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Diacylglycerol Kinase Regulates Tyrosinase Expression and Function in Human Melanocytes

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Abstract

Diacylglycerol increases the melanin content of human melanocytes in vitro and increases the pigmentation of guinea pig skin in vivo, but the mechanism(s) underlying those effects remain unknown. In this study, we characterized the role of diacylglycerol kinase (DGK), which phosphorylates diacylglycerol to generate phosphatidic acid, in the regulation of pigmentation. Ten isoforms of DGK have been identified, and we show that DGKζ is the most abundant isoform expressed by human melanocytic cells. Melanin content, tyrosinase activity and tyrosinase protein levels were significantly reduced by a DGK inhibitor, but tyrosinase and MITF mRNA levels were not changed by that inhibition, and there were no effects on the expression of other melanogenesis-related proteins. Isoform-specific siRNAs showed that knockdown of DGKζ decreased melanin content and tyrosinase expression in melanocytic cells. Over-expression of DGKζ increased tyrosinase protein levels, but did not increase tyrosinase mRNA levels. Glycosidase digestion revealed that inhibition of DGK reduced only the mature form of tyrosinase and the decrease of tyrosinase resulting from DGK inhibition could be blocked partially by protease inhibitors. These results suggest that DGK regulates melanogenesis via modulation of the post-translational processing of tyrosinase, which may be related with the protein degradation machinery.

Keywords

diacylglycerol kinase; tyrosinase; melanogenesis

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CONFLICT OF INTEREST

The authors state no conflict of interest.

INTRODUCTION

Many hormones, growth factors and other cell stimuli evoke a transient increase in levels of cellular diacylglycerol (DAG) through hydrolysis of phosphoinositides by phospholipase C [reviewed in (Sakane *et al.*, 2007; 2008)]. A possible role for DAG in regulating human pigmentation was first reported in a study which showed that DAG increases the melanin content of human melanocytes in culture (Gordon and Gilchrest, 1989). The melanin content induced by DAG could be completely blocked by a protein kinase C (PKC) inhibitor. It has been reported that topical application of DAG increases pigmentation in guinea pig skin in a dose-dependent manner (Allan *et al.*, 1995). Ultraviolet radiation (UVR) induces the formation of DAG in human melanocytes (Carsberg *et al.*, 1995) and therefore, DAG is considered to be an important factor that may regulate melanogenesis in the skin.

To date, 10 isoforms of diacylglycerol kinase (DGK) have been identified and characterized in mammals. DGK phosphorylates DAG to generate phosphatidic acid (PA). DAG and PA are second messengers, which are involved in several major intracellular signal transduction pathways. DGKs differ in their activators, expression patterns, substrate specificities and structural domains. DGKs can be divided into 5 subfamilies according to their distinctive structural motifs and functions, as reviewed in (Mérida *et al.*, 2008; Sakane *et al.*, 2007; 2008). Various DGKs regulate cell cycle progression, apoptosis, migration, protein degradation, endocytosis and protein trafficking from the endoplasmic reticulum (ER) to the Golgi. Recently, it was shown that DGK isoforms are involved in regulating the activation of Akt, Ras, PKC and mitogen-activated protein kinase (MAPK) (Chibalin *et al.*, 2008; Olenchock *et al.*, 2006). DGKs are also known to associate with receptor for activated C-kinase-1 (RACK-1) (Imai *et al.*, 2009). Interestingly, many of those signaling pathways have been shown to regulate melanogenesis (Hemesath *et al.*, 1998; Khaled *et al.*, 2003; Park *et al.*, 2004).

DAG exerts its effects by modulating the activity of a number of enzymes, such as PKC, DGK, α- and β-chimerins, Ras guanyl releasing protein (RasGRP), and protein kinase D (Blumberg *et al.*, 2008). PKCβ has been shown to induce melanogenesis via the activation of tyrosinase (Park *et al.*, 1993; 1999). Moreover, when PKCβ is activated, it binds RACK-1 and translocates to the melanosome membrane. The suppression of RACK-1 by siRNA decreases tyrosinase activity (Park *et al.*, 2004). These results demonstrate that PKCβ is involved in the regulation of tyrosinase activity. However, the role of PKC in melanogenesis remains controversial. Previous reports demonstrated that pretreatment with a PKC inhibitor or a high concentration of 12-*O*-tetradecanoylphorbol 13-acetate, which down-regulates specific isoforms of PKC including PKCα and PKCβ, had no significant effect on DAG-induced melanogenesis. These findings suggest that DAG modulates melanogenesis via a PKC-independent pathway (Carsberg *et al.*, 1994).

DGK phosphorylates DAG to generate phosphatidic acid (PA). DAG and PA are second messengers, which are involved in several major intracellular signal transduction pathways. PA has been shown to activate a number of enzymes involved in signal transduction, including PKCζ, mammalian target of rapamycin (mTOR), Ras GTPase-activating protein (RasGAP), Raf-1 kinase, phosphatidylinositol-4-phosphate 5-kinase (PI5K) and sphingosine

kinase (Sakane *et al.*, 2007; 2008). Therefore, DGK is thought to occupy a central position in signal transduction by controlling the balance between DAG and PA.

To assess the potential melanocyte specific-functions of DGK isoforms, we examined the expression levels of DGK isoforms in normal human epidermal melanocytes (NHEMs) and in several melanoma cell lines. We also examined the effects of DGK on melanogenesis using a DGK inhibitor (R59949), siRNAs and adenovirus-mediated over-expression of DGK. We now report that DGK regulates melanogenesis through modulation of the post-translational processing of tyrosinase.

RESULTS

Expression of DGK isoforms in melanocytic cells

We first examined the expression levels of the various DGK isoforms in pigmented NHEMs, SK-Mel-23 human melanoma cells and B16F10 mouse melanoma cells using RT-PCR (Figure 1a). DGKη and DGKζ were the most abundant DGK isoforms expressed in NHEMs and SK-Mel-23 cells, and DGK† was expressed at a moderate level. In B16F10 murine melanoma cells, several other DGK isoforms in addition to DGKη and DGKζ were also highly expressed. We also confirmed the expression levels of DGK isoforms using real-time RT-PCR (data not shown). Because *DGK*η and DGKζ were the most abundantly expressed isoforms in all melanocytic cell lines examined, we focused our attention on them. Two bands of DGKζ were detected in 293A cells (Figure 1b, arrowheads), which correspond to 2 splicing variants, as previously reported (Ding *et al.*, 1997). Interestingly, the 130 kDa form is expressed at a higher level in NHEMs and in B16F10 cells, whereas the smaller (115 kDa) form predominates in SK-Mel-23 and 293A cells, suggesting cell-specific expression of the two splicing variants.

Effect of a DGK inhibitor on melanin synthesis and tyrosinase activity in NHEMs

To examine the effects of DGK on melanogenesis, we measured the melanin content of NHEMs in the presence of the DGK inhibitor, R59949. This inhibitor is known to inhibit the activity of several DGK isoforms, such as DGKα (Jiang *et al.*, 2000), DGK† (Miele *et al.*, 2007) and DGKζ (Avila-Flores *et al.*, 2005), in a cell-specific manner. Recently, an in vitro assay against a panel of protein kinases demonstrated that R59949 is a specific inhibitor of DGK and does not affect other protein kinases (Chibalin *et al.*, 2008). NHEMs were cultured with R59949 (1 or 2.5 μM) for 72 h, and at those concentrations, R59949 does not affect their proliferation (Suppl Fig 1). However, the melanin content of NHEMs was significantly inhibited by treatment with R59949 at 1 or 2.5 μM (Figure 1c).

To examine the mechanism(s) responsible for the decreased pigmentation induced by the DGK inhibitor, we assayed tyrosinase activity by quantifying L-DOPA oxidation. Consistent with the decrease in melanin content, tyrosinase activity was significantly reduced by R59949 as early as 24 h (Figure 1d). Thus, inhibiting DGK decreases tyrosinase activity, which subsequently decreases melanin synthesis in NHEMs. To examine whether the inhibition of DGK also regulates other melanogenesis-related proteins, NHEMs were treated with R59949 at 1 or 2.5 μM for 24 and for 48 h. Protein expression levels were then

assessed by Western blotting (Figure 2a). The DGK inhibitor diminished tyrosinase expression, but had no effect on levels of TYRP1, DCT or Pmel17 proteins; note that MITF levels at 48 h were slightly decreased.

We next examined MITF and tyrosinase mRNA levels after treatment with the DGK inhibitor. MITF and tyrosinase mRNA levels were not changed by treatment with R59949 for 3-24 h (Figure 2b). These results show that the DGK inhibitor does not decrease melanin synthesis by down-regulating MITF or tyrosinase mRNA levels, but instead suggest that the regulation occurs at a post-translational level.

Involvement of protein degradation in the DGK inhibitor-induced reduction of tyrosinase

We next examined whether the DGK inhibitor-induced reduction of tyrosinase was due to its proteolytic degradation. We used 2 proteasome inhibitors, MG132 (a membrane permeable proteasome inhibitor) and ALLN (a neutral cysteine protease inhibitor that also blocks proteasome-mediated proteolysis). The optimal concentration of each proteasome inhibitor was determined previously (Ando *et al.*, 2004). Treatment with MG132 or ALLN did not rescue the DGK inhibitor-reduced tyrosinase expression completely, but the decrease of tyrosinase induced by R59949 could be partially blocked by either inhibitor after co-incubation for 48 h (Figure 3a).

Inhibition of DGK regulates the degradation of tyrosinase after processing in the ER

The trafficking and stability of tyrosinase is modulated by its glycosylation. Thus, glycosidase digestion was used to evaluate whether the post-translational processing of tyrosinase is disrupted in DGK inhibitor-treated melanocytic cells. EndoH cleaves tyrosinase with early high mannose when it is immature in the ER, but does not affect mature forms of tyrosinase containing complex carbohydrates once it has been processed through the Golgi (Ando *et al.*, 2006). Although the total amount of tyrosinase protein was decreased by treatment with R59949, the inhibitor did not affect the amount of EndoH-sensitive tyrosinase in B16F10 cells or in NHEMs (Figure 3b), showing that it had no effect of de novo synthesis of tyrosinase or its early processing.

Inhibition of DGK diminishes the induction of melanogenesis by αMSH

To investigate the possible effect of the DGK inhibitor on cAMP-induced melanogenesis, we treated B16F10 melanoma cells with αMSH (100 nM) in the presence or absence of R59949 (10-20 μM) and then measured melanin content and tyrosinase protein levels. As expected, melanin content was dramatically increased by αMSH treatment alone. However, melanin content was greatly reduced in cells treated with αMSH in the presence of 20 μM R59949 (Figure 3c). Treatment of B16F10 cells with αMSH also increased levels of tyrosinase protein (Figure 3d), but pretreatment with the DGK inhibitor diminished the level of tyrosinase protein, and blocked the αMSH-induced increase in tyrosinase level. These results indicate that the inhibition of DGK affects cAMP-induced pigmentation and has a potent effect to reduce melanin through its effects on tyrosinase.

Effect of PD98059 and Gö6983 on tyrosinase levels

Some DGK isoforms are known to be involved in the extracellular signal-regulated kinase (ERK) or PKC signaling pathways. To investigate whether the ERK and/or the PKC signaling pathways could interfere with the DGK inhibitor-induced reduction of tyrosinase, we examined tyrosinase protein levels in the presence of R59949 and PD98059, a selective inhibitor of MEK (MAPK/ERK kinase, a specific upstream activator of ERK) or Gö6983 (a PKC inhibitor). NHEMs were treated with R59949 for 24 h in the absence or presence of PD98059 or Gö6983 (added 1 h before addition of the DGK inhibitor). Neither of those inhibitors abrogated the DGK inhibitor-induced reduction of tyrosinase (Figure 3e), which suggests that another signaling pathway could be involved in the DGK inhibitor-induced reduction of tyrosinase.

Effects of DGK silencing on melanogenesis

Treatment with the DGK inhibitor was effective in reducing melanogenesis, but as noted above R59949 is not a DGK isoform-specific inhibitor (Sakane *et al.*, 2008). To determine which DGK isoform(s) is involved in the DGK inhibitor-induced reduction of melanogenesis, we used isoform-specific siRNAs to inhibit the activity of each DGK isoform. First, we examined whether R59949 affects melanin content, tyrosinase activity and/or tyrosinase expression in SK-Mel-23 melanoma cells, which are much easier to transfect than NHEMs. As expected, melanin content, tyrosinase activity and protein levels were all reduced by the DGK inhibitor (Suppl. Figure 2), which is consistent with its effects on NHEMs. SK-Mel-23 cells were then transiently transfected with siRNAs for DGKη and DGKζ or with a control non-specific siRNA. These experiments were performed using 2 different DGK isoform-specific duplexes for each isoform. We examined the expression of DGK isoforms using real-time RT-PCR 48 h after transfection. The results of that analysis confirmed that the mRNA level of each DGK isoform was significantly reduced in cells transfected with the DGK siRNA (Figure 4a, left) and Western blotting confirmed the decreased protein level of each isoform (Figure 4a, right). We observed a reduction of tyrosinase protein level 4 d after transfection with the DGKη or DGKζ siRNAs but there was no significant effect of those 2 DGK isoforms on MITF protein levels (Figure 4b). To confirm the functional role of DGKη and DGKζ in regulating melanin synthesis, we assayed melanin content 6 d after the initial transfection of siRNA, because it takes a longer time to reduce melanin content once tyrosinase activity is decreased. Melanin content was dramatically reduced only by the two DGKζ siRNAs, and not by the DGKη siRNAs (Figure 4c). DGKη siRNA also reduced tyrosinase expression, but the effect was slight compared to the DGKζ siRNA. These data show that DGKζ is specifically involved in the regulation of melanogenesis.

Adenovirus-mediated over-expression of DGKζ increases tyrosinase protein levels

To further characterize the role of DGKζ in modulating tyrosinase function, we examined whether the over-expression of DGKζ affects tyrosinase expression in NHEMs. NHEMs were transduced with 25 or 50 MOI of adenovirus expressing DGKα or DGKζ (Ad-DGKs) for 24 h. Cells received fresh medium and were then further incubated for 3 d. Western blot analysis was then performed to confirm the adenovirus-mediated expression of DGKs.

Protein levels of DGKα or DGKζ increased in dose-dependent manners (Suppl Fig. 3). On the basis of these results, NHEMs were used for the following experiments 72 h after transduction with 25 or 50 MOI Ad-DGKs. Consistently, the adenovirus-mediated over-expression of DGKζ was the only DGK isoform that increased tyrosinase protein levels in NHEMs (and MITF to some extent) (Figure 5a), but without a substantial increase of tyrosinase mRNA levels (Figure 5b). Based on these results, we conclude that DGKζ regulates tyrosinase at the post-transcriptional level.

DISCUSSION

DGKs contribute to diverse activities in various tissues and cells (Sakane *et al.*, 2007; 2008). DAG is known to regulate melanogenic functions in PKC- dependent and independent manners (Allan *et al.*, 1995; Carsberg *et al.*, 1994; 1995; Gordon and Gilchrest, 1989; Park *et al.*, 1993; 1999; 2004). In this study, we investigated the effects of DGKs on melanogenesis in NHEMs and in melanoma cell lines. First, we demonstrate that inhibition of DGK activity reduces melanin content in NHEMs at a concentration where it has no effect on cell number or cell viability. Inhibition of DGK reduced levels of tyrosinase protein and melanin content in all melanocytic cell lines examined. One difference between the cell lines is that melanoma cell lines (B16F10 and SK-Mel-23) are much less sensitive to R59949 than are NHEMs and require higher concentrations to reduce levels of melanin content and tyrosinase compared with NHEMs (10-20 μM vs 1-2.5 μM).

Our results with glycosidase digestion by EndoH reveal that a post-transcriptionally mediated decrease in mature tyrosinase protein accounts for the DGK inhibitor-elicited reduction in tyrosinase activity. As shown in Fig 5c, our model suggests that the DGK inhibitor treatment disrupts the amount of mature tyrosinase that reaches melanosomes, which results in a dramatic decrease of functional tyrosinase and melanin synthesis. The decrease of tyrosinase protein levels induced by the DGK inhibitor could be blocked slightly by proteasome inhibitors, which suggests that it reduces melanin synthesis by accelerating the degradation of tyrosinase.

Levels of proteins are regulated by a balance between their synthesis and degradation. The maturation of tyrosinase is inefficient with only approximately 50% of protein passing the quality control test and reaching its mature form under optimal conditions (Halaban *et al.*, 1997). Since the DGK inhibitor did not reduce MITF or tyrosinase mRNAs, and reduced only the mature form of tyrosinase, the DGK inhibitor-induced decrease of tyrosinase is a post-transcriptionally mediated decrease in the mature form of tyrosinase, which is involved with the protein degradation machinery. However, little attention has been paid to regulating tyrosinase degradation, which depends on tyrosinase processing and maturation in the ER and Golgi, and its degradation via the ubiquitin proteasome system and/or the endosomal/lysosomal system (Ando *et al.*, 2007). Tyrosinase destined for degradation in the ER is proteolyzed by proteasomes via ER-associated protein degradation (ERAD). Fatty acids (Ando *et al.*, 2004; 2006), phospholipase D2 (Kageyama *et al.*, 2004) and p38 (Bellei *et al.*, 2010) are known to accelerate the degradation of tyrosinase by increasing the proteasomal degradation of tyrosinase. In addition, tyrosinase degradation also occurs following its complete maturation in the Golgi. In fact, tyrosinase processed beyond the post-Golgi stage

can be rapidly degraded by integration into ERAD, as observed in linoleic acid-treated melanoma cells (Ando *et al.*, 2006), or by the endosomal/lysosomal system, as observed in phenylthiourea (PTU)-treated melanoma cells (Hall and Orlow, 2005).

The retention of tyrosinase in the ER/Golgi also occurs for mutant forms of tyrosinase and TYRP1 that cause types I and 3 oculocutaneous albinism, respectively, (Halaban *et al.*, 2000; Toyofuku *et al.*, 2001) and in human amelanotic melanoma cells (Halaban *et al.*, 1997). The sum of those studies suggests that tyrosinase degradation is an effective and physiological way to regulate melanogenesis. Interestingly, fatty acids are known to regulate the growth factor induced activation of DGKs in vascular smooth muscle cells (Du *et al.*, 2001). DGKα is the major DGK isoform in vascular smooth muscle, and growth factors increase DGKα activity. Fatty acids inhibit the DGK activity induced by the growth factors. On the other hand, the DGK inhibitor increases DAG levels, and the accumulation of DAG increases PKC activity (Chibalin *et al.*, 2008). PKCα is known to enhance phospholipase D (PLD) activity in human melanoma cells (Oka *et al.*, 2003). PKC inhibitors increase melanin content and tyrosinase protein levels in B16F10 cells. over-expression of PKCα prevents basal and forskolin induced-tyrosinase promoter activity in B16F10 cells (Bertolotto *et al.*, 1998). Therefore, DGK might act as a downstream signal for fatty acids, or as an upstream signal for PLD, regulating melanogenesis through the degradation of tyrosinase.

The presence of PKCα and absence of PKCβ in melanoma cell lines, including B16 and SK-Mel-23, has been reported (Oka and Kikkawa, 2005). Therefore, differences in the expression of PKC isoforms could be one reason for different responses between cell lines. Further, DGKζ$^{-/-}$ cells demonstrated impaired recruitment of PKCβ to the membrane (Olenchock *et al.*, 2006). PKCβ is known to be involved in regulating melanogenesis in NHEMs, and therefore DGK might function in part by regulating the activity of PKCβ.

To examine the isoform-specific effect of DGK in melanogenesis, we used siRNAs to inhibit DGK activity. We found that the knockdown of DGKζ decreased melanin content and levels of tyrosinase protein in SK-Mel-23 cells. To characterize the role of DGKs in modulating tyrosinase levels, we examined whether the over-expression of DGKζ affects tyrosinase levels in NHEMs. Interestingly, the adenovirus-mediated over-expression of DGKζ increased levels of tyrosinase protein in NHEMs, but did not affect tyrosinase mRNA levels. Based on these results, we speculate that DGKζ regulates tyrosinase function at the protein level. Since DGK does not regulate the expression of tyrosinase directly, the increase of tyrosinase protein associated with over-expression of DGKζ appears to be due to inhibition of tyrosinase degradation. In addition, we examined whether the over-expression of DGKζ rescues the DGK inhibitor-induced decrease of tyrosinase in NHEMs. However, over-expression of DGKζ did not rescue the DGK inhibitor-induced decrease of tyrosinase. These data suggest that not only DGKζ but also other DGK isoform(s) may be involved in the DGK inhibitor-induced decrease of tyrosinase.

It has been demonstrated that DAG and PA are lipids with important functions in membrane trafficking (Haucke and Di, 2007; Roth, 1999). DAG binds to and activates various proteins needed for vesicle formation, such as protein kinase D, ADP-ribosylation factor (Arf) and GTPase-activating protein (Haucke and Di, 2007; Roth, 1999). PA can also bind to and

activate enzymes, such as coatomer, Arf and kinesin (Manifava *et al.*, 2001). It has also been demonstrated that DGKδ suppresses ER to Golgi trafficking (Nagaya *et al.*, 2002) and DGKδ is also involved in endocytosis through its binding to adaptor protein 2α (AP2α) (Kawasaki *et al.*, 2008; Pelkmans *et al.*, 2005). In addition, DGKζ is known to interact with several PDZ (post synaptic density protein, disc-large, and zonula occludens-1)-containing proteins, including syntrophin, PSD-95 and sorting nexin 27 (Kim *et al.*, 2010). Functionally, DGKζ over-expression increases spine density in cultured neurons in a manner that requires DGK catalytic activity and PSD-95 binding. Conversely, knockdown of DGKζ in cultured neurons decreases spine density, an effect that is rescued by co-expression of wild-type DGKζ, but not by a DGKζ mutant that lacks catalytic activity or PSD-95 binding (Kim *et al.*, 2010). Therefore DGK activity is thought to be necessary for the interaction between DGK and other proteins. In melanocytes, the PDZ domain protein GIPC is known to interact with TYRP1 in the Golgi region and it was proposed that this interaction is involved in the trafficking of TYRP1 along the endosomal pathway (Kedlaya *et al.*, 2011). The identification of target molecules for DGK would allow us to more fully understand the detailed machinery that regulates melanogenesis.

In summary, we demonstrate that DGK is involved in the regulation of melanogenesis through the modulation of tyrosinase protein levels, which is regulated by the protein degradation machinery. The DGKζ isoform is clearly involved in that process, and it remains possible that other DGK isoforms might also play a role. Our study sheds new light on the dynamic regulation of tyrosinase and provides additional evidence on the important role of DGK in post-translational processing of tyrosinase in melanocytes.

MATERIALS AND METHODS

Chemicals and antibodies used

R59949, PD98059, Gö6983, MG-132, ALLN and αMSH were from Calbiochem (San Diego, CA) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), synthetic melanin and L-DOPA were from Sigma Aldrich (St. Louis, MO).

DGKα and DGKη antibodies were from ProteinTech (Chicago, IL). The DGKζ (N-19) antibody was from Santa Cruz Biotech (Santa Cruz, CA), the microphthalmia-associated transcription factor (MITF) antibody (C5) was from Thermo Scientific (Fremont, CA) and the β-actin antibody was from Abcam (Cambridge, MA). Antibodies to tyrosinase (αPEP7h, αPEP7), tyrosinase-related protein-1 (TYRP1) (αPEP1h), DOPAchrome tautomerase (DCT) (αPEP8h) and Pmel17 (αPEP13h) were from our own laboratory (Virador *et al.*, 2001).

Cell culture

Lightly pigmented NHEMs were obtained from Cascade Biologics (Portland, OR) and were cultured in melanocyte growth medium consisting of Medium 254 and human melanocyte growth supplement (Cascade Biologics) with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO_2 in air at 37°C. NHEMs from the 3rd to 7th passages were used in these experiments. B16F10 mouse melanoma cells and SK-Mel-23 human pigmented melanoma cells were cultured in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). The medium was changed every second day. 293A cells (Invitrogen) were cultured according to the manufacturer's instructions.

PCR primers

Pre-designed primer sets used in this study are listed in the Supplemental Information.

RNA extraction, RT-PCR and real-time quantitative RT-PCR

Details of the RNA extraction and real-time RT-PCR procedures and data analysis are listed in the Supplemental Methods.

Glycosidase digestion

Cell extracts (5 μg protein) were digested with 1,000 U EndoH (New England Biolabs, Beverly, MA) for 3 h at 37°C. After the digestion, cell extracts were mixed with Tris-glycine SDS sample buffer (Invitrogen) supplemented with 2-mercaptoethanol and were boiled for 5 min. Samples were subjected to SDS-PAGE, and immunoreactive bands were detected by Western blotting using the αPEP7 or αPEP7h antibodies.

Western blotting analysis

Details of the Western blotting analysis are provided in the Supplemental Methods.

Melanin content and tyrosinase assays

Details of these assays are provided in the Supplemental Methods.

Targeted inhibition using siRNA

Details of the small interfering RNA (siRNA) procedures are listed in the Supplemental Methods.

Recombinant adenoviral vectors construction and infection

Details of the lentiviral vectors used and infection procedures are provided in the Supplemental Methods.

Statistical analysis

The results were averaged over 3 or 4 independent experiments. *P* values were calculated using Student's *t* test, and a $P < 0.05$ is considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DAG	diacylglycerol
DGK	diacylglycerol kinase
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
MAPK	mitogen-activated protein kinase
NHEMs	normal human epidermal melanocytes
PA	phosphatidic acid
PKC	protein kinase C
UVR	ultraviolet radiation

REFERENCES

Allan AE, Archambault M, Messana E, et al. Topically applied diacylglycerols increase pigmentation in guinea pig skin. J Invest Dermatol. 1995; 105:687–692. [PubMed: 7594645]

Ando H, Kondoh H, Ichihashi M, et al. Approaches to identify inhibitors of melanin biosynthesis via the quality control of tyrosinase. J Invest Dermatol. 2007; 127:751–761. [PubMed: 17218941]

Ando H, Watabe H, Valencia JC, et al. Fatty acids regulate pigmentation via proteasomal degradation of tyrosinase - a new aspect of ubiquitin-proteasome function. J Biol Chem. 2004; 279:15427–15433. [PubMed: 14739285]

Ando H, Wen Z-M, Kim H-Y, et al. Intracellular composition of fatty acid affects the processing and function of tyrosinase through the ubiquitin-proteasome pathway. Biochem J. 2006; 394:43–50. [PubMed: 16232122]

Avila-Flores A, Santos T, Rincon E, et al. Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. J Biol Chem. 2005; 280:10091–10099. [PubMed: 15632115]

Bellei B, Maresca V, Flori E, et al. p38 regulates pigmentation via proteasomal degradation of tyrosinase. J Biol Chem. 2010; 285:7288–7299. [PubMed: 20053998]

Bertolotto C, Bille K, Ortonne JP, et al. In B16 melanoma cells, the inhibition of melanogenesis by TPA results from PKC activation and diminution of microphthalmia binding to the M-box of the tyrosinase promoter. Oncogene. 1998; 16:1665–1670. [PubMed: 9582014]

Blumberg PM, Kedei N, Lewin NE, et al. Wealth of opportunity - the C1 domain as a target for drug development. Curr Drug Targets. 2008; 9:641–652. [PubMed: 18691011]

Carsberg CJ, Ohanian J, Friedmann PS. Ultraviolet radiation stimulates a biphasic pattern of 1,2-diacylglycerol formation in cultured human melanocytes and keratinocytes by activation of phospholipases C and D. Biochem J. 1995; 305:471–477. [PubMed: 7832762]

Carsberg CJ, Warenius HM, Friedmann PS. Ultraviolet radiation-induced melanogenesis in human melanocytes. Effects of modulating protein kinase C. J Cell Sci. 1994; 107:2591–2597. [PubMed: 7531203]

Chibalin AV, Leng Y, Vieira E, et al. Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. Cell. 2008; 132:375–386. [PubMed: 18267070]

Ding L, Bunting M, Topham MK, et al. Alternative splicing of the human diacylglycerol kinase zeta gene in muscle. Proc Natl Acad Sci U S A. 1997; 94:5519–5524. [PubMed: 9159104]

Du X, Jiang Y, Qian W, et al. Fatty acids inhibit growth-factor-induced diacylglycerol kinase alpha activation in vascular smooth-muscle cells. Biochem J. 2001; 357:275–282. [PubMed: 11415460]

Gordon PR, Gilchrest BA. Human melanogenesis is stimulated by diacylglycerol. J Invest Dermatol. 1989; 93:700–702. [PubMed: 2794552]

Halaban R, Cheng E, Zhang Y, et al. Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. Proc Natl Acad Sci USA. 1997; 94:6210–6215. [PubMed: 9177196]

Halaban R, Svedine S, Cheng E, et al. Endoplasmic reticulum retention is a common defect associated with tyrosinase-negative albinism. Proc Natl Acad Sci USA. 2000; 97:5889–5894. [PubMed: 10823941]

Hall AM, Orlow SJ. Degradation of tyrosinase induced by phenylthiourea occurs following Golgi maturation. Pigment Cell Res. 2005; 18:122–129. [PubMed: 15760341]

Haucke V, Di PG. Lipids and lipid modifications in the regulation of membrane traffic. Curr Opin Cell Biol. 2007; 19:426–435. [PubMed: 17651957]

Hemesath TJ, Price ER, Takemoto C, et al. MAP kinase links the transcription factor microphthalmia to c-kit signalling in melanocytes. Nature. 1998; 391:298–301. [PubMed: 9440696]

Imai S, Yasuda S, Kai M, et al. Diacylglycerol kinase delta associates with receptor for activated C kinase 1, RACK1. Biochim Biophys Acta. 2009; 1791:246–253. [PubMed: 19416640]

Jiang Y, Sakane F, Kanoh H, et al. Selectivity of the diacylglycerol kinase inhibitor 3-[2-(4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl]-2, 3-dihydro-2-thioxo-4(1H)quinazolinone (R59949) among diacylglycerol kinase subtypes. Biochem Pharmacol. 2000; 59:763–772. [PubMed: 10718334]

Kageyama A, Oka M, Okada T, et al. Down-regulation of melanogenesis by phospholipase D2 through ubiquitin proteasome-mediated degradation of tyrosinase. J Biol Chem. 2004; 279:27774–27780. [PubMed: 15067002]

Kawasaki T, Kobayashi T, Ueyama T, et al. Regulation of clathrin-dependent endocytosis by diacylglycerol kinase delta: importance of kinase activity and binding to AP2alpha. Biochem J. 2008; 409:471–479. [PubMed: 17880279]

Kedlaya R, Kandala G, Liu TF, et al. Interactions between GIPC-APPL and GIPC-TRP1 regulate melanosomal protein trafficking and melanogenesis in human melanocytes. Arch Biochem Biophys. 2011; 508:227–233. [PubMed: 21291857]

Khaled M, Larribere L, Bille K, et al. Microphthalmia associated transcription factor is a target of the phosphatidylinositol-3-kinase pathway. J Invest Dermatol. 2003; 121:831–836. [PubMed: 14632202]

Kim K, Yang J, Kim E. Diacylglycerol kinases in the regulation of dendritic spines. J Neurochem. 2010; 112:577–587. [PubMed: 19922438]

Manifava M, Thuring JW, Lim ZY, et al. Differential binding of traffic-related proteins to phosphatidic acid- or phosphatidylinositol (4,5)- bisphosphate-coupled affinity reagents. J Biol Chem. 2001; 276:8987–8994. [PubMed: 11124268]

Mérida I, vila-Flores A, Merino E. Diacylglycerol kinases: at the hub of cell signalling. Biochem J. 2008; 409:1–18. [PubMed: 18062770]

Miele C, Paturzo F, Teperino R, et al. Glucose regulates diacylglycerol intracellular levels and protein kinase C activity by modulating diacylglycerol kinase subcellular localization. J Biol Chem. 2007; 282:31835–31843. [PubMed: 17675299]

Nagaya H, Wada I, Jia YJ, et al. Diacylglycerol kinase delta suppresses ER-to-Golgi traffic via its SAM and PH domains. Mol Biol Cell. 2002; 13:302–316. [PubMed: 11809841]

Oka M, Kageshita T, Ono T, et al. Protein kinase C alpha associates with phospholipase D1 and enhances basal phospholipase D activity in a protein phosphorylation-independent manner in human melanoma cells. J Invest Dermatol. 2003; 121:69–76. [PubMed: 12839565]

Oka M, Kikkawa U. Protein kinase C in melanoma. Cancer Metastasis Rev. 2005; 24:287–300. [PubMed: 15986138]

Olenchock BA, Guo R, Silverman MA, et al. Impaired degranulation but enhanced cytokine production after Fc epsilonRI stimulation of diacylglycerol kinase zeta-deficient mast cells. J Exp Med. 2006; 203:1471–1480. [PubMed: 16717114]

Park HY, Perez JM, Laursen R, et al. Protein kinase C-β activates tyrosinase by phosphorylating serine residues in its cytoplasmic domain. J Biol Chem. 1999; 274:16470–16478. [PubMed: 10347209]

Park HY, Russakovsky V, Ohno S, et al. The β isoform of protein kinase C stimulates human melanogenesis by activating tyrosinase in pigment cells. J Biol Chem. 1993; 268:11742–11749. [PubMed: 7685020]

Park HY, Wu H, Killoran CE, et al. The receptor for activated C-kinase-I (RACK-I) anchors activated PKC-β on melanosomes. J Cell Sci. 2004; 117:3659–3668. [PubMed: 15252133]

Pelkmans L, Fava E, Grabner H, et al. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. Nature. 2005; 436:78–86. [PubMed: 15889048]

Roth MG. Lipid regulators of membrane traffic through the Golgi complex. Trends Cell Biol. 1999; 9:174–179. [PubMed: 10322451]

Sakane F, Imai S, Kai M, et al. Diacylglycerol kinases: why so many of them? Biochim Biophys Acta. 2007; 1771:793–806. [PubMed: 17512245]

Sakane F, Imai S, Kai M, et al. Diacylglycerol kinases as emerging potential drug targets for a variety of diseases. Curr Drug Targets. 2008; 9:626–640. [PubMed: 18691010]

Toyofuku K, Wada I, Valencia JC, et al. Oculocutaneous albinism (OCA) types 1 and 3 are ER retention diseases: Mutations in tyrosinase and/or Tyrp1 influence the maturation, degradation of calnexin association of the other. FASEB J. 2001; 15:2149–2161. [PubMed: 11641241]

Virador V, Matsunaga N, Matsunaga J, et al. Production of melanocyte-specific antibodies to human melanosomal proteins: Expression patterns in normal human skin and in cutaneous pigmented lesions. Pigment Cell Res. 2001; 14:289–297. [PubMed: 11549113]

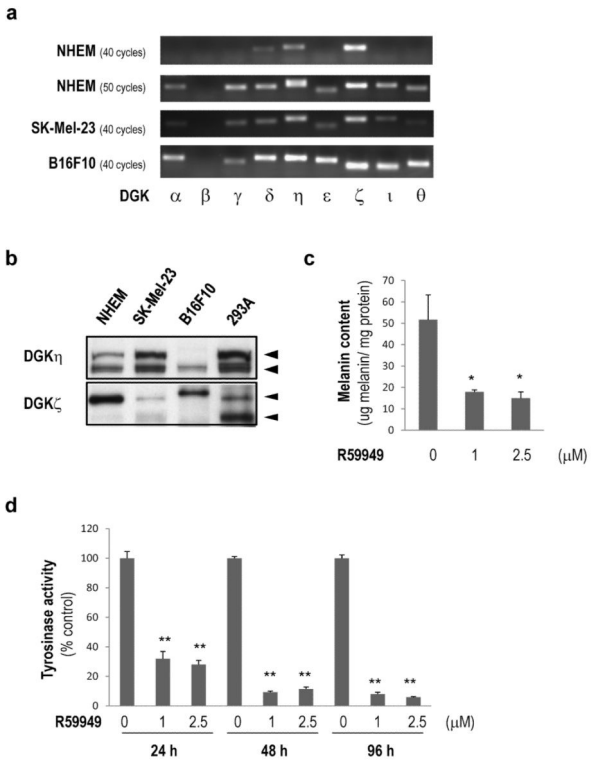

Figure 1. Expression of DGK isoform mRNAs and proteins in melanocytic cells and effects of levels of tyrosinase and melanin

(a) Expression levels of DGK isoform mRNAs examined by RT-PCR. **(b)** Expression of DGKη and DGKζ examined by Western blot analysis using anti-DGK antibodies. Each lane represents lysates from NHEMs, SK-Mel-23 cells, B16F10 cells and A293 cells; arrowheads indicate the bands of interest. **(c)** Melanin content in NHEMs treated with the DGK inhibitor. Results are averages of 3 independent experiments ± S.D. *P<0.05 compared to the control. **(d)** NHEMs were cultured with 1-2.5 μM DGK inhibitor and tyrosinase activity was examined; results are averages of 3 independent experiments ± S.D. **P<0.01 compared to the control at each time point.

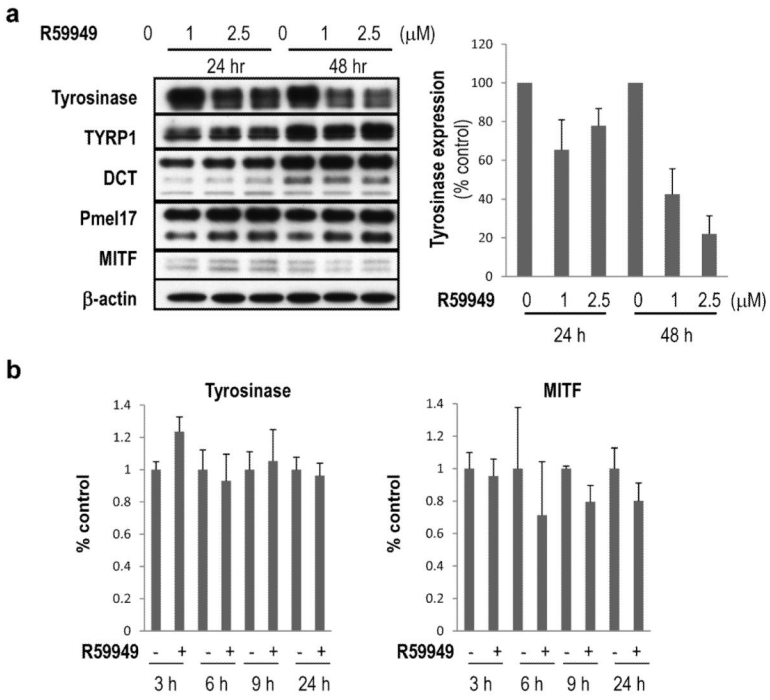

Figure 2. Effect of the DGK inhibitor on melanogenesis and on the expression of melanocyte-specific factors in NHEMs

melanogenesis-related proteins analyzed by Western blot **(a)** and real-time RT-PCR analysis **(b)** to characterize tyrosinase and MITF mRNA levels in DGK inhibitor-treated cells. Results are averages of 3 independent experiments ± S.D.

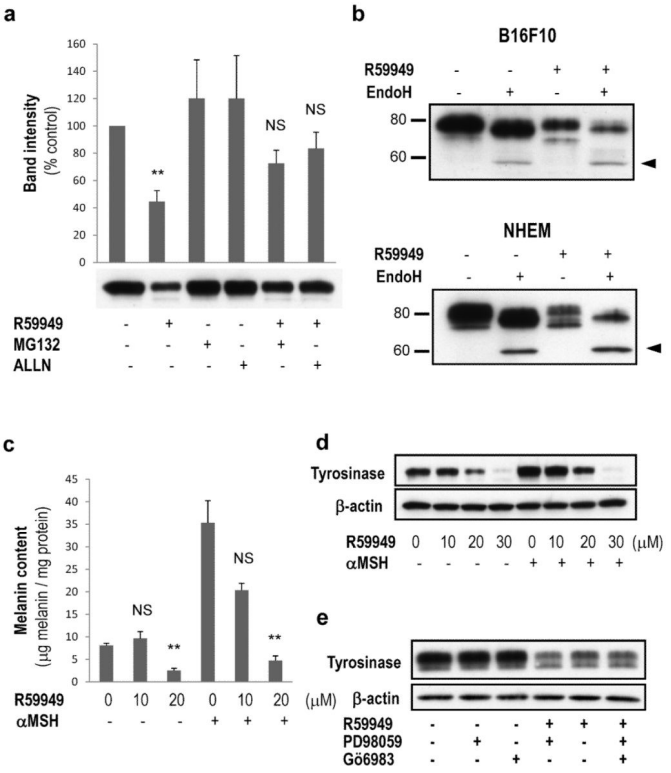

Figure 3. Effect of the DGK inhibitor on the post-translational processing of tyrosinase and proteasomal degradation

(a) Levels of tyrosinase in B16F10 cells after treatment with the DGK inhibitor (20 μM) for 48 h in the presence or absence of proteasome or lysosomal protease inhibitors (120 nM MG132 or 3 μM ALLN); results are expressed as the % of the untreated control in each experiment and are means ± S.D. for triplicate determinations. **P<0.01 compared to the relevant control without the inhibitor; NS = not significant. **(b)** B16F10 cells or NHEMs (5 μg protein/lane) were treated with the DGK inhibitor and extracts of those cells were then digested with or without EndoH as noted, after which immunoreactive tyrosinase bands were detected by Western blotting. Arrowheads indicate the position of the unglycosylated tyrosinase band. Each experiment was performed 3 times with similar results. **(c)** Melanin content in B16F10 cells treated with αMSH in the presence or absence of the DGK inhibitor (10 to 30 μM) for 48 h; αMSH was added to the culture medium 1 h after treatment with the DGK inhibitor. Results are averages of 3 independent experiments ± S.D. **P<0.01 compared to the relevant control with or without αMSH; NS = not significant. **(d)** Effect of αMSH and R59949 on tyrosinase protein levels; experiments were carried out as detailed for 3c above, and were repeated 3 times with similar results. **(e)** NHEMs were treated with the DGK inhibitor (1 μM) for 24 h in the absence or presence of PD98059 (20 μM) or Gö6983 (1 μM), and tyrosinase protein levels were examined. This experiment was performed 3 times with similar results.

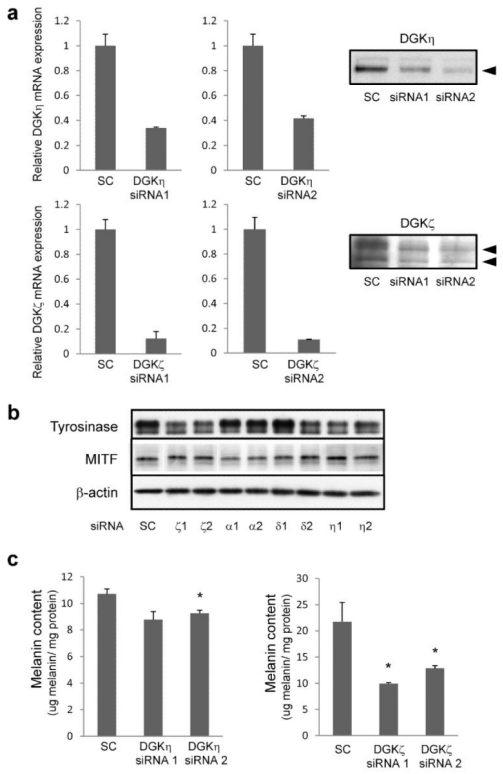

Figure 4. Effect of the DGK inhibition on melanogenesis in SK-Mel-23 cells

(a) Efficiency of siRNAs on the expression of DGKζ or DGKζ isoforms in SK-Mel-23 cells. The expression level of each DGK isoform was determined by real-time RT-PCR. Western blots on the right show protein levels of DGKζ or DGKζ isoforms in the siRNA-treated cells as noted. **(b)** Effect of DGK isoform silencing on tyrosinase and MITF protein levels. **(c)** Effect of DGKη or DGKζ silencing on melanin content. Results are averages of 3 independent experiments ± S.D. *P< 0.05 compared to the control (SC).

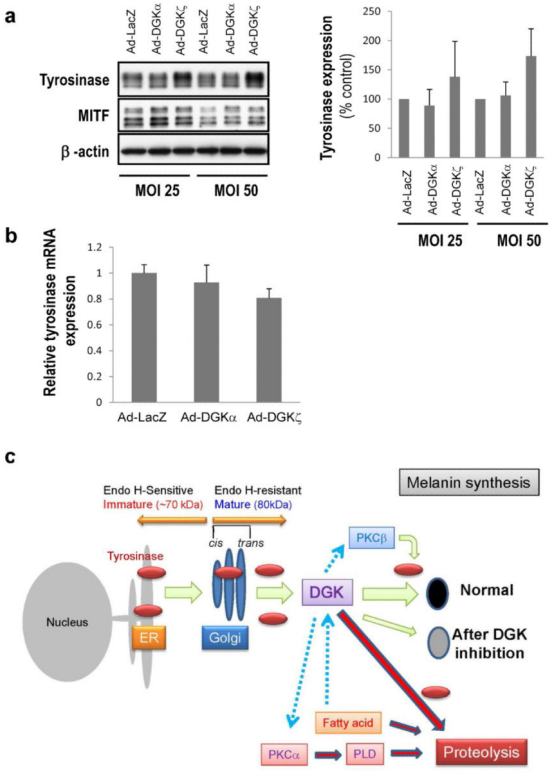

Figure 5. Effect of over-expression of DGKζ on tyrosinase levels

(a) NHEMs were transduced with 25 or 50 MOI of adenovirus expressing DGKα or DGKζ for 24 h, and 48 h after transduction, tyrosinase and MITF levels were examined by Western blotting; experiments were performed 3 times with similar results. **(b)** Real-time RT-PCR analysis of tyrosinase mRNA levels; results are averages of 3 independent experiments ± S.D. no significant differences were noted. **(c)** Scheme summarizing the action of DGK to regulate the function of tyrosinase. Tyrosinase enters the *cis*-Golgi as an EndoH-sensitive (~70 kDa) protein and exits the *trans*-Golgi network as an EndoH-resistant (~80 kDa) protein. After maturation in the Golgi, tyrosinase is trafficked either to melanosomes for melanin synthesis or to the proteolytic degradation machinery. The proteolysis of tyrosinase is divided into two pathways, one that is integrated into the ER associated degradation in the ubiquitin proteasome system, while the other is integrated into the endosomal/lysosomal degradation system. Inhibition of DGK activity disrupts the post-translational processing of tyrosinase from the Golgi, which leads to a dramatic decrease of functional tyrosinase. Dotted arrows represent processes reported in other studies as discussed in the text