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Expression profiles of phosphatidylinositol phosphate kinase genes during normal human *in vitro* erythropoiesis

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ABSTRACT. Phosphatidylinositol phosphate kinases (PIPKs) are enzymes that participate in diverse intracellular signaling pathways. They are classified into 3 functionally distinct subfamilies - PIPKI (α, β, γ), PIPKII (α, β, γ), and PIPKIII - located in various subcellular compartments. Recently, the PIPKIIα and β-globin genes were found to be overexpressed in reticulocytes from 2 siblings with hemoglobin H disease, suggesting a possible relationship between PIPKIIα and the production of globins. The main aim of this study was to determine the expression profiles of PIPK genes in healthy individuals during *in vitro* erythropoiesis using quantitative real-time polymerase chain reaction and to compare these profiles with profiles of globin genes. Our results showed that expression of all PIPKs increases as the cells differentiate, coinciding with the expression profiles of globins. Analysis of the effects of globins on PIPK genes revealed that they varied significantly

between the globins, the most noticeable being the effect of α-globin on PIPKIIα ($P < 0.0001$) and γ-globin on PIPKIIγ ($P < 0.0001$). The relationship between the expression of PIPKs and globin genes was statistically significant, particularly between PIPKIIα and α-globin ($P = 0.0002$) and PIPKIIγ and β-globin ($P < 0.0001$). Linear correlation analysis revealed a strong relationship between PIPKIIα and α-globin genes. This study is the first to establish the expression profiles of PIPK genes during *in vitro* erythropoiesis in healthy individuals and suggests a parallel between the expression of PIPK and globin genes, reinforcing the hypothesis that they may be related.

Key words: Phosphatidylinositol phosphate kinases; Globin genes; Gene expression; Human erythropoiesis

INTRODUCTION

Phosphatidylinositol phosphate kinases (PIPKs) are lipid kinases responsible for the production of a variety of lipid second messengers, including phosphatidylinositol-4,5-biphosphate ($PI4,5P_2$). $PI4,5P_2$ regulates various cellular activities, including modulation of the actin cytoskeleton, vesicle trafficking, secretion, focal adhesion formation, and gene expression (Anderson et al., 1999; Doughman et al., 2003; Heck et al., 2007; van den Bout and Divecha, 2009).

PIPKs are divided into 3 functionally distinct subfamilies located in various subcellular compartments according to their signaling specificity: PIPKI or PIP5K, PIPKII or PIP4K, and PIPKIII (Anderson et al., 1999; Doughman et al., 2003; Heck et al., 2007). PIPKI and PIPKII have 3 isoforms (α, β, and γ) and produce $PI4,5P_2$ by catalyzing the phosphorylation of phosphatidylinositol-4-phosphate and phosphatidylinositol-5-phosphate, respectively (Hinchliffe and Irvine, 2006; Wilcox And Hinchliffe, 2008; Mellman and Anderson, 2009). PIPKIs are found in the plasma membrane, focal adhesions, Golgi, and nucleus, and their activities appear to be necessary in endocytosis, adhesion, secretion, and actin rearrangements. PIPKIIs have been found in the cytosol, nucleus, and endoplasmic reticulum, but their physiological functions have not been well defined to date. Both PIPKI and PIPKII seem to be implicated in the regulation of gene expression, pre-messenger RNA (mRNA) processing, and mRNA export. PIPKIIIs are found in the cellular internal membranes and maintain vesicle membrane integrity (Anderson et al., 1999; Doughman et al., 2003; Mellman et al., 2008). PIPKIIα was the first to be cloned from human erythrocytes and is found in abundance in these cells (Bazenet et al., 1990; Boronenkov and Anderson, 1995).

In a recent study, the PIPKIIα gene was shown to be differentially expressed in reticulocytes from 2 siblings with the same genotype who had hemoglobin (Hb) H disease. Expression of both PIPKIIα and β-globin genes was higher in the patient with the higher Hb H level, suggesting a relationship between PIPKIIα and the production of globins, particularly β-globin (Wenning et al., 2009).

Few studies of PIPKs and erythroid cells appear in the literature (Bazenet et al., 1990; Boronenkov and Anderson, 1995; Wenning et al., 2009), and to our knowledge, none has evaluated the expression profiles of these molecules in normal erythroid cells. The main aim of this study was therefore to determine gene expression profiles for PIPK family members

during erythroid differentiation in cultured human $CD34^+$ cells from healthy subjects and compare them with those of human globin genes (α, β, and γ).

MATERIAL AND METHODS

Hematopoietic cell culture

Peripheral blood samples from 11 healthy adult volunteers were used to establish liquid cultures of $CD34^+$ cells to differentiate these cells into erythroblasts (Ugo et al., 2004). All subjects gave written consent to participate in the study in accordance with the Declaration of Helsinki. The study was approved by the local Ethics Committee.

Mononuclear cells were obtained from the blood samples (54 mL) via centrifugation over a Ficoll-Paque gradient (GE Healthcare Bio-Sciences, Sweden). $CD34^+$ cells were then separated through double-positive selection using a magnetic cell sorting system (Human Indirect CD34 MicroBead Kit, MACS Miltenyi Biotec GmbH, Germany) according to manufacturer instructions. The purity of the recovered $CD34^+$ cells was determined with flow cytometry (FACSCalibur, Becton Dickinson, USA) using the monoclonal mouse anti-human CD34 antibody class III/retinal pigment epithelium Clone BIRMA-K3 (DakoCytomation, Denmark).

Fresh $CD34^+$ cells were cultured at 37°C in 5% CO_2 and 95% humidity for 13 days in Iscove's modified Dulbecco's medium (Invitrogen, USA) supplemented with 2.5 µg/mL amphotericin B (Invitrogen), 50 µg/mL streptomycin-penicillin (Invitrogen), 1% deionized bovine serum albumin (USBiological, USA), lipid solution (Sigma Aldrich, USA), human apo-transferrin (Sigma Aldrich), fetal calf serum (Invitrogen), 50 ng/mL recombinant human stem cell factor (PeproTech, USA), 5 ng/mL interleukin-3 (PeproTech), and 1 U/mL human erythropoietin 3000 IU (Eprex, Vetter Pharma-Fertigung GmbH, Germany).

Cell samples were collected on the 7th, 10th, and 13th days of culture to determine cell viability using the Trypan blue exclusion test, carry out total RNA extraction and complementary DNA (cDNA) synthesis, and analyze cell differentiation using flow cytometry with cell surface marker antibodies (CD71 and glycophorin A; Caltag Laboratories, USA), cytoplasmic antibodies (fetal Hb; Caltag Laboratories), and cytospin slides.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA samples were isolated using a commercial kit (RNeasy Mini and Micro kit, Qiagen, GmbH, Germany) according to the manufacturer protocol. RNA integrity was assessed using electrophoresis on a 1% agarose gel. Two micrograms of RNA from the cell culture samples was subjected to DNAse I treatment (Invitrogen) and used to synthesize cDNA with SuperScript III™ Reverse Transcriptase (Invitrogen) according to manufacturer instructions. The RNA samples were quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., USA).

Gene expression assays were carried out using qRT-PCR (Step One Plus Real-Time PCR System, Applied Biosystems) with the SYBR Green System (Invitrogen). cDNAs from cell culture samples of 5 µg PIPK and 0.5 µg globin were used with SYBR Green qPCR Supermix-UDG and ROX (Invitrogen) in a 12-µL volume to detect amplified products. PCR primers were designed using the Primer Express software (Applied Biosystems) to amplify

mRNA from the gene of interest as follows: PIP5K1α_F, 5'-GGGGAACACAAGGCACAAG T-3' and PIP5K1α_R, 5'-TAAAACATCAGGACGACCAAGG-3'; PIP5K1β_F, 5'-GATACGG AAACATACAACGCG-3' and PIP5K1β_R, 5'-CCATGATCTTGAAGCTTTCTAGC-3'; PIP5K1γ_F, 5'-CTGTCGCCTTCCGCTACTTC-3' and PIP5K1γ_R, 5'-GGTCATTGCACAG GGAGTAC-3'; PIP4K2α_F, 5'-CCACCGTTTGTCTGTGTATAGGA-3' and PIP4K2α_R, 5'-TTCAGAGTTGGCAGTTCTTTGG-3'; PIP4K2β_F, 5'-TGCTGCCAAAACGGTGAAA-3' and PIP4K2β_R, 5'-CGTTGAAGCGTTTGGAGTACTG-3'; PIP4K2γ_F, 5'-CCAGTGAGG ACATTGCTGACAT-3' and PIP4K2γ_R, 5'-CAGAAGCGTGTTGCCATGG-3'; PIP5K3_F, 5'-ACTCTGAGCCATCCTGGTTTAA-3' and PIP5K3_R, 5'-ATGTGCGCTTGCTAGGACT G-3'; α-globin_F, 5'-GGCGAGTATGGTGCGGAG-3' and α-globin_R, 5'-TGCGGGAAGTA GGTCTTGGT-3'; β-globin_F, 5'-CACTGTGACAAGCTGCACGTG-3' and β-globin_R, 5'-GCACACAGACCAGCACGTTG-3'; γ-globin_F, 5'-TGTGGAAGATGCTGGAGGAGA -3' and γ-globin_R, 5'-CAAAGAACCTCTGGGTCCATG-3'. The housekeeping genes β-actin (BAC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous controls: BAC (F, 5'-AGGCCAACCGCGAGAAG-3' and R, 5'-ACAGCCTGGATAGCAACG TACA-3') and GAPDH (F, 5'-GCACCGTCAAGGCTGAGAAC-3' and R, 5'-CCACTTGAT TTTGGAGGGATCT-3'. All relative quantification (RQ) experiments were performed in duplicate. RQ of gene expression was calculated using the equation $RQ = 2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

The expression profiles of the PIPK (Iα, Iβ, Iγ, IIα, IIβ, IIγ, and III) and globin (α, β, and γ) genes were compared on the 10th and 13th days of the erythroid culture. The data were normalized using the average expression level of 2 endogenous controls, GAPDH and BAC, which are normally expressed during erythroid differentiation. The samples from the 7th day were used as calibrators to calculate RQ. As the ΔΔCt of the calibrator is always equal to 0, the gene expression relative to the calibrator, which is given by $RQ = 2^{-\Delta\Delta Ct}$, is always equal to 1 (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis of the data was carried out with Origin 5.0 (MicroCal Software Inc., USA) and SAS System for Windows 9.1.3 (SAS Institute Inc., USA). The mean and 95% confidence interval were calculated for all variables studied. The generalized estimating equation test with a rank transformation and the Spearman correlation coefficient between the areas under the curves were used to determine the relationship between PIPKs and globin genes. Correlations were considered to be significant at $P < 0.05$.

RESULTS

The qRT-PCR analysis showed increased expression of PIPKIIα and other PIPK genes during erythroid differentiation (10th and 13th days), coinciding with the expression profiles of globin genes (Figure 1). Additionally, we determined the relationship between the PIPK genes and globin genes using the generalized estimating equation test. We found significant correlations, particularly between the effect of α-globin on PIPKIIα ($P < 0.0001$) and that of γ-globin on PIPKIIγ ($P < 0.0001$; Table 1). We also analyzed the effect of PIPKs on globin genes and found that PIPKIIα had an effect on the α-globin gene ($P = 0.0002$), and PIPKIIγ

had an effect on the β-globin gene ($P < 0.0001$; Table 2). Linear correlation analysis revealed a strong correlation between PIPKIIα and α-globin gene expression (Table 3). Figure 2 shows the statistically significant correlations found between the expression of the various PIPKs and globin genes.

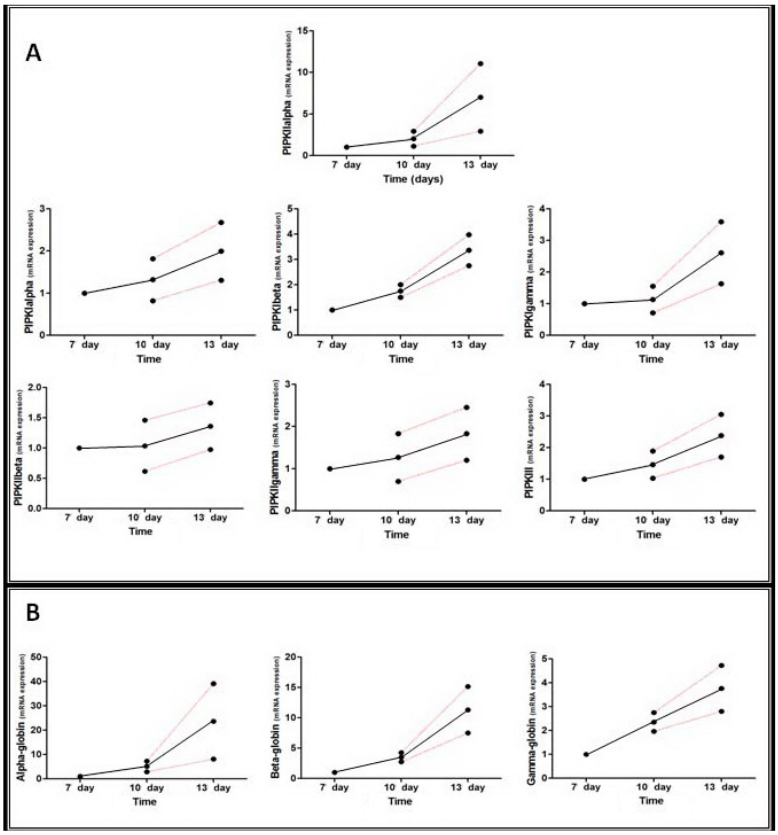

Figure 1. Expression profiles of the PIPK (IIα, Iα, Iβ, Iγ, IIβ, IIγ, and III - **A**) and globin (α, β and γ - **B**) genes in the study population. The red and black lines correspond, respectively, to the 95% confidence interval and mean expression for the study population. Recorded values were normalized according to the quantity of BAC and GAPDH mRNA measured in each sample. PIPK = phosphatidylinositol phosphate kinase.

Table 1. Analysis of the effect of globins on PIPK genes (N = 11).

	α-globin P*	β-globin P*	γ-globin P*
PIPKIα	0.045	0.552	0.365
PIPKIβ	0.581	0.0008	0.045
PIPKIγ	0.012	0.536	0.158
PIPKIIα	<0.0001	0.663	0.816
PIPKIIβ	0.316	0.525	0.003
PIPKIIγ	0.015	0.0005	<0.0001
PIPKIII	0.002	0.734	0.908

*Generalized estimating equation test. PIPK = phosphatidylinositol phosphate kinase.

Table 2. Analysis of the effect of PIPKs on globin genes (N = 11).

	PIPKIα P*	PIPKIβ P*	PIPKIγ P*	PIPKIIα P*	PIPKIIβ P*	PIPKIIγ P*	PIPKIII P*
α-globin	0.008	0.131	0.031	0.0002	0.365	0.673	0.041
β-globin	0.697	0.004	0.407	0.964	0.201	<0.0001	0.975
γ-globin	0.256	0.452	0.067	0.507	0.340	0.078	0.802

*Generalized estimating equation test.

Table 3. Linear correlation analysis of the areas under the expression curves for PIPK and globin genes (N = 11).

	α-globin		β-globin		γ-globin	
	r	P*	r	P*	r	P*
PIPKIα	**0.636**	**0.035**	0.081	0.811	-0.150	0.659
PIPKIβ	0.582	0.060	**0.609**	**0.046**	0.150	0.659
PIPKIγ	**0.609**	**0.046**	-0.027	0.936	-0.328	0.324
PIPKIIα	**0.800**	**0.003**	0.536	0.089	-0.127	0.708
PIPKIIβ	0.500	0.117	-0.027	0.936	0.168	0.620
PIPKIIγ	0.372	0.258	-0.227	0.501	-0.478	0.136
PIPKIII	0.472	0.142	0.100	0.769	-0.200	0.554

*Spearman correlation coefficient. PIPK = phosphatidylinositol phosphate kinase. Values in bold mean statistically significant correlations (P < 0.05).

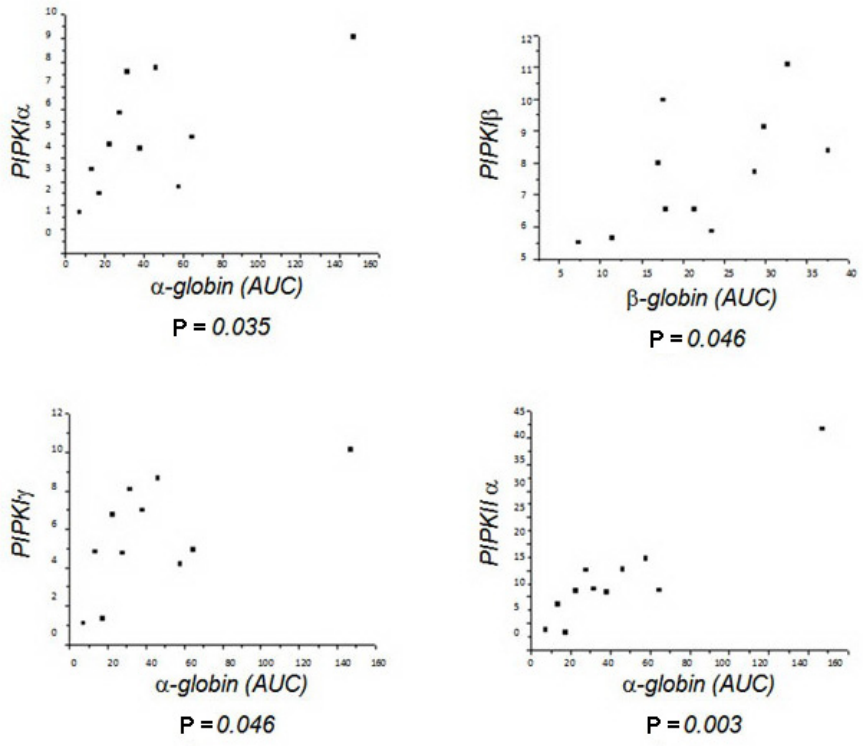

Figure 2. Graphic representation of the significant correlations found between the expression of phosphatidylinositol phosphate kinases (PIPKs) and globin genes. AUC = area under the curve.

DISCUSSION

PIPKs are lipid kinases that participate in diverse intracellular signaling pathways and signal transduction (Anderson et al., 1999). The membrane-bound form of PIPK was purified from erythrocyte membranes, which helped elucidate some of the biochemical properties of these enzymes (Ling et al., 1989). Bazenet et al. (1990) have characterized PIPK types I and II in erythrocytes, demonstrating that the former is a red blood cell membrane protein and the latter is cytosolic. PIPKIIα was the first to be cloned from erythrocytes and is abundant in these cells (Bazenet et al., 1990; Boronenkov and Anderson, 1995). However, few studies of PIPKs in erythroid cells appear in the literature.

As mentioned earlier, PIPKs are present in various subcellular compartments, including the plasma membrane, cytosol, endoplasmic reticulum, cytoskeleton, and nucleus (Loijens et al., 1996; Anderson et al., 1999). Boronenkov et al. (1998) have demonstrated that PIPKIα and PIPKIIα are associated with subnuclear domains, identified as "nuclear speckles", which contain factors that participate in mRNA processing. This finding suggests that these kinases are involved in gene expression. Although some attempts have been made to elucidate the regulation of gene expression by these nuclear phosphoinositides (Mellman et al., 2008; Mellman and Anderson, 2009; Keune et al., 2011), the mechanism by which they directly regulate these functions remains unknown. Recently, Wenning et al. (2009) have suggested that PIPKIIα may be involved in the regulation of β-globin gene expression in α-thalassemia patients; however, none of the studies in the literature to date have investigated the normal profiles of PIPKs in erythroid cells from healthy individuals. This study is the first to establish the expression profiles of PIPK genes during *in vitro* human erythroid differentiation in cells from a group of healthy individuals.

Our results clearly show an increase in the expression levels of PIPKIIα and other PIPK genes during *in vitro* erythropoiesis, coinciding with an increase in the expression of globin genes, particularly α- and β-globin. The analysis of the effect of PIPKs on globin genes and of globins on PIPK genes revealed a statistically significant relationship between them, as did the correlation analysis. We have established the expression profiles of PIPKs in erythroid cell cultures originating from healthy individuals and have shown that 1) the expression of these genes increases gradually during *in vitro* erythropoiesis, and 2) each gene has a characteristic profile, as is the case with the globin genes (Mahajan et al., 2009). These findings provide evidence of a relationship between these enzymes and Hb synthesis and reinforces the hypothesis that they may be related. Further studies should be carried out using cells in which the PIPK genes are either overexpressed or have been knocked down to gain a better understanding of the roles of these enzymes in erythroid cells and their possible relationship with globin genes.

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