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Molecular Cloning and Characterization of the Human Diacylglycerol Kinase β (DGKβ) Gene

ALTERNATIVE SPLICING GENERATES DGKβ ISOTYPES WITH DIFFERENT PROPERTIES*

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Diacylglycerol kinases are key modulators of levels of diacylglycerol, a second messenger involved in a variety of cellular responses to extracellular stimuli. A number of diacylglycerol kinases encoded by separate genes are present in mammalian genomes. We have cloned cDNAs encoding several isoforms of the human homologue of the rat diacylglycerol kinase β gene and characterized two such isoforms that differ at their carboxyl terminus through alternative splicing and the usage of different polyadenylation signals. Quantitative analysis of gene expression in a panel of human tissue cDNAs revealed that transcripts corresponding to both isoforms are co-expressed in central nervous system tissues and in the uterus, with one variant being expressed at relatively higher levels. As green fluorescent protein fusions, the two isoforms displayed localization to different subcellular compartments, with one variant being associated with the plasma membrane, while the other isoform was predominantly localized within the cytoplasm. Differences were also observed in their subcellular localization in response to phorbol ester stimulation. Enzymatic assays demonstrated that the two isoforms display comparable diacylglycerol kinase activities. Therefore, the human diacylglycerol kinase β gene can generate several enzyme isoforms, which can display different expression levels and subcellular localization but similar enzymatic activities *in vitro*.

Diacylglycerol (DG)[1] represents a key signaling intermediate downstream of Gq/phospholipase C-β coupled receptors. It is synthesized by phospholipase C-β from phosphoinositides as a response to a variety of molecules involved in intercellular communication, including hormones, neurotransmitters, and growth factors. The best known target of DG action is protein kinase C (PKC), whose activity plays a central role in the control of proliferation and differentiation of many different cell types (1). In addition, DG can also modulate the activity of intracellular proteins such as members of the Rho and Ras families, thus potentially affecting other cellular functions such as cytoskeletal organization (2). The control of steady-state cellular levels of DG is therefore crucial to normal cellular physiology. The signaling properties of DG are terminated by its conversion to phosphatidic acid (itself a second messenger, reviewed in Ref. 3, through the action of diacylglycerol kinases (DGKs), a class of evolutionary conserved enzymes presently counting nine mammalian subtypes encoded by separate genes (α, β, γ, δ, ϵ, ζ, η, θ, ι; reviewed in Ref. 4). The cloning of DGK genes from several mammalian species (5–19) has allowed an analysis of the domain architecture of these proteins. Structurally, mammalian DGK subtypes are characterized by the presence of conserved domains, common to all subtypes and of additional subtype-specific functional domains, which allow their grouping into five separate classes (3, 4). The functional domains shared by all DGK subtypes comprise the catalytic (kinase) domain and a cysteine-rich domain with homology to the C1A and C1B motifs of PKC. By analogy with the C1A and C1B motifs in PKC, the cysteine-rich domains of DGK have been proposed to bind DG and present it to the catalytic domain (4, 20). However, a functional analysis of DGKα, DGKβ, and DGKγ demonstrated that the catalytic domain in these proteins lies in the COOH-terminal region, outside the zinc finger domain (21). The subtype-specific functional domains (present in different DGK classes) include motifs of known function (*e.g.* calcium binding motifs, pleckstrin homology domains, myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation site domains) as well as domains showing significant sequence conservation but whose function remains unknown (*e.g.* the C1 domain of Class I DGKs) (4). Very recently, plant DGK isoforms generated from the same gene through alternative splicing have been characterized which differ at their COOH terminus for the presence of a calmodulin-binding domain (22); however, the presence of calmodulin-binding domains in mammalian DGK enzymes has not been described to date. The precise function of the subtype-specific protein motifs is, in many instances, still unclear. However, the diversity and complexity of DGK enzymes indicates the potential for regulation of DGK activity at several levels, including subcellular localization (e.g. see Refs. 12, 19, and 23), substrate specificity (*e.g.* see Refs. 11, 15, and 16), and modulation of kinase activity (*e.g.* see Refs. 24–26).

While the expression of DGK genes in non-central nervous system tissues is relatively limited (except perhaps for the

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[1] The abbreviations used are: DG, diacylglycerol; PKC, protein kinase C; DGK, diacylglycerol kinase; CRD, cysteine-rich domain; RT, reverse transcription; EST, expressed sequence tag; DGKβ STD, standard human DGKβ isoform; DGKβ SV3′, splice variant 3′ human DGKβ isoform; GFP, green fluorescence protein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

genes encoding DGKε and DGKθ), the brain is a major site of DGK gene expression (4, 27, 28), implicating functional diversification of DGK enzymes in this organ. Recently, a detailed analysis has been carried out of DGK expression in rat brain, with special reference to DGKα, DGKβ, and DGKγ (28), showing localized expression in distinct brain regions for these DGK subtypes. DGKβ is a member of class I DGKs. Like the other members of this subgroup (the DGKα and DGKγ subtypes), it is characterized by the presence of a calcium binding (EF-hand) motif and an additional 70-amino acid domain of uncharacterized function, in addition to the kinase domain and the cysteine-rich domain motifs shared with all DGKs. To date, the only mammalian DGKβ gene for which a full-length cDNA is available has been cloned from the rat (8). In that study, expression of rat DGKβ was found to be predominant in the adult brain, with lower expression levels in spleen, adrenal, small intestine, and heart. In the rat brain, DGKβ expression localizes to the caudate putamen, the nucleus accumbens, the olfactory bulb and tubercle, and the hippocampal pyramidal cell layer (28). The enzymatic properties of DGKβ and its expression pattern make it an important modulator of PKC activation in response to signaling downstream of G protein-coupled receptors, in brain regions representing important glutamatergic, dopaminergic, acetylcholinergic, and serotonergic terminal fields (29). Given the important role proposed for PKC in the control of mood, learning, and memory (*e.g.* see Ref. 30), modulation of the DG pool in these brain areas by DGKβ may be relevant to cognitive and emotional aspects of central nervous system function.

We have previously reported the identification and characterization of the human DGKβ locus using bioinformatics tools and presented RT-PCR evidence supporting the expression of mRNAs from this locus and the existence of alternative splicing events capable of generating a number of isoforms (31). In particular, one of the transcripts arising from the human DGKβ locus corresponds to an EST annotated in GenBank™ as differentially expressed in bipolar disorder patients (31), which can encode a DGKβ protein displaying a COOH-terminal truncation downstream of the catalytic domain. This EST potentially implicates the control of DGKβ splicing in mood disorders, although nothing is known of the properties of the encoded protein variant and of the relative abundance of its transcripts. Here we report the cloning of cDNAs encoding multiple DGKβ isoforms from human brain, confirming that alternative splicing of the DGKβ locus yields a number of transcripts encoding different DGKβ isoforms. We have then focused our analysis on the alternative splicing event potentially associated with mood disorders, which can generate DGKβ isoforms differing at their COOH terminus, and show that the encoded proteins display comparable enzymatic activities but different expression levels, responses to increased substrate availability and subcellular localization. The potential implications of these findings for DG signaling in the context of normal and aberrant neuronal physiology are discussed.

EXPERIMENTAL PROCEDURES

PCR Amplification Employing a Proofreading Thermostable DNA Polymerase, Molecular Cloning, and Automated DNA Sequencing—PCR amplification was carried out employing the GeneAmp XL PCR kit (PE Biosystems, Branchburg, NJ), using human adult and fetal brain cDNA (see below) as a template. Reaction conditions (for primers, dNTP, and enzyme) were according to manufacturer's protocol, with a final $Mg(OAc)_2$ concentration of 0.8 mM. Primer sequences were as follows: primer 5HDAGKFOR (5′-primer), 5′-ATGACAAACCAGGAAAAATGG-3′; DAGKF2REV (3′-primer for STD isoform), 5′-AGGATTATTCCTTGCTTCGG-3′; DAGK7REV (3′-primer for SV3′ isoform), 5′-AGCTAAATCATTGCCAAGGG-3′. Reaction details were as follows: 94 °C/3 min; 45×(94 °C/30 s; 55 °C/30 s; 72 °C/5 min); 72 °C/20 min. PCR products were analyzed by electrophoresis on a 1% agarose gel poured and run in 1 × TAE (Tris acetate-EDTA) buffer (32). The product was purified on a Qiaex PCR chromatography column (Qiagen GmbH, Hilden, Germany) and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted from several clones using Quiaex Miniprep columns (Qiagen) and subjected to automated DNA sequencing by standard protocols using an ABI377 machine (PE Biosystems).

Tissue Culture, RNA Isolation, and Northern Blotting—Total RNA was extracted from rat tissues as described (33). Neuro2-A, PC12, and NB-OK-1 cells were cultured as described previously (34). Polyadenylated RNA was extracted from subconfluent monolayers employing a modified oligo(dT)-cellulose binding protocol (31), and 3 μg of each sample were loaded on denaturing gels and subjected to Northern blot analysis according to standard procedures (31). Blots were exposed on Kodak XAR-5 films at −80 °C with intensifying screens for 10 days. Human polyadenylated RNA from various human tissues was purchased from CLONTECH (Palo Alto, CA).

Real Time Quantitative PCR (TaqMan) Analysis—Real time quantitative PCR analysis of DGKβ isoform and β-actin expression was carried out with the aid of an ABI7700 machine (PE Biosystems). A 2× stock mixture of reagents comprising all necessary TaqMan PCR components except primers and probe was purchased from PE Biosystems and employed according to manufacturer's instructions. TaqMan primers specific for each human DGKβ isoform were as follows: DGKβ STD 5′primer, 5′-CACAAGAACCAAGCCCCAA-3′; 3′-primer, 5′-GAGGGAGCAGAATAAACCGGT-3′. The TaqMan DGKβ STD probe was 5′-CTGATGGGCCCGCCTCCAAA-3′ and was carboxyfluorescein-labeled at its 5′ end. TaqMan DGKβ SV3′ primers were as follows: 5′-primer, 5′-TGCATTTCAAAAACATGCTAGGA-3′; 3′-primer, 5′-TGAAGGAAATTACACTGTTGGTCTTG-3′. The TaqMan DGKβ SV3′ probe was 5′-TGCTTTTGGCACTGGGAGTAGACACATGA-3′ and was carboxyfluorescein-labeled at its 5′ end. For TaqMan analysis of rat DGKβ expression, the primers were ratDGKβFOR 5′-GGATGCAGACCCCATGCA-3′ (forward) and ratDGKβREV 5′-AGGTGGAGGTCCCATCAGC-3′ (reverse), in combination with the carboxyfluorescein-labeled probe ratDGKβPROBE 5′-AAAAATTACACACAAGAACCAGGCCCCAA-3′. Final primers and probe concentrations were 300 nM each primer and 200 nM, respectively. Reaction parameters were 50 °C/2 min; 95 °C/10 min; 35×(95 °C/15 s; 53 °C/1 min). Three measurements per sample were carried out in each of two independent experiments. Results were analyzed with the ABI sequence detector software version 1.6.3 (PE Biosystems). Quantitation was carried out relative to a standard curve of the corresponding DGKβ cDNA. For β-actin quantitation, a β-actin detection kit was purchased (PE Biosystems) and employed according to manufacturer's instructions.

In Vitro Translation—The cDNAs for hDGKβ STD and hDGKβ SV3′ (GenBank™ accession numbers AX032742 and AX032745, respectively) were subcloned into expression vector, pcDNA3.1/V5/His-TOPO. The resulting constructs were used as templates for an *in vitro* transcription reaction coupled to translation reaction (TNT kit, Promega, Madison, WI). Control reactions contained no template or a luciferase containing plasmid vector as template. 7.5% SDS-PAGE coupled to Western blot analysis using anti-V5 antibody as a probe was used to identify the protein produced in TNT reactions.

Enzymatic Assays—The cDNAs for hDGKβ STD and hDGKβ SV3′ in pcDNA3.1/V5/His-TOPO or the vector alone were transfected into COS cells by use of LipofectAMINE (Invitrogen). After 3 days the cells were harvested and lysed by sonication in lysis buffer (8). Immunoblotting was carried out with anti-epitope tag antibody (anti-V5 antibody). The amounts of the expressed proteins (hDGKβ STD and hDGKβ SV3′) were equalized by means of densitometric indication of the corresponding bands, and the protein concentrations were also adjusted. Equal amount of each lysate was used to measure DG kinase activity in the presence of calcium (0.1 mM) by the octylglucoside mixed-micelle assay toward 1-stearoyl-2-linoleoyl-*sn*-glycerol (18:0/18:2 DG (Sigma)) as described previously (8, 9, 12).

Subcellular Localization Studies in HEK-293T Cells—Mammalian expression vectors encoding human DGKβ variants were constructed in pEGFP-C1 (CLONTECH). Full-length DGKβ STD and DGKβ SV3′ were fused to a green fluorescent protein (GFP) sequence attached at their NH_2 termini using a combination of cDNA fragments and engineered PCR fragments that ensured reading frame conservation.

All PCR-based constructs were verified by dideoxy chain termination sequencing with dye terminators using an ABI PRISM 6700 Automated nucleic acid work station (PE Biosystems).

GFP-tagged cDNA were transfected into HEK-293T cells, cultured in Dulbecco's modified Eagle's F-12 medium containing heat-inactivated 10% fetal bovine serum, using LipofectAMINE (Invitrogen), and trans-

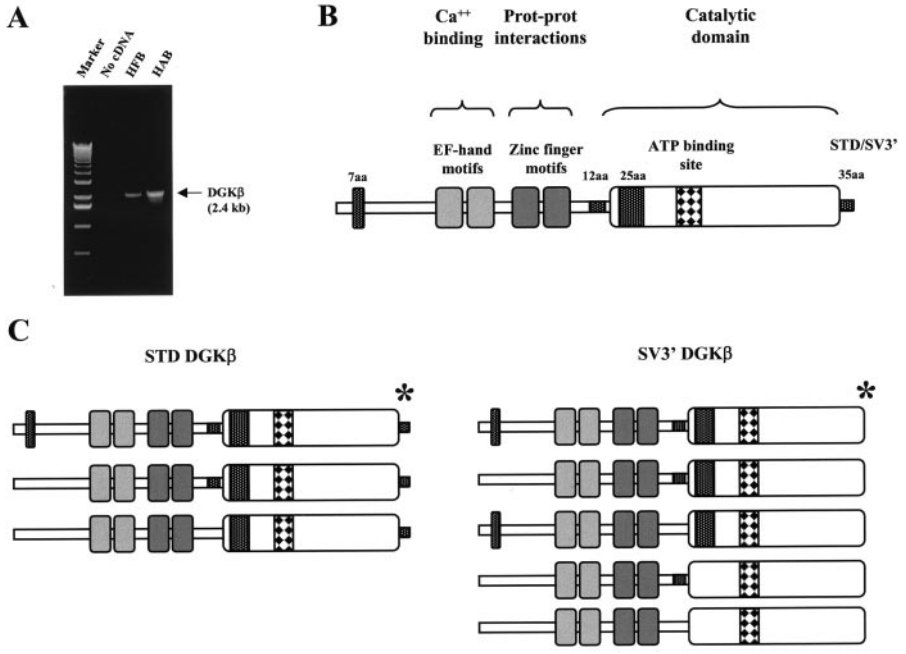

FIG. 1. **Cloning and sequence analysis of multiple human DGKβ cDNAs.** *A*, agarose gel electrophoresis of the products resulting from RT-PCR amplification of human DGKβ cDNAs from fetal (*HFB*) and adult brain (*HAB*) cDNA. DNA products of approximately 2.4 kb were obtained from each cDNA source. *B*, domain architecture of human DGKβ proteins. The conserved functional domains are indicated, as well as the protein regions relevant to the alternative splicing and the alternative polyadenylation events (*stippled areas*). Due to the existence of these events the human DGKβ locus can potentially generate up to 16 different isoforms. *C*, summary and classification of the obtained full-length human DGKβ cDNAs, demonstrating the existence of transcripts capable of encoding multiple DGKβ isoforms. The isoforms indicated with an *asterisk* refer to the DGKβ STD and SV3′ isoforms analyzed in this manuscript.

fected cells were plated onto poly-D-lysin-coated glass slides (Lab-Tek Flaskette Glass Slide, Nalge Nunc International, Naperville, IL).

Living cells transfected with GFP constructs were observed with a confocal laser scanning microscope (Axioplan 2, LSM 510, Carl Zeiss, Göttingen, Germany) at 24 or 48 h after transfection.

In translocation experiments, the culture medium was replaced after transfection with medium without fetal bovine serum for 1 day before tetradecanoylphorbol 13-acetate (TPA) addition. TPA was purchased from Sigma.

RESULTS

Cloning of Human DGKβ cDNAs—We have previously reported (31) the identification of the human DGKβ locus, characterized its genomic structure, and determined through bioinformatics and RT-PCR approaches that the locus is transcribed as a complex series of mRNAs due to alternative splicing and the use of different polyadenylation signals. This analysis predicted the existence of three alternatively spliced exons internal to the coding sequence and of a polyadenylation signal in the intron immediately downstream of the penultimate coding exon of the gene. A series of RT-PCR studies, which employed primers spanning each of the three putative alternatively spliced exons or were specific for the alternative 3′ regions of DGKβ, confirmed the existence of these alternative splicing events (31). These studies were carried out on different preparations of human tissue cDNA, prepared from independent batches of commercially obtained mRNA. In particular, the existence of an alternatively spliced 3′ exon can result in the generation of transcripts predicted to encode two human DGKβ variants. One of such variants is fully homologous to the predicted rat DGKβ amino acid sequence (and is thus referred to as "standard human DGKβ isoform," or DGKβ STD), and the other represents a truncated variant lacking part of the coding sequence present within the last exon (and is thus referred to as "splice variant 3′ human DGKβ isoform," or DGKβ SV3′). The existence of three internal alternatively spliced exons results in further complexity in the predicted coding capacity of the human DGKβ locus, potentially resulting in DGKβ STD or SV3′ proteins with alternative amino acid regions. To physically clone human DGKβ cDNAs, we elected to amplify by proofreading PCR the entire predicted coding sequences from cDNA derived from fetal and adult human brain, based on the nucleotide sequence of the human DGKβ genomic locus and the predicted rat and human DGKβ coding sequences (31). To obtain cDNAs for the predicted variants, we employed a single forward primer anchored 5′ to the predicted DGKβ initiation codon and two alternative reverse primers anchored 3′ to the stop codons predicted for the STD and SV3′ DGKβ variants. The resulting PCR products were cloned and subjected to DNA sequencing. This analysis confirmed the cloning of three DGKβ STD cDNAs and five DGKβ SV3′ cDNAs. Collectively, these cDNA clones represented 8 of the 16 DGKβ isoforms potentially generated from the human DGKβ locus (Fig. 1) and confirmed the predicted cDNA structure and splicing events previously reported by us (31). To further confirm the identification of the human homologue of the previously reported rat DGKβ, a Northern blot comprising a panel of polyadenylated RNAs from adult rat tissues comparable with the one reported in Ref. 8 was probed with one of the obtained human DGKβ cDNA clones. The results (Fig. 2*A*) revealed a single transcript of ~6.5 kb present exclusively in the brain. In their report, Goto and co-workers (8) observed DGKβ transcripts expression in the heart and adrenal; our inability to detect DGKβ expression in these rat tissues employing a human probe may be due to lower sensitivity of our Northern blotting assay, differences in mRNA quality, or in the use of a probe from a different species (human *versus* rat). To address this discrepancy, we performed a quantitative RT-PCR (TaqMan) analysis on a panel of cDNAs prepared from various rat tissues, using a set of primers and probe specific for the published rat DGKβ sequence (Fig. 2*B*). The results confirmed that, among the adult rat tissues sampled in our experiments, the brain appears to be the only organ expressing DGKβ transcripts, in accordance with the Northern blotting data obtained using a human DGKβ probe (Fig. 1). The apparent size of the transcript and the predominance of expression in brain tissue are in accordance with the data reported for the rat DGKβ gene (8).

The cloning of human DGKβ cDNAs with different structures indicates that the human locus can generate transcripts potentially encoding several protein isoforms, which differ in the presence or absence of four amino acid insertions of 7, 12, 25, and 35 residues, respectively (see Fig. 1). Of particular interest are DGKβ variants displaying a deletion of the COOH-terminal 35 amino acids, comprising a region 3′ of the catalytic

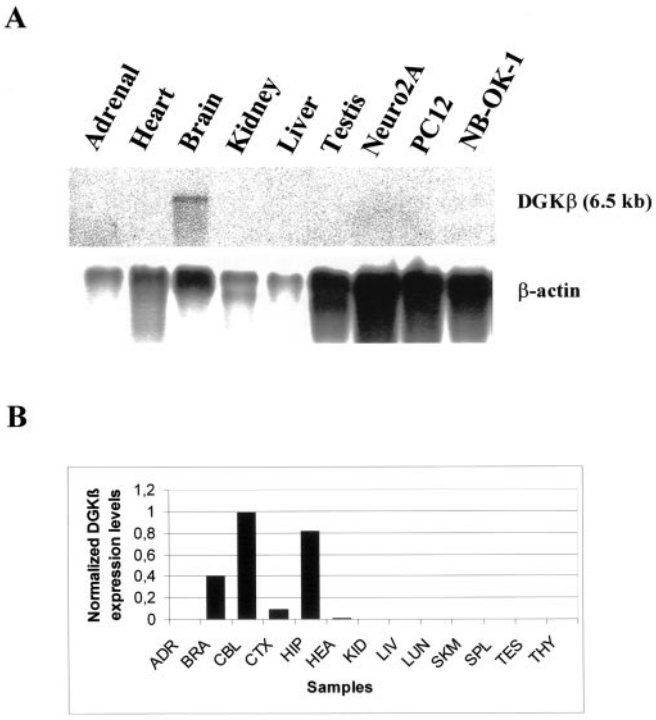

FIG. 2. **Expression of DGKβ transcripts in rat tissues.** *A*, Northern blot of polyadenylated RNA (3 μg) from adult rat tissues and from rodent and human neuronal cell lines. The blot was probed with a full-length human DGKβ cDNA, revealing a single band of approximately 6.5 kb in the brain. The blot was subsequently stripped and reprobed with a β-actin cDNA to control for RNA integrity. *B*, quantitative (TaqMan) RT-PCR analysis of DGKβ expression in a panel of adult rat tissues, demonstrating the specificity of expression in the central nervous system. Abbreviations used: *ADR*, adrenal gland; *BRA*, brain; *CBL*, cerebellum; *CTX*, cortex; *HIP*, hippocampus; *HEA*, heart; *KID*, kidney; *LIV*, liver; *LUN*, lung; *SKM*, skeletal muscle; *SPL*, spleen; *TES*, testis; *THY*, thymus.

domain. In fact, deletion of this domain is associated with a human DGKβ EST (dbEST Id: 1371986 GenBank™ accession number AF019352) annotated as differentially expressed in mood disorder patients (31) and suggests that DGKβ variants bearing this truncation may be overrepresented in at least some cases of bipolar affective disorder. Given the importance of DG levels for PKC activity, the role of PKC in modulating aspects of cognitive and emotional behavior, and the expression of DGKβ in relevant areas of the rat central nervous system (8, 27, 28), further studies on DGKβ isoforms were focused on the comparative analysis of the expression, function, and subcellular localization of DGKβ proteins lacking the COOH-terminal coding exon.

Quantitative Expression Patterns of DGKβ STD and DGKβ SV3′—To determine the pattern of expression of DGKβ transcripts potentially encoding the DGKβ STD and DGKβ SV3′ isotypes, a series of quantitative RT-PCR (TaqMan) studies were carried out on a panel of cDNAs synthesized from human tissue samples, including several distinct brain regions (*e.g.* see Ref. 34). Primers and probes were designed to recognize transcripts encoding either the DGKβ STD or the DGKβ SV3′ isoforms, independently of the presence or absence of the internal alternatively spliced exons. The results (Fig. 3) indicate that the human DGKβ locus is expressed predominantly in the central nervous system and uterus, with much lower levels of expression in other tissues. Increased DGKβ transcript levels in adult brain relatively to fetal brain indicate that DGKβ expression may be developmentally regulated. Within the adult brain, the amygdala, caudate nucleus, and hippocampus express the highest levels of DGKβ transcripts, in agreement with data previously reported for the rat DGKβ gene (8, 27, 28). Although the tissue-specific profiles of expression for transcripts encoding the COOH terminus variants are essentially superimposable, important differences are observed in the relative levels of expression. In fact, transcripts encoding DGKβ STD proteins are expressed in the adult brain at levels about 10–100-fold higher than transcripts encoding DGKβ SV3′ isotypes (Fig. 3). An important observation is the expression of DGKβ transcripts in the uterus, suggesting a role for this enzyme in aspects of uterine function. The apparent discrepancy between the absolute and β-actin-normalized expression profiles of DGKβ SV3′ transcripts may be due to the low levels of expression of DGK SV3′ mRNAs in normal human tissue.

Enzymatic Properties of DGKβ STD and DGKβ SV3′ Isotypes—The existence of transcripts capable of encoding DGKβ isotypes differing at their COOH terminus may indicate that the encoded proteins differ in their enzymatic activities. To address this aspect of DGKβ function, experiments were carried out in which the enzymatic activities of DGKβ STD and SV3′ isotypes (comprising the three internal alternatively spliced exons) were assayed as previously described for the rat DGKβ enzyme (8). First, an *in vitro* translation experiment was set up to determine whether the DGKβ STD and SV3′ cDNAs can be translated into proteins. The results (Fig. 4*A*) indicated that DGKβ STD and SV3′ cDNAs can direct the synthesis of proteins with an apparent size in accordance with the predicted molecular mass of 90.5 and 87.0 kDa, encoded by their respective open reading frames. Second, a series of DG kinase assays were carried out to investigate the catalytic properties of the two isoforms. These enzymatic assays demonstrated that the DGKβ STD and SV3′ display comparable DG kinase activites under *in vitro* conditions (Fig. 4*B*). Therefore, the COOH-terminal region missing in the DGKβ SV3′ does not appear to influence the kinase activity of the enzyme, despite its vicinity to the protein's catalytic domain.

Differential Localization and Pharmacological Responses of DGKβ STD and DGKβ SV3′ Proteins—An important aspect of the regulation of DGK activity is its subcellular localization. Although many DGKs are predominantly cytosolic, some DGK isoforms (notably the α, ζ, ι, and θ isotypes) have been reported to be present in the nucleus (reviewed in Ref. 4) where they are thought to modulate levels of nuclear DG. Nuclear DGK activity has been associated with the control of cell division and may even affect mRNA synthesis and splicing (reviewed in Ref. 4). When overexpressed in COS-7 cells, the rat DGKβ enzyme was found exclusively in the membrane fraction (8). The discovery and cloning of several isoforms of the human DGKβ enzyme (Ref. 31 and this work) prompted an investigation into the possibility of enzyme localization to different subcellular compartments. To this end, translational fusions were constructed between the GFP and STD or SV3′ variants. GFP-DGKβ fusions were then overexpressed in HEK-293T cells and their subcellular distribution investigated under standard culture conditions using confocal microscopy. The results (Fig. 5*A*) indicated that the DGKβ STD variant localized predominantly to the plasma membrane (in a manner consistent with previous observations on rat DGKβ; Ref. 8), while the SV3′ variant was predominantly cytosolic. Notably, neither variant was found in the nucleus. GFP fusion of DGKβ STD was associated with the plasma membrane only in growing cells. Indeed, quiescent cells cultured without serum did not show plasma membrane localization of DGKβ STD (Fig. 5*B*, *upper left panel*). Next, the response of GFP-DGKβ isoform fusions to treatment with the phorbol ester TPA in serum-starved HEK-293T cells was investigated (Fig. 5*B*). Membrane translocation in response to phorbol esters is a well established phenomenon for various

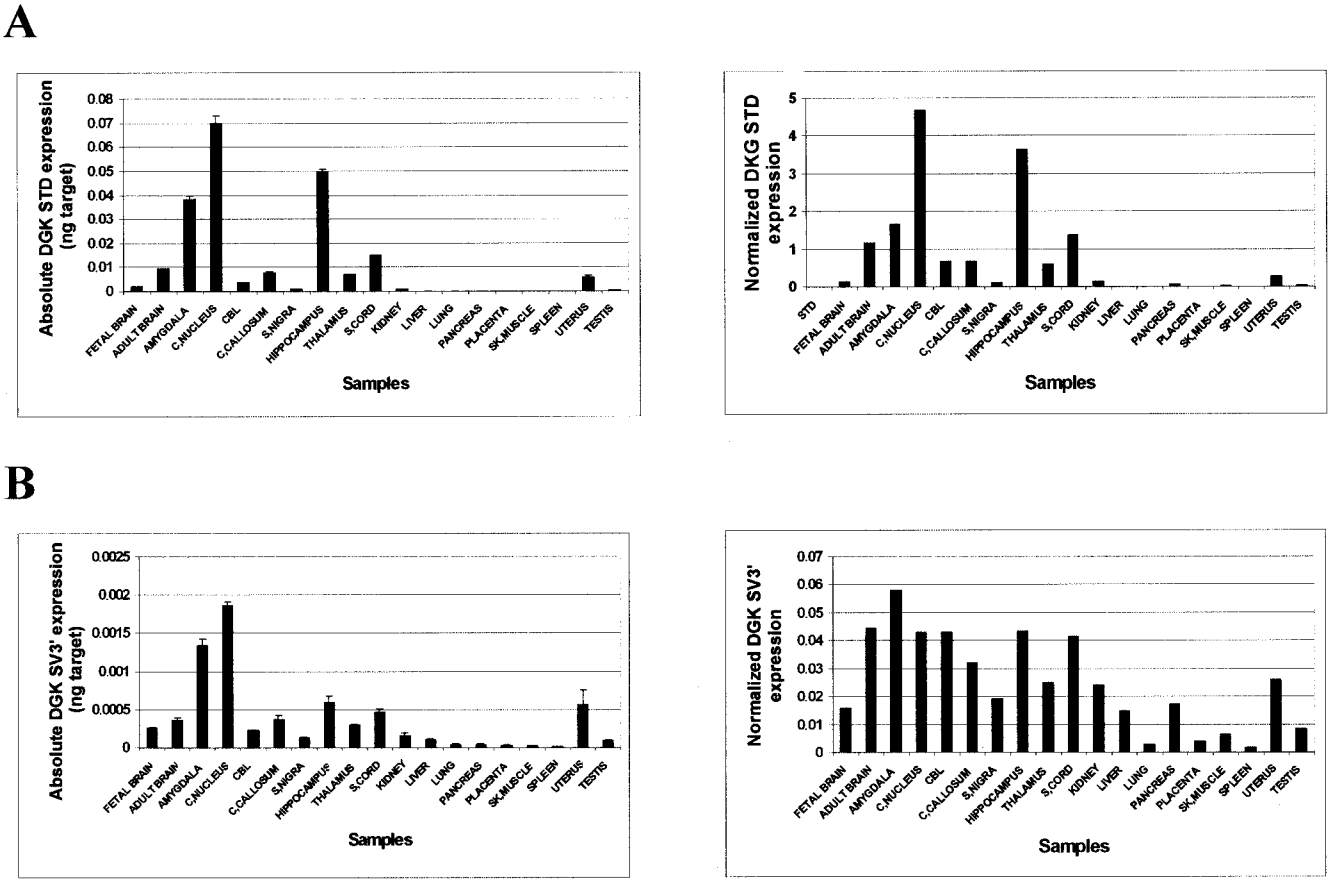

FIG. 3. **Quantitative (TaqMan) expression analysis of DGKβ isoforms in a panel of human tissue cDNAs.** In each case, absolute levels are provided in the *left-hand histogram* (the means and S.E. of three measurements per sample are indicated), while levels normalized relatively to β-actin are provided in the *right-hand histogram*. *A*, analysis of STD DGKβ expression in multiple human tissues. *B*, analysis of SV3′ DGKβ expression in multiple human tissues.

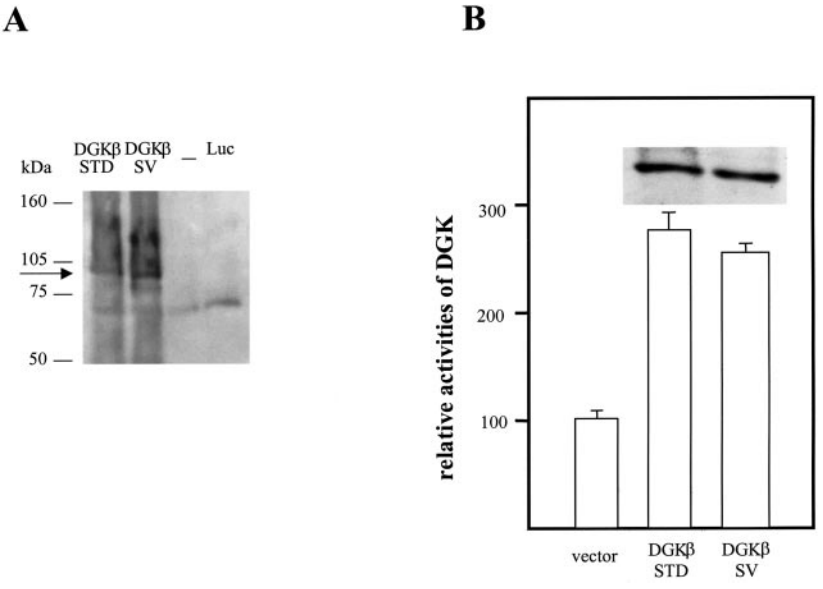

FIG. 4. **Characterization of human STD and SV3′ DGKβ proteins.** *A*, *in vitro* translation of cDNAs encoding DGKβ STD (*first lane*) and SV3′ (*second lane*) proteins tagged to the V5 epitope was performed using TNT kit. Control reactions contained no template (*third lane*) or a luciferase coding plasmid vector (*fourth lane*). Western blot analysis of the produced proteins was performed using anti-V5 antibody as a probe. *B*, enzymatic assay demonstrating the comparable DG kinase activity of the two proteins. The photograph *inset* shows the Western blot analysis with anti-V5 antibody to normalize protein levels.

DG-binding proteins such as DGKs (35) PKCs (1) and Munc 13-1 (37), although the exact mechanism of TPA-induced translocation remains to be clarified. Quiescent HEK-293T cells transfected with the DGKβ STD isoform-GFP fusion showed a predominantly cytosolic distribution of the fusion protein. When challenged with TPA, a rapid (10 min) redistribution of the protein to the membrane was observed (Fig. 5*B*). The possible presence of a membrane-targeting signal in the COOH-terminal tail of DGKβ STD was investigated using various protein motif data bases and software tools such as Pfam (38), PRINTS (39) ProDom (40), Prosite (41), PSORT II (42), and SMART (36). However, no membrane-targeting signal was discovered in DGKβ STD. In contrast, the distribution of the DGKβ SV3′ isoform-GFP fusion was identical in cycling and quiescent HEK-293T cells, with no significant effects of TPA on its subcellular distribution. In conclusion, these results suggest

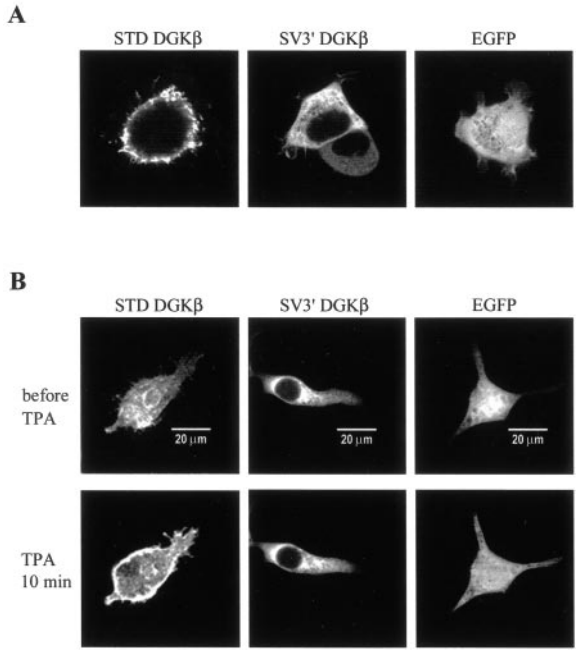

FIG. 5. **Subcellular localization of human DGKβ STD and SV3′ proteins in mammalian cells.** *A*, expression constructs comprising the human DGKβ STD and SV3′ cDNAs translationally fused with GFP or the empty EGFP vector were transfected into HEK293 cells and the subcellular localization of the encoded GFP fusion protein analyzed by confocal microscopy. *B*, effects of TPA treatment on the subcellular localization of human DGKβ STD and SV3′ proteins.

that the DGKβ STD and SV3′ isoforms differ in their subcellular localization and in their response to increased substrate concentration.

DISCUSSION

We report for the first time the identification and functional characterization of cDNAs encoding different isoforms of the human DGKβ gene. The results reported here support the data previously reported using bioinformatics tools (31) and confirm that the human DGKβ gene is transcribed as a complex series of mRNAs, as a result of alternative splicing and differential polyadenylation signal usage. This is only the second analysis concerning a mammalian DGKβ gene and its encoded proteins and the first report providing evidence for the existence of multiple DGKβ isoforms with different properties. The isolation of full-length cDNAs with the potential to encode different DGKβ isoforms suggests the existence of a degree of diversification of human DGKβ activity, which may involve the modulation of isoform expression, enzymatic activity, and/or of subcellular localization. The predicted human DGKβ isoforms differ in the presence of three internal, alternatively spliced coding exons and a COOH-terminal region, which is differentially present due to alternative polyadenylation signal usage (31). As a result, a total of 16 different isoforms can be generated from the human DGKβ locus. From fetal and adult human brain, we have cloned and sequenced cDNAs encoding eight of these, which presumably represent the most abundantly transcribed DGKβ mRNA species. Evidence for the existence of transcripts capable of encoding the remaining DGKβ isoforms has been described elsewhere (31). The lack of isoform-specific DGKβ antibodies renders a confirmation of the existence of different DGKβ isoforms *in vivo* rather difficult, but the cloning and functional data presented here and the expression data reported previously by us indicate that DGKβ transcripts capable of encoding different isoforms are at least transcribed and that these transcripts can be translated *in vitro* and *in vivo* (as V5 or GFP fusions) to yield protein products of the expected size.

We focused our analysis on a comparison of the properties of human DGKβ isoforms differing for the presence of the COOH-terminal portion of the gene (that is, the DGKβ STD and SV3′ isoforms). In fact, a human DGKβ EST (dbEST Id: 1371986; GenBank™ accession number AF019352) is annotated in GenBank™ as being differentially expressed in bipolar disorder patients and corresponds to the 3′ region of human DGKβ transcripts encoding the isoforms lacking the COOH-terminal region. This EST does not extend sufficiently toward the 5′ end of the coding sequence to determine the representation of the other alternatively spliced exons and is therefore uninformative toward a possible association between mood disorders and DGKβ alternative splicing events other than that influencing the COOH-terminal coding sequence. Transcripts encoding different protein isoforms of the same gene may differ in their prevalence and/or tissue distribution. Our TaqMan analysis of the expression of mRNAs encoding the DGKβ STD and SV3′ isoforms indicates that the two DGKβ transcript classes are expressed at substantially different levels (with transcripts encoding the STD variants being present at 10–100-fold higher levels than those encoding the SV3′ variants), but shows an essentially superimposable pattern of tissue distribution. Therefore, in normal human tissue transcripts encoding the SV3′ isoforms are expressed at far lower levels than those encoding the STD isoforms. It would be extremely interesting to confirm the possible differential expression of the SV3′ isoforms in tissues from bipolar disorder patients. To understand the physiological significance of the existence of DGKβ isoforms differing at their COOH terminus, we have attempted a functional comparison of the DGKβ STD and SV3′ isoforms and investigated their diacylglycerol kinase activities *in vitro* and their subcellular localization *in vivo*. The results indicate that, despite showing similar enzymatic activity, the two isoforms may display differences in their subcellular localization and in their response to increased substrate availability. These differences may bear important consequences for the activity of the enzyme *in vivo*. In fact, it has been shown that DGK activity does not occur ubiquitously within the cell. Experiments have shown that membrane translocation has been described to be a necessary (but not sufficient) step for DGK activation and that DGK activity seems to be localized at membrane sites where DG is produced upon receptor-mediated phospholipase C-β activation, rather than acting on DG randomly produced at membrane sites (reviewed in Ref. 4). In line with this view, DGKβ SV3′ isoforms may not be active *in vivo*, despite showing a relatively normal kinase activity *in vitro*, due to their inability to associate with the cell membrane. It would therefore be interesting to determine whether these isoforms can show diacylglycerol kinase activity in intact cells *in vivo*. Taken together, our data suggest that an altered balance in the relative level of STD and SV3′ DGKβ transcripts (such as may be the case in some mood disorder syndromes) may lead to abnormal DG turnover within neurons in the amygdala, caudate nucleus, and hippocampus, regions that are known to be involved in the control of emotional and cognitive behavior.

Although the functional significance for the existence of multiple DGKβ isoforms remains unknown, it is likely that the alternatively spliced exons encoding the 7, 12, 25, and 35 amino acid domains will influence regulatory aspects of the protein's kinase activity and/or subcellular localization (as shown here for the 35 amino acid COOH-terminal region) or even a coupling with other intracellular signaling pathways. For instance, a recent study demonstrated that an alternatively spliced COOH-terminal domain of tomato DGK functions as a calmodulin-binding domain, thus providing a coupling between calcium and phospholipid signaling in this plant (22). The

identification of multiple DGKβ isoforms described here may spur interest on the possible existence of multiple isoforms of other DGKs. Furthermore, it sheds a novel light on the functional and regulatory aspects of an important enzyme modulating the levels of DG and phosphatidic acid, two key intracellular messengers, in neuronal cells.

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Molecular Cloning and Characterization of the Human Diacylglycerol Kinase β (DGK β) Gene: ALTERNATIVE SPLICING GENERATES DGKβ ISOTYPES WITH DIFFERENT PROPERTIES

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