear matrix is a dynamic structure, mostly composed of nonhistone proteins, which has been proposed to serve as a sort of nuclear skeleton or scaffold (Berezney and Wei, 1998; Cremer *et al.,* 2000; Nickerson, 2001; Martelli *et al.,* 2002). Many of the enzymes related to nuclear lipid-dependent signaling pathways are found associated with the nuclear matrix, which conceivably plays a critical role in the regulation of these complex networks (Maraldi *et al.,* 1999). The nuclear matrix contains different enzymes of phosphoinositide metabolism such as DGK that are preferentially detected in the internal matrix (Martelli *et al.,* 2004).

The presence of DGK in membrane-free rat liver cell nuclei was subsequently reported by Previati *et al.* (1994). This group demonstrated that nuclear, but not microsomal, DGK activity was completely inhibited by the pharmacological inhibitor, R59022. The results suggested that the nuclear enzyme was different from the microsomal one. It should be considered here that R59022 only inhibits class I DGK isotypes (Van Blitterswijk and Houssa, 2000). Therefore, we might speculate that the isoform present in membrane-deprived rat liver nuclei was DGK-α. However, in these very early reports there was no clear indication of the DGK isozyme(s) present within the nucleus, because at that time our general knowledge about the different isotypes was quite limited. Due to the progessive identification of the various isoforms and the availability of specific antibodies, in the following years it became clear that almost all DGK isoforms may be nucleus-associated (Table 1). As to the subnu-

clear localization of DGK isoforms, DGK-ι has been detected in the nucleus of several cell types, where it associates with the nuclear matrix and concentrates in the speckles (Tabellini *et al.,* 2003), a nuclear subdomain which is enriched in factors important for mRNA splicing and components of inositide metabolism (Lamond and Spector, 2003; Martelli *et al.,* 2004).

Functions of nuclear DAG kinases

The evidence so far collected indicates that, at least in some cases, nuclear DGKs are involved in the regulation of DG levels that fluctuate during the cell cycle progression. There are numerous examples of treatment with agonists that cause a rise in the intranuclear DG mass (D'Santos *et al.,* 1998; D'Santos et al. 2000; Cocco *et al.,* 2001). One of the functions of nuclear DG seems to be the attraction and/or the activation of DG-dependent PKC isoforms such as -βII and -α (Divecha *et al.,* 1991; Mallia *et al.,* 1997; Sun *et al.,* 1997; Neri *et al.,* 1998; Martelli *et al.,* 1999). Nuclear DGK-ζ seems to be tightly related to the regulation of DNA replication. It was demonstrated that when A172 cells, a glioblastoma cell line with high levels of intranuclear DGK-ζ, were challenged with epidermal growth factor (EGF), nuclear DAG mass rose two- to three-fold above the baseline, whereas total cell levels did not change (Topham *et al.,* 1998). As a step towards the elucidation of the function of nuclear DGK-ζ, (Topham *et al.,* 1998) compared nuclear DG levels in control A172 cells with levels in cells treated with phorbol esters that have a high amount of nuclear DGK-ζ. When A172 cells were treated with EGF for 10 min, nuclear DG mass in cells not pre-treated with phorbol esters increased about 2.5-fold above the basal levels, whereas nuclear DG levels in cells pre-treated with phorbol esters increased only 1.3-fold. Moreover, in cells overexpressing an inducible form of DGK-ζ, the doubling time increased about two-fold over controls. In addition, cells transfected with DGK-ζ cDNAs encoding either a kinase-dead mutant or a mutant that did not localize to the nucleus, accumulated in the G_0/G_1-phase of the cell cycle. These findings convincingly demonstrated that DGK-ζ acts as a negative regulator of cell proliferation through the control of nuclear DG mass. To this end, both its enzymatic activity and localization in the nucleus were found to be essential. Our group has analyzed endogenous nuclear DGK activity in response to insulin-like growth factor-1 (IGF-1) stimulation of quiescent Swiss 3T3 cells. Indeed, this is a classic experimental model in which there is a rise in nuclear (but not whole cell) DG mass (Divecha *et al.,* 1991; Neri *et al.,* 1998). Exposure to IGF-1 provoked stimulation of a nuclear DGK activity, but not of DGK activity present in whole cell homogenate. An inverse relationship between nuclear DG mass and DGK activity levels was shown by time course analysis. If 3T3 cells were pre-treated with DGK pharmacological inhibitors, the IGF-1-evoked rise in nuclear DGK activity was blocked and intranuclear levels of DG remained elevated for a period longer than in control cells. Also nuclear PKC-α activity stayed higher in cells treated with the DGK inhibitors than in untreated cells, suggesting that nuclear DG is important for regu-

Table 1. DGK isoforms present in the nucleus. n.d.: not determined.

Isoform	*Tissue or cell line*	*References*
n.d.	NIH 3T3 cells	(Payrastre et al., 1992)
n.d. (α?)	rat liver	(Previati et al., 1994)
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)
n.d. (α?)	Swiss 3T3 cells	(Martelli et al., 2000)
γ	CHO-K1 cells	(Shirai et al., 2000)
δ	IIC9 cells	(Bregoli et al., 2001)
ζ	COS-7 cells, A172 cells, Hela cells, rat hippocampal and cerebellar neurons, rat lung alveolar type IIcells and macrophages	(Goto et al., 1996; Topham et al., 1998; Hogan et al., 2001; Hozumi et al., 2003; Ali et al., 2004)
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, MCF-7 cells, PC12 cells	(van Blitterswijk and Houssa, 2000; Bregoli et al. 2001; Walker et al., 2001; Tabellini et al., 2003)

lating PKC-α function. Moreover, DGK pharmacological inhibitors markedly potentiated the mitogenic effect of IGF-1, supporting the idea that nuclear DGK activity is essential to control DNA replication (Martelli *et al.,* 2000). However, we do not know how DGK-ζ negatively affects DNA and future experiments should address this issue.

DGK-ζ is present also within the nucleus of neural cells (Hozumi *et al.,* 2003) and it was recently found to translocate from the nucleus to the cytoplasm in response to transient ischemia in pyramidal cells in CA1 and in CA3 areas of hippocampus. However, the significance of this relocalization has not yet been established (Ali *et al.,* 2004).

Another nuclear DGK isoform which has been investigated quite extensively is the -ι one. Indeed, it has been shown that nuclear DGK-θ activity increased in either α-thrombin-stimulated IIC9 cells (Bregoli *et al.,* 2001) or in nerve growth factor-challenged PC12 cells (Tabellini *et al.,* 2004). In both cases, activation of nuclear DGK-θ activity has been related to down-regulation of nuclear DG levels.

However, it could not be ruled out that one of the functions of nuclear DGKs is the production of PA. The function of PA (if any) in the nucleus is completely unknown. Certainly, we need to know more about nuclear PA, also taking into account that its synthesis has been reported to be in relationship with the cell cycle in murine erythroleukemia cells (D'Santos *et al.,* 1999). Since the nuclear matrix is considered by some investigators to be the equivalent of the cytoskeleton and contains actin (Gedge *et al.,* 2005), it might be that nuclear PA is some how involved in regulating the structure of this nuclear scaffold.

Concluding remarks and future perspectives

DGKs deeply influence signaling events by metabolizing DG and/or by generating PA. The accumulating evidence indicates that the structural diversity of DGK isotypes is mirrored by the range of different functions and sites of action of these isozymes. Regulation of DGK activity appears to be precisely tuned through localization, lipid and non-lipid cofactors, post-translational modifications (e.g. phoshorylation), availability of substrate. Additional specificity is achieved by binding regulatory proteins (Luo *et al.,* 2004). Our knowledge of nuclear DGK isoform is at present quite sketchy. Forced overexpression of isotypes with deletions in their putative regulatory domains should provide important data about the mechanisms controlling the activity of nuclear DGK. Our understanding at the molecular level of nuclear lipid-dependent signaling pathways is at present evolving quite rapidly. A better knowledge of nuclear DGK isotypes appears desirable mostly because, if it will be determined that they are indeed regulated in a way peculiar to the nuclear compartment, this should then enable rationale drug design to selectively inhibit the relevant nuclear isotypes while sparing those operating at the plasma membrane.

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