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Alternative Splicing Generates a Diacylglycerol Kinase α Transcript That Acts as a Dominant-Negative Modulator of Superoxide Production in Localized Aggressive Periodontitis

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Abstract

Background—Diacylglycerol (DAG), levels of which are tightly regulated by diacylglycerol kinases (DGKs), is a lipid mediator linked to key biologic functions. Members of the DGK family undergo alternative splicing, generating the protein diversity necessary to control different intracellular DAG pools. DGK α function is altered in polymorphonuclear neutrophils (PMNs) of patients with localized aggressive periodontitis (LAgP), suggesting a genetic basis. Here, the authors assess DGK α spliced transcripts in human LAgP neutrophils.

Methods—In an expression library of a patient with LAgP, PMNs were screened for different DGK α transcripts. Real-time polymerase chain reaction and in vitro expression assays were performed to assess the fate of different transcripts on protein translocation and superoxide production in human leukemia cells (HL-60) and COS-7 cells.

Results—A DGK α transcript that lacks exon 10 (DGK α 10) and generates a premature stop codon and a truncated protein was identified as being upregulated in LAgP neutrophils. In vitro assays revealed that DGK α 10 translocation occurred even in the absence of important regulatory motifs. Transfection of HL-60 neutrophil-like cells with the DGK α 10 spliced variant induced an increase in the stimulated production of superoxide anion replicating the phenotype of LAgP PMNs.

Conclusion—DGK α 10 can act as a dominant-negative transcript that can modulate superoxide production and provides an example of genetic regulation of the inflammatory response that may be relevant to human inflammatory diseases such as LAgP. *J Periodontol* 2014;85:934-943.

Keywords

Aggressive periodontitis; diacylglycerol kinase; inflammation; neutrophils; phosphoprotein phosphatases; protein kinases

Localized aggressive periodontitis (LAgP) is a relatively rare (0.1% to 5% of the population) syndrome of periodontal bone loss that is inherited as an autosomal dominant trait.^{1,2} LAgP is characterized by infection with *Aggregatibacter actinomycetemcomitans*, hyperimmunoglobulinemia, and polymorphonuclear neutrophil (PMN) functional abnormalities including reduced chemotaxis and increased superoxide generation.³ Focus on the biochemical basis of the PMN abnormalities revealed a series of intracellular signaling abnormalities in the phosphoinositide pathway, including accumulation of diacylglycerol (DAG), but not inositol triphosphate, suggesting a blockade at the level of diacylglycerol kinase (DGK).⁴ Further investigation revealed reduced activity of DGK α (the active isoform in PMNs) in LAgP.⁵

DAG is a lipid mediator with a diverse array of functions involving glycerolipid biosynthesis and signal transduction.⁶ DAG is generated from different sources in response to cell stimulation and downstream activation of pathways involving phospholipases.⁷ Phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate DAG.⁸ The intracellular Ca²⁺ regulator inositol triphosphate is also produced,⁹ which, in concert with DAG, activates protein kinase C (PKC).¹⁰ DAG activity is regulated by DGKs, a family of isozymes that convert DAG to phosphatidic acid (PA), thus exerting negative regulatory feedback in DAG-dependent signal transduction.¹¹ The different DGK isoforms characterized thus far are classified according to structural features, and each group is involved in the regulation of DAG in different cell compartments.¹²

DAG and DGK regulate different functions including proliferation, differentiation, apoptosis, and activation of cells of acquired and innate immunity.¹³ Evidence for the importance of DAG-/DGK-mediated pathways in human neutrophils is illustrated by incubation of these cells with DGK pharmacologic blockers, which increase oxidase activation, demonstrating that accumulation of DAG and superoxide anion production are linked.¹⁴ Among the expressed isoforms, DGK α is significantly upregulated throughout differentiation of granulocytes.¹⁵ This finding suggests an important role for the DGK α isoform in the regulation of DAG pools generated in cells phenotypically committed to the granulocyte lineage. Notably, the DAG/DGK pathway is related to the activation of PKC and the nicotinamide adenine dinucleotide phosphate (NADPH) complex assembly, leading to the production of superoxide anion. Superoxide is a potent molecule that is extremely important in innate immune defense against microbial pathogens and is capable of promoting tissue disorganization when produced in excess.¹⁶ Previous evidence supports a role for imbalances of DAG pools in LAgP neutrophils, thus increasing the potency and duration of superoxide production and tissue breakdown elicited by these cells.^{4,5,17}

Alternative splicing is an important process that accounts for protein diversity and regulation of gene expression. Alternatively spliced variants may have enzymatic function or be abnormally spliced nonfunctional proteins that, once expressed, can act as dominant-

negative forms.¹⁸ A number of members of the DGK family undergo alternative splicing.^{19,20} The aim of the present investigation is to determine the role of alternatively spliced variants of DGK α transcripts expressed by LAgP PMNs and to characterize function-related changes induced by spliced isoforms that impact PMN function in LAgP.

MATERIALS AND METHODS

Patients were recruited from the patient population of the Clinical Research Center at Boston University. Medical and dental histories were obtained to ensure absence of systemic conditions that could impact PMN function. Patients taking anti-inflammatory drugs were excluded from the study. This study was approved by the Institutional Review Board of Boston University and informed consent forms signed by the patients were obtained before the beginning of the study. Diagnosis of LAgP was based on bone loss, deep probing depths (PD), and attachment loss limited to first molars and incisors as previously described. Patients with LAgP also exhibited reduced PMN chemotaxis as determined by protocols previously reported^{2,21} and excessive production of superoxide anion.⁵ Bone loss was detected and evaluated through peri-apical radiographs and PD. Age- and sex-matched individuals without signs of any form of periodontitis except mild gingivitis served as controls. A total of 10 patients with LAgP (five males and five females, mean age: 29 ± 4.6 years) and 10 matching controls (mean age: 30 ± 3.2 years) participated in this investigation.

Isolation of Peripheral Blood Neutrophils

Approximately 50 mL peripheral venous blood was collected into blood collection tubes containing 25 units/mL heparin. PMNs were isolated by gradient centrifugation as previously described.²² The monocyte layer was transferred to a separate tube, and PMNs were collected by gentle pipetting. PMNs were subjected to isotonic lysis of contaminating red blood cells for 5 minutes. After two washes in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS) and determination of cell counts/milliliter, PMNs were resuspended in medium containing 5% heat-inactivated fetal bovine serum and preincubated for 10 minutes at 37 °C in a 5% CO_2 atmosphere. PMNs were stimulated with 5 nM n-formylmethionyl-leucylphenylalanine (fMLP)[‡] for the indicated times and processed for downstream assays accordingly. Human leukemia cells (HL-60)[§] were cultured in Iscove medium supplemented with 10% heat-inactivated fetal bovine serum and differentiated into neutrophil-like cells with 1.25% dimethylsulfoxide (DMSO)^{||} for 7 days at 37 °C in a 5% CO_2 atmosphere.

Construction of a Complementary DNA Library From LAgP PMN

An mRNA-enriched mixture obtained from total human PMN RNA isolated from PMNs of patients with LAgP was used to construct an expression library. RNA quality was assessed by staining of 1.2% denaturing gels.[¶] First-strand synthesis was carried out with ≈ 500 ng total RNA. RNA was primed at its 3' end with a modified oligo(dT) primer containing a *Sfi* I restriction site. In the same reverse transcriptase mixture, a 3' poly-G-containing

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oligonucleotide sequence was added. This oligo pairs with the deoxycytidine nucleotide region incorporated at the 5' end of the first strand by the terminal transferase activity of the RT. Second-strand synthesis was carried out with 2 μ L of the first-strand reaction; complementary DNA (cDNA) was incubated with primers complementary to the homopolymeric sequences incorporated into the cDNA during reverse transcription, amplified with a proofreading DNA polymerase,[#] and treated with *Sfi* I restriction enzyme.^{**} The cDNA was ligated into a vector and electroporated into DH5 α -E *Escherichia coli* cells^{††} that were propagated on Luria-Bertani (LB) agar plates. Probes for clone screening were synthesized by reverse transcription-polymerase chain reaction (RT-PCR) amplification of a 340-bp fragment of DGK α from human brain tissue isotopically labeled. Positive clones were propagated and sequenced.

Coupled In Vitro Transcription/Translation Reticulocyte Assay

Plasmids containing the spliced and wild-type (WT) inserts were used as templates and reamplified, using a single forward primer (5'-TAATACGACTCACTATAGGAGACCACCATGGCCAAGGAGAGGGGCCTAATAAG-3') containing transcription/translation consensus sequences. The reverse primer was designed to anneal to a sequence downstream to exon 10, at the boundaries of exons 16 and 17 (5'-CTGGCAAGTTAGCTTTGT-CAATG-GTCTC-3'). PCR was carried out using pDNR-LIB α 10 or pDNR-LIB α . For the in vitro transcription/translation assay, $\approx$ 500 ng of the gel-purified amplicon containing either DGK α 10 or DGK α was mixed with rabbit reticulocyte lysate.^{‡‡} [³⁵S]-methionine (40 μ Ci) was added, and the samples were loaded onto precast sodium dodecyl sulfate–polyacrylamide gels and electrophoresed, dried, and exposed on x-ray film.

Quantitative PCR for Detection of DGKs

The expression of a panel of DGKs (α , δ , γ , ϵ , and ζ) was assessed in 10 LAgP and 10 age- and sex-matched control neutrophils. Total RNA was isolated^{§§} and on-column treated with DNase. RNA quality was assessed by denaturing gel electrophoresis and staining. Purity and concentration of RNA was assessed by spectroscopy at 260/280 and 260 nm, respectively. Only samples whose 260/280 ratios were $\geq$ 1.8 were selected for the analysis. FAM-MGB-labeled probes and primers were custom-designed to anneal to exons 9 to 10 (DGK α) and 9 to 11 (DGK α 10). The primers and probes synthesized^{||||} were forward 5'-CGACCAGTGTGCCATGAAAG-3'/reverse 5'-CTTC CGAGACTTGGCATAGGT-3'/probe 5'-CCCTGCCT TGTGAAGTC-3' and forward 5'-GTCTTGCGAAA-CAGGGACTGA-3'/reverse 5'-CCTCCTTGCACCC-ACACAT-3'/probe 5'-CTGTAACCGTCCAATCAC-3' for DGK α and DGK α 10, respectively. Primers and probes for the remainder of the DGKs (δ , γ , ϵ , and ζ) were also assessed^{¶¶} and measured by relative expression. Validation of the assay was performed as previously described.²³ The

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^{††}Invitrogen, Thermo Fisher Scientific.

^{‡‡}TnTT7 PCR DNA Quick, Promega, Madison, WI.

^{§§}RNeasy, Qiagen, Valencia, CA.

^{||||}Applied Biosystems, Thermo Fisher Scientific.

^{¶¶}TaqMan gene expression inventoried assays, Applied Biosystems, Thermo Fisher Scientific.

approach used for preparation of standards for absolute quantification of the spliced transcript was customized using in vitro transcription and synthesis of complementary RNA. Different human tissues and cancer cell lines were evaluated for the expression of DGK α and DGK α 10 as well. cDNA synthesis was performed using a kit^{##} from 100 ng total RNA primed with random hexamers and reverse transcribed using avian myeloblastosis leukemia virus reverse transcriptase in 10- μ L reaction mixtures (final concentration 20 ng/ μ L cDNA). Reverse-transcription reaction mixtures were incubated at 25 °C for 10 minutes, 48 °C for 30 minutes, and 95 °C for 5 minutes in a thermal cycler.^{***} Reverse-transcription products were prepared in 10-ng duplicates, and real-time PCR was performed using primers and TaqMan probes labeled with MGB-FAM dye. β -Actin was selected as an endogenous control and amplified using pre-formulated VIC-TAMRA-labeled probe (endogenous control).^{†††} Quantification was performed in an automated thermal cycler,⁺⁺⁺ and results were analyzed through a software interface for calculation of relative expression (DGK/ β -actin). Data were obtained from three separate experiments run in duplicate for each individual or three separate batches of HL-60 cells in culture.

Subcloning of DGK α and DGK α D10 Expression Vectors and Transfection of COS-7 Cells

Vectors containing DGK α and DGK α 10 were digested with *Sfi* I, blunt ended, and subcloned in an amino-terminus polyhistidine-tagged, eukaryotic expression plasmid^{\$\$\$} used to transform *E. coli* cells. Aliquots of $\approx 3 \times 10^5$ cells were plated (RNA and protein isolation) and transferred to coverslip chambers (fluorescence microscopy). Plasmid transfection was carried out^{||||} for pcDNA α 10 or pcDNA α . For fluorescence microscopy, cells were fixed with 4% paraformaldehyde in PBS, and cells were transfected with pcDNA α 10, incubated with mouse fluorescein isothiocyanate-conjugated anti-V5 antibody, and analyzed on a fluorescence optical microscope.^{¶¶¶} Quantitative PCR (qPCR) was performed as described above using primers for DGK α .

Synthesis of Dominant-Negative DGK α Using Site-Directed Mutagenesis

Plasmid (100 ng) containing the WT DGK α was methylated and used in a PCR mix containing 300 nM mutagenesis primers (forward 5'-ATAGCCGG-ATTTTGGTGTGTGACGGAGACGGCACAG-3'; reverse 5'-ACACACAAAATCCGGCTAT-CAGGAACATCC-TTG-3'). Approximately 2 ng of the mutagenesis reaction was used to transform DH5 α -T1 *E. coli* cells. Plasmid DNA was subjected to sequencing to confirm mutagenesis.

^{##}High Capacity Reverse Transcription with RNase inhibitor, Applied Biosystems, Thermo Fisher Scientific.

^{***}ABI 9700, Applied Biosystems, Thermo Fisher Scientific.

^{†††}TaqMan probe, Thermo Fisher Scientific.

⁺⁺⁺ABI 7000, Applied Biosystems, Thermo Fisher Scientific.

^{\$\$\$}pcDNA 3.1, Invitrogen, Thermo Fisher Scientific.

^{||||}Fugene 6, Roche Molecular, Pleasanton, CA.

^{¶¶¶}Carl Zeiss MicroImaging, Thornwood, NY.

Translocation Assay and Confocal Microscopy

COS-7 cells^{###} were transfected and phorbol myristate acetate was added to a final concentration of 1 μ M. Slides were stored protected from light; 10 positive cells were randomly selected in each slide and analyzed in a confocal laser scanning microscope.^{****}

Transient Transfection and Assessment of Superoxide Production

HL-60 cells were incubated in 1.25% DMSO. After 3 days, the cell suspension was electroporated with plasmid DNA (no insert), DGK α 10, or DGK α MUTA (negative control mutant construct). Cells were then lysed for RNA isolation or assessed for superoxide production and CD11b/18 expression. Superoxide production was assessed using the cytochrome C and superoxide dismutase assay after incubation of cells with fMLP.

Data Analyses

Kolmogorov-Smirnov and Levene tests were carried out to assess normality and variance. Based on this analysis, one-way and two-way analysis of variance tests were used for multiple comparisons of parametric data, and Bonferroni post hoc test was used to assess inter- and intragroup variation. Real-time relative expression was assessed by Kruskal–Wallis and Dunn post hoc tests. Data were processed using a statistical package,^{††††} and significance was set at $P < 0.05$.

RESULTS

Differential Expression of Select DGKs in LAgP and Control PMNs

Quantitative assessment of the expressed DGK isoforms in LAgP and controls was carried out using real-time fluorescence-based PCR. The results presented in Figure 1 demonstrate no major differences of the expressed isoforms between LAgP and controls with the exception of DGK α , which was significantly increased ($P < 0.05$) after exposure to 5 μ M fMLP. After 180 minutes, the levels of DGK α RNA increased in the control group, but remained significantly lower ($P < 0.05$) compared with LAgP. In the samples analyzed, the isoforms δ , γ , ϵ , and ζ were significantly lower than isoform α and did not present significant variations that pointed to a specific expression profile caused by fMLP stimulation. PBS and incubation time did not induce major changes in the levels of mRNA in either group. Throughout the incubation process with fMLP, an increase in the levels of DGK α was observed in both LAgP and control groups.

Human PMN DGK α RNA Transcripts

Two transcripts were identified based on size. Alignment of the sequenced clones with the original sequence revealed that LAgP PMN expressed the native sequence of DGK α and a transcript that lacked 89 bp, which comprised exon 10, creating a unique change in the exon–exon boundary of DGK α , from exons 9 and 10 (5'-CTGAGCTG-TAAACC and TCTGTAAGTACA-3') to exons 9 and 11 (CTGAGCTGTAAACC and GTC-

^{###}ATCC.

^{****}Leica, Bannockburn, IL.

^{††††}Prism 6 for Mac, GraphPad Software, La Jolla, CA.

CAATCACATG-3'). This new sequence was submitted (Genbank AY930112), herein referred to as DGK α 10. The nucleotide sequence encoded by exon 10 codes for a stretch of 29 amino acids; from these, 16 matched a cysteine-rich motif conserved among species known as DAG/phorbol-ester binding motif. Skipping of exon 10 introduced a frame shift in the open reading frame, leading to a premature stop codon at amino acid 248 along with a stretch of an additional 10 amino acids that did not match the data bank protein sequence (see supplementary Figure 1 in online *Journal of Periodontology*).

Characterization of DGK α D10 Premature Stop Codon

Using the coupled in vitro transcription/translation reticulocyte assay, the plasmids containing the cloned sequences with DGK α and DGK α 10 were reamplified using primers containing transcription/translation consensus sequences as described above. There were three possible outcomes (Fig. 2A): 1) a 50-KDa protein compatible with the WT; 2) a 46-KDa protein lacking only exon 10 with no consequences to the remainder of the protein size downstream to exon 10; and 3) a 28-KDa band compatible with the absence of exon 10 and a frame shift leading to a premature stop codon at amino acid 248. The results show that skipping of exon 10 indeed leads to the translation of a truncated protein of $\approx$ 28 KDa (outcome 3) (Fig. 2A). Once translated, the spliced transcript contains the two amino-terminus EF-hands and only one of the presumed DAG-binding motifs present in the WT protein (Fig. 2B).

Expression of DGK α and Spliced Transcripts in Human Tissue and Cell-Line Panels

Expression of the transcripts was initially assessed in a panel of human cancer cell lines and tissues using qPCR and end-point RT-PCR. Expression of the spliced transcript DGK α 10 was observed in tissues (Fig. 3A) and cell lines (Fig. 3B). The cell lines presented significantly higher relative levels of DGK α and DGK α 10 compared to the analyzed tissues. Among the cell lines analyzed, levels were higher in RAJI and T-47 cell lines. The levels of the non-spliced transcript were significantly higher in every tissue and cell line observed, except the cell line U-937, where the spliced transcript accounted for nearly 70% of the expressed DGK α .

Expression of DGK α and DGK α D10 in PMN from LAgP and Controls

Real-time PCR was used to evaluate possible variations in the levels of DGK α transcripts. To that end, primers and Taqman probes were designed to anneal to each of the transcripts and were used as described in *Materials and Methods*. The expression of DGK α and DGK α 10 in PMN from control and LAgP individuals is shown in Figure 4. The results revealed that overall, the levels of the spliced transcript DGK α 10 were increased in LAgP PMNs, but only reached significance at the later time point of stimulation. In control PMNs, the predominant transcript was the full-length DGK α , here referred to as WT.

Expression of DGK α -Tagged Fusion Proteins in Mammalian Cells

The sequences of DGK α and DGK α 10 were subcloned downstream to a cytomegalovirus promoter of mammalian expression vectors (pcDNA 3.1). Optimization of the conditions was confirmed by fluorescent microscopy and qPCR (see supplementary Figure 2 in on-line

Journal of Periodontology). Stimulation of transfected cells with PMA caused both DGK α and DGK α 10 constructs to translocate to the cell membrane. Stimulation of cells transfected with plasmid only did not respond to PMA and did not translocate to the membrane (Fig. 5). PMA induced an increase in fluorescence on the surface of cells transfected with either DGK α or DGK α 10 compared to PBS-containing DMSO (PMA-negative group).

Transfection of HL-60 Cells and Superoxide Production

The HL-60 cell line was used as previously described;¹⁵ primary neutrophils are end cells that cannot be cultured to sufficient periods for transfection. HL-60 cells were differentiated with DMSO for 3 days and transfected with the vector (pcDNA), spliced transcript (DGK α 10), or mutagenic dominant-negative construct (MUTA). These conditions were effective in inducing expression of the constructs after 48 hours (Fig. 6A). Because the TaqMan primer-probe used to detect the MUTA construct also detects native DGK α , cells transfected with plasmid only (pcDNA) were used to represent the basal levels of native DGK α expressed by the cell. The overexpressed constructs DGK α 10 and MUTA caused higher levels of stimulated superoxide generation than the native DGK α . Incubation of HL-60 cells with 1.25% DMSO for 6 days increased superoxide anion production in cells transfected with plasmid alone (pcDNA). Transfection with either DGK α 10 or the dominant-negative construct MUTA induced a significant increase in fMLP-induced production of superoxide compared to cells transfected with plasmid only (Fig. 6B).

DISCUSSION

LAgP PMNs accumulate DAG as a result of decreased DGK activity. In this study, the authors sought to quantify and characterize DGK levels and isoforms in LAgP PMNs. The isoform distribution and quantity of DGK in LAgP was abnormal in a single isoform, DGK α , which was surprisingly elevated in spite of reduced activity in the same cells. The authors identified and characterized DGK α 10, a spliced, non-functional variant of DGK α that lacks exon 10, which was elevated in LAgP PMNs. Translation of DGK α 10 generated a truncated, dominant-negative form that regulates superoxide generation. This is the first report of DGK α alternative splicing, although other DGK isoforms have been reported to present spliced variants.²² The α isoform was the first DGK to be characterized²³ and was classified as type I DGK, a member of the Ca²⁺-dependent group.¹³ It has six conserved motifs, two EF hands in the NH₂ terminus, two zinc-finger or DAG/phorbol-ester binding domains, and two catalytic domains located at the carboxyl-terminus. In DGK α 10, splicing out of exon 10 completely abrogated the second predicted DAG/ phorbol-ester binding domain and the catalytic domains. The change in the open reading frame altered the last 11 amino acids and introduced a premature stop codon at methionine 248.

Many investigations have identified and characterized novel transcripts that produce truncated proteins, which may or may not retain their ability to function properly,^{24,25} and a number of other members of the DGK family undergo alternative splicing.^{19,20} Splicing has been shown to impact proteins related to different structures, thus modulating protein binding,²⁶ intracellular receptor domains,²⁷ and localization of proteins.²⁸ Inclusion of

premature stop codons by alternative splicing is a powerful natural mechanism that changes enzymatic affinity, substrate specificity, and catalytic properties.²⁹ The excluded motifs may be directly related to catalysis and substrate recognition, and often, inactive variants act as dominant-negative forms over active proteins.¹⁸ Splicing that leads to translation of truncated proteins has also been linked to protein functional defects, which may cause genetic diseases.³⁰ Other members of the DGK family have been shown to undergo splicing, generating proteins with diverse functions and expression patterns.

Expression of DGK α 10 was not restricted to human neutrophils. Although human tissues express the spliced transcript in comparatively low levels, cancer cell lines presented overall higher expression. This finding can be explained by the fact that in normal conditions, spliced transcripts leading to truncated proteins are monitored by RNA surveillance and targeted for degradation.³⁰ Expression of truncated isoforms in human tissues and cancer-derived cells has also been reported by others.³¹ DGK α levels are elevated in neutrophils of individuals with LAgP, and yet, the intracellular levels of DAG are higher, indicating a limited ability of the DGK α pools to regulate the conversion to PA in LAgP.⁴ The present findings revealed significant upregulation of the spliced transcript in LAgP PMNs only after a longer period of stimulation, which is essentially the mirror image of the ratio of WT to spliced variant in healthy individuals without LAgP. Although the *ex vivo* conditions used may not exactly mimic the *in vivo* milieu, there was a trend for upregulation of the spliced isoform at all times, even though a relevant variation among individuals was observed.

In contrast, a previous study found lower levels of total DGK α RNA levels in LAgP PMNs compared with controls.⁵ This difference may be related to the fact that those authors used primers that probably annealed to different regions of the open reading frame and not the spliced variant, as well as the cell challenge conditions. Furthermore, the findings of both studies call attention to the great variability in PMN response observed among different individuals and conditions. It is noteworthy that transcript levels do not always reflect protein levels and/or function and thus, despite the upregulation of the spliced variant in LAgP PMNs, the actual levels of the truncated protein within the cells could not be assessed since antibodies against this isoform are not available. Nevertheless, if the DGK spliced transcripts are in fact translated into truncated proteins, then a genetic defect related to the mechanism that performs surveillance and degradation of transcripts that may give rise to truncated proteins, i.e., RNA non-sense-mediated decay,³² may be responsible, at least in part, for LAgP PMN response. A number of diseases have been linked to truncated proteins associated with spliced transcripts and failure of the surveillance system to target them for degradation, which greatly alters transcript levels.^{31,33}

The potential of the truncated protein to hamper regulation of DAG in LAgP was assessed by a series of experiments where the spliced transcript was expressed in other cell lines. The first such assessment was related to the ability of the protein to translocate; confocal microscopy analysis of the cloned DGK α 10 expressed in COS-7 cells showed that translocation occurred upon stimulation with PMA, an analog to DAG. The presumed truncated DGK α lacks one of the two DAG binding motifs and the catalytic domains, but it retains two EF-hand motifs at the NH₂-terminus. It has been previously shown in lymphocytes that enzymatic activity is not required for DGK α to translocate to the plasma

membrane.³⁴ Kinetically, however, the catalytically inactive isozyme remains in the membrane longer compared to WT, suggesting that PA generated by DAG phosphorylation has an important function in enzyme relocation to the cytosol. Nevertheless, it is important to emphasize that the mechanism of efficiency of translocation has not been addressed here.

A role has long been suggested for DGK α in the control of PKC-mediated NADPH-oxidase assembly, which mediates the ability of neutrophils to produce superoxide anion.¹⁴ The importance of DGK α in granulocyte function is corroborated by the expression profile of DGKs observed during differentiation of HL-60 cells; the present authors have previously shown that expression of different DGK isoforms is dynamically regulated in HL-60 cells, a model for granulocyte differentiation, and that DGK α mRNA levels increase as differentiation into a neutrophil-like phenotype proceeds.¹⁵ The increased expression levels of DGK α coincide with the ability of the cells to produce superoxide anion upon stimulation, further calling attention to the regulatory function of this isoform. This is critical, since LAgP PMN altered function has been associated with an exacerbation of reactive oxygen species production in response to bacterial products,⁵ which, in turn, produce more tissue breakdown. The present results suggest that the truncated protein can diffuse throughout the cytosol, forming heterodimers with the WT DGK α , thus suppressing its function and acting as a dominant negative. To assess that possibility, a dominant-negative construct was used as a positive control to block the expression of native DGK α induced by differentiation. After transfection of neutrophil-like cells with DGK α 10, there was an increase in the levels of fMLP-induced superoxide production compared to cells transfected with vector alone. The levels of superoxide produced by the cells transfected with the truncated construct reached those of the DGK α -dominant-negative created with site-directed mutagenesis. There is a clear link between DGK α expression and regulation of superoxide generation in neutrophils, offering a possible explanation for the hyper-responsive trait observed in LAgP PMN.

Accumulating evidence points to redundancy in the activation of the NADPH oxidase system leading to superoxide production. Select PKC isoforms have been identified as key intermediates in this process, although the use of PKC inhibitors does not inhibit fMLP-induced superoxide production.³⁵ The type of agonist-specific phospholipase activated (C or D) in the generation of DAG has also been implicated because after 5 minutes, DAG levels remain high even in the absence of superoxide production, suggesting a temporally orchestrated process involving multiple pathways.³⁶ This work further substantiates the importance of the generation of lipid mediators and their regulation in signal transduction. Although diverse, DGK-mediated modulatory functions appear to be relevantly linked to immune and inflammatory responses.

CONCLUSIONS

This study provides novel evidence for a protective regulatory role played by DGK α in the modulation of agonist-mediated activation of superoxide anion production by PMNs, a key bactericidal component that also accounts for oxidative stress and tissue breakdown. Also, the findings suggest a potential role for DGK α alternative splicing in LAgP pathogenesis through the generation of a truncated protein that can act as a dominant-negative isoform.

Future studies must address the participation of accessory DAG pools involved in this pathway as well as detection of the truncated protein in LAgP neutrophils and cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hart TC, Shapira L, Van Dyke TE. Neutrophil defects as risk factors for periodontal diseases. *J Periodontol.* 1994; 65(Suppl. 5):521–529. [PubMed: 8046568]
2. Van Dyke TE, Schweinebraten M, Cianciola LJ, Offenbacher S, Genco RJ. Neutrophil chemotaxis in families with localized juvenile periodontitis. *J Periodontol Res.* 1985; 20:503–514. [PubMed: 2934535]
3. Kantarci A, Oyaizu K, Van Dyke TE. Neutrophil-mediated tissue injury in periodontal disease pathogenesis: Findings from localized aggressive periodontitis. *J Periodontol.* 2003; 74:66–75. [PubMed: 12593599]
4. Tyagi SR, Uhlinger DJ, Lambeth JD, Champagne C, Van Dyke TE. Altered diacylglycerol level and metabolism in neutrophils from patients with localized juvenile periodontitis. *Infect Immun.* 1992; 60:2481–2487. [PubMed: 1316876]
5. Gronert K, Kantarci A, Levy BD, et al. A molecular defect in intracellular lipid signaling in human neutrophils in localized aggressive periodontal tissue damage. *J Immunol.* 2004; 172:1856–1861. [PubMed: 14734770]
6. Kent C, Carman GM, Spence MW, Dowhan W. Regulation of eukaryotic phospholipid metabolism. *FASEB J.* 1991; 5:2258–2266. [PubMed: 1860617]
7. Gilbert JJ, Pettitt TR, Seatter SD, Reid SD, Wakelam MJ, Harnett MM. Antagonistic roles for phospholipase D activities in B cell signaling: While the antigen receptors transduce mitogenic signals via a novel phospholipase D activity, phosphatidylcholine-phospholipase D mediates antiproliferative signals. *J Immunol.* 1998; 161:6575–6584. [PubMed: 9862684]
8. Berridge MJ. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J.* 1984; 220:345–360. [PubMed: 6146314]
9. Berridge MJ, Irvine RF. Inositol phosphates and cell signalling. *Nature.* 1989; 341:197–205. [PubMed: 2550825]
10. Myers MA, McPhail LC, Snyderman R. Redistribution of protein kinase C activity in human monocytes: Correlation with activation of the respiratory burst. *J Immunol.* 1985; 135:3411–3416. [PubMed: 2995492]
11. Sanjuán MA, Jones DR, Izquierdo M, Mérida I. Role of diacylglycerol kinase alpha in the attenuation of receptor signaling. *J Cell Biol.* 2001; 153:207–220. [PubMed: 11285286]
12. van Blitterswijk WJ, Houssa B. Properties and functions of diacylglycerol kinases. *Cell Signal.* 2000; 12:595–605. [PubMed: 11080611]
13. Topham MK, Prescott SM. Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J Biol Chem.* 1999; 274:11447–11450. [PubMed: 10206945]
14. Rider LG, Nidel JE. Diacylglycerol accumulation and superoxide anion production in stimulated human neutrophils. *J Biol Chem.* 1987; 262:5603–5608. [PubMed: 3032939]

15. Batista EL Jr, Warbington M, Badwey JA, Van Dyke TE. Differentiation of HL-60 cells to granulocytes involves regulation of select diacylglycerol kinases (DGKs). *J Cell Biochem.* 2005; 94:774–793. [PubMed: 15578578]
16. Kobayashi T, Tsunawaki S, Seguchi H. Evaluation of the process for superoxide production by NADPH oxidase in human neutrophils: Evidence for cytoplasmic origin of superoxide. *Redox Rep.* 2001; 6:27–36. [PubMed: 11333112]
17. Dennison DK, Van Dyke TE. The acute inflammatory response and the role of phagocytic cells in periodontal health and disease. *Periodontol* 2000. 1997; 14:54–78. [PubMed: 9567966]
18. Alberti L, Bachelot T, Duc A, Biota C, Blay JY. A spliced isoform of interleukin 6 mRNA produced by renal cell carcinoma encodes for an interleukin 6 inhibitor. *Cancer Res.* 2005; 65:2–5. [PubMed: 15665272]
19. Ding L, Bunting M, Topham MK, McIntyre TM, Zimmerman GA, Prescott SM. Alternative splicing of the human diacylglycerol kinase zeta gene in muscle. *Proc Natl Acad Sci USA.* 1997; 94:5519–5524. [PubMed: 9159104]
20. Murakami T, Sakane F, Imai S, Houkin K, Kanoh H. Identification and characterization of two splice variants of human diacylglycerol kinase eta. *J Biol Chem.* 2003; 278:34364–34372. [PubMed: 12810723]
21. Van Dyke TE, Horoszewicz HU, Genco RJ. The poly-morphonuclear leukocyte (PMNL) locomotor defect in juvenile periodontitis. Study of random migration, chemokinesis and chemotaxis. *J Periodontol.* 1982; 53:682–687. [PubMed: 6960166]
22. Shulga YV, Topham MK, Epand RM. Regulation and functions of diacylglycerol kinases. *Chem Rev.* 2011; 111:6186–6208. [PubMed: 21800853]
23. Sakane F, Yamada K, Kanoh H, Yokoyama C, Tanabe T. Porcine diacylglycerol kinase sequence has zinc finger and E-F hand motifs. *Nature.* 1990; 344:345–348. [PubMed: 2156169]
24. Kherrouche Z, De Launoit Y, Monté D. Human E2F6 is alternatively spliced to generate multiple protein iso-forms. *Biochem Biophys Res Commun.* 2004; 317:749–760. [PubMed: 15081404]
25. Kim HJ, Woo IS, Kang ES, et al. Identification of a truncated alternative splicing variant of human PPARgamma1 that exhibits dominant negative activity. *Biochem Biophys Res Commun.* 2006; 347:698–706. [PubMed: 16842753]
26. Indraccolo S, Minuzzo S, Zamarchi R, Calderazzo F, Piovan E, Amadori A. Alternatively spliced forms of Igalpha and Igbeta prevent B cell receptor expression on the cell surface. *Eur J Immunol.* 2002; 32:1530–1540. [PubMed: 12115635]
27. Aigner A, Juhl H, Malerczyk C, Tkybusch A, Benz CC, Czubayko F. Expression of a truncated 100 kDa HER2 splice variant acts as an endogenous inhibitor of tumour cell proliferation. *Oncogene.* 2001; 20:2101–2111. [PubMed: 11360194]
28. Pasqualini C, Guivarc'h D, Barnier JV, Guibert B, Vincent JD, Vernier P. Differential subcellular distribution and transcriptional activity of sigmaE3, sigmaE4, and sigmaE3–4 isoforms of the rat estrogen receptor-alpha. *Mol Endocrinol.* 2001; 15:894–908. [PubMed: 11376109]
29. Stamm S, Ben-Ari S, Rafalska I, et al. Function of alternative splicing. *Gene.* 2005; 344:1–20. [PubMed: 15656968]
30. Garcia-Blanco MA, Baraniak AP, Lasda EL. Alternative splicing in disease and therapy. *Nat Biotechnol.* 2004; 22:535–546. [PubMed: 15122293]
31. Lualdi S, Di Rocco M, Corsolini F, et al. Identification of nine new IDS alleles in mucopolysaccharidosis II. Quantitative evaluation by real-time RT-PCR of mRNAs sensitive to nonsense-mediated and nonstop decay mechanisms. *Biochim Biophys Acta.* 2006; 1762:478–484. [PubMed: 16495038]
32. Hentze MW, Kulozik AE. A perfect message: RNA surveillance and nonsense-mediated decay. *Cell.* 1999; 96:307–310. [PubMed: 10025395]
33. Montfort M, Chabás A, Vilageliu L, Grinberg D. Analysis of nonsense-mediated mRNA decay in mutant alleles identified in Spanish Gaucher disease patients. *Blood Cells Mol Dis.* 2006; 36:46–52. [PubMed: 16326120]
34. Flores I, Jones DR, Ciprés A, Díaz-Flores E, Sanjuan MA, Mérida I. Diacylglycerol kinase inhibition prevents IL-2-induced G1 to S transition through a phosphatidylinositol-3 kinase-independent mechanism. *J Immunol.* 1999; 163:708–714. [PubMed: 10395661]

35. Pongracz J, Lord JM. Superoxide production in human neutrophils: Evidence for signal redundancy and the involvement of more than one PKC isoenzyme class. *Biochem Biophys Res Commun.* 1998; 247:624–629. [PubMed: 9647744]
36. Thompson NT, Tateson JE, Randall RW, Spacey GD, Bonser RW, Garland LG. The temporal relationship between phospholipase activation, diacylglycerol formation and superoxide production in the human neutrophil. *Biochem J.* 1990; 271:209–213. [PubMed: 2171498]

Figure 1.

Quantitative expression of DGK isoforms in LAgPand control PMNs. Cells were incubated with PBS or 5 μ M fMLP for 15 (f5 15) and 180 (f5 180) minutes, and expression of DGK isoforms was analyzed. No significant differences were observed in the expression of isoforms δ , γ , ϵ , and ζ upon stimulation. DGK α mRNA levels underwent a significant upregulation in LAgP PMNs after stimulation for 30 minutes and remained upregulated until 90 minutes. Means and SD of three paired, matched patients run in duplicate (Kruskal–Wallis and Dunn post hoc tests).

Figure 2.

Protein truncation analysis predicted three possible outcomes assessed by coupled in vitro transcription/translation of the PCR-amplified fragments: a 50-KDa protein amplified from DGK α full-length sequence and for DGK α 10 either a 46-KDa fragment in the absence of the stop codon or a 28-KDa fragment in the truncated form (**A**). Gel analysis confirmed that skipping exon 10 introduced a premature stop codon that generated a 28-KDa protein fragment (**A**, right panel). The predicted primary structure of DGK α and DGK α 10 shows that the truncated protein is approximately one-third the size of the native protein and lacks key presumed catalytic functional domains, although the two amino-terminus EF-hands and one of the DAG-binding motifs are maintained (**B**).

Figure 3.

A) Expression of DGKα was significantly higher in human tissues compared with the spliced transcript. **B)** In human cancer cell lines, expression of DGKα was also substantially higher for all cells with the exception of U-937 cultures, where DGKα 10 reached >60% of DGKα levels.

Figure 4.

Expression of DGK α (WT) and DGK α 10 spliced (Spl) in LAGP and control PMNs. Cells were incubated with fMLP (10 nM) for 30 and 180 minutes (30'/180'). LAGP PMNs showed increased levels of DGK α 10 compared with control, which was significant after a longer period of stimulation. DGK α (WT) was the predominant transcript in control PMNs, which also expressed the spliced transcript, but at lower levels. (Mean and SD from 10 pairs of age- and sex-matched control and LAGP individuals.) * $P < 0.05$, LAGP relative to control of same condition.

Figure 5.

COS-7 cells were transfected with pcDNA (vector alone), DGK α , or DGK α 10 and incubated with either 1 mM PMA or DMSO-containing PBS (PMA–) for 30 minutes. Confocal microscopy revealed that incubation with PMA induced a shift in the fluorescence from the cytosol to the cell membrane in DGK α - and DGK α 10-transfected cells (arrows), but not in cells transfected with the empty vector.

Figure 6.

Efficiency of transfection was assessed through fluorescence microscopy (**A** and **B**; **original magnification** $\times 100$) and qPCR (**C** and **D**), which showed overexpression of DGK α 10 (DGK spliced [Spl]) and DGK α MUTA (MUTA) by the respectively transfected cells.

Superoxide anion production assessment (**E**) showed that expression of the spliced transcript (alpha spl) induced an increase in the production of superoxide anion, which was higher than in differentiated cells transfected with plasmid alone (pcDNA), but similar to the dominant negative mutagenic construct (MUTA). Non-differentiated HL-60 cells (ND) produced some superoxide as well, but less than DMSO-incubated cells (pcDNA-transfected). * $P < 0.01$.