

Suppression of proline-directed protein kinase F_A expression inhibits the growth of human chronic myeloid leukaemia cells

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Summary Initial studies revealed that proline-directed protein kinase F_A (PDPK F_A) was overexpressed in various cancerous tissues relative to normal controls. However, the functional role of overexpressed PDPK F_A in cancer remains to be established. In this report, we explore the potential role of PDPK F_A in leukaemia cell growth by investigating the effects of partial inhibition of this kinase on the malignant phenotype of human chronic myeloid leukaemia cells (K562). Cloning of PDPK F_A cDNA and its recombinant antisense expression vector and PDPK F_A -specific antibody were successfully developed. Two stable antisense clones of K562 cells were subcloned which expressed 70% and 45% of PDPK F_A respectively, compared with control-transfected clone in both immunoprecipitate activity assay and immunoblot analysis. In sharp contrast, these two antisense clones expressed no significant suppression of any other related PDPK family members, indicating the specificity of these two antisense clones. Moreover, these antisense clones proportionally and potentially exhibited cell growth retardation, poor clonogenic growth in soft agar and loss of serum independence. The results demonstrate that specific antisense suppression of PDPK F_A is sufficient to interfere with the growth of K562 cells, indicating that PDPK F_A is essential for human chronic myeloid leukaemia cell growth. © 2000 Cancer Research Campaign

Keywords: chronic myeloid leukaemia cells K562; PDPK F_A ; cell growth

Proline-directed protein kinase F_A (PDPK F_A) was originally identified as type-1 protein phosphatase activating factor/glycogen synthase kinase-3 α (Vandenhede et al, 1980; Hemmings et al, 1981; Woodgett, 1990), but has subsequently been demonstrated as a multisubstrate PDPK possibly involved in the regulation of diverse cell functions (for reviews, see Plyte et al, 1992; Yang, 1994). Initial clinical studies revealed that PDPK F_A was overexpressed many-fold in various human cancerous tissues relative to normal controls and the expression of PDPK F_A activity appeared to be significantly correlated with the progression states of various human cancers (Lee et al, 1995; Yang et al, 1995, 1996, 1998). This provided an initial evidence that overexpression of PDPK F_A may be involved in tumour promotion and progression. However, the exact functional role of overexpressed PDPK F_A in cancer has not yet been directly demonstrated and remains to be established.

In this report, we use a more direct approach to investigate the potential role of PDPK F_A in controlling the growth of K562 cells which were developed from leukaemia cells of a chronic myeloid leukemia blastic phase patient by Lozzio and Lozzio (1975). We have successfully cloned a partial sequence of PDPK F_A cDNA and constructed a recombinant antisense expression vector. The antisense expression vector could be stably transfected into K562 cells. This results in a specific suppression of PDPK F_A expression, and concomitantly leads to cell growth retardation, decreased serum independence and poor clonogenic growth in soft agar. The

results presented here demonstrate that specific antisense suppression of PDPK F_A expression is sufficient to interfere with the growth properties of K562 cells, indicating that PDPK F_A is essential for human chronic myeloid leukaemia cell growth. Suppression of overexpressed PDPK F_A may provide a useful clinic target for therapeutic intervention aimed at inhibiting the malignant behaviour of human chronic myeloid leukaemia.

MATERIALS AND METHODS

Materials

Human chronic myeloid leukaemia cells (K562), derived from leukaemia cells of a chronic myeloid leukaemia blastic phase patient, were supplied by American Type Culture Collection (ATCC, Rockville, MD, USA). The cells within passages 10–30 were used for all the experiments in this text. [γ - 32 P]ATP and enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased from Amersham (Buckinghamshire, UK). RPMI-1640, fetal bovine serum and geneticin (G418) were from Gibco (Paisley, UK). Benzamidine, *n*- α -tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulphonyl fluoride (PMSF), sodium fluoride (NaF), Triton X-100, LiCl and Tween-20 were from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium orthovanadate and 3-(cyclohexylamino)-1-propane-sulphonic acid (CAPS) were from Sigma (St Louis, MO, USA). BCA (bacteriochlorine *a*) protein assay reagent was from Pierce (Rockford, IL, USA). Protein A-Sepharose CL-4B was from Pharmacia (Uppsala, Sweden). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was from Millipore (Bedford, MA, USA).

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Phospho GS-2, mouse monoclonal anti-GSK-3 β and rabbit polyclonal anti-Erk-1/-2 antibodies were from UBI (Lake Placid, NY, USA). Goat anti-rabbit and anti-mouse IgG antibodies conjugated with peroxidase were from Bio-Rad (Hercules, CA, USA). Aprotinin and DOTAP transfection kit were from Boehringer Mannheim (Mannheim, Germany). X-ray film was from Fuji (Tokyo, Japan).

Production of anti-PDPK F_A antibody

The peptide QAPDATPTLTNSS, corresponding to the carboxyl terminal region from amino acids 471–483 of the sequence of F_A (Woodgett, 1990) was synthesized by peptide synthesizer (model 9050, Milligen, Bedford, MA, USA). The cysteine residue was added to the NH₂ terminus in order to facilitate coupling of the peptide to bovine serum albumin according to the procedure described by Reichlin (1980) using glutaraldehyde as the cross-linker. The detailed procedure for production and affinity-purification of this antibody was as described in previous reports (Yu and Yang, 1993, 1994; Yang et al, 1998).

Cloning of PDPK F_A cDNA and construction of recombinant antisense expression vector

A partial sequence (~1.0 kb fragment starting from 3' end of F_A cDNA) was cloned from human fibroblast cells by reverse transcriptase polymerization chain reaction using 5'-CGCGCCT-GGAAGAGGCCAG-3', 5'-ACTGGAGGTGGGGACAGGGA-3', 5'-AAGCTAGCGCCTGTGCTCGGCGCCATGA-3' and 5'-TTG-AATTCGCCCTCAGGAGGAGTTAGTG-3', as primers (Woodgett, 1990; He et al, 1995). The cloned cDNA fragment was constructed into pBK-CMV vector in an antisense orientation downstream of the cytomegalovirus (CMV) promoter using *EcoRI*-*NheI* as the ligation sites. The neomycin-resistant gene placed downstream of the simian virus 40 origin was used as the second open reading frame for initial screening of the transfected clones. The developed antisense construct named as AtF_ApBK-CMV was put into mass production in *Escherichia coli* and plasmid was purified by alkaline lysis method.

Cell culture and selection of stably-transfected clones

K562 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 25 U ml⁻¹ penicillin and 25 μ g ml⁻¹ streptomycin. Cells were incubated at 37°C in a 95% air/5% carbon dioxide (CO₂) and water-saturated atmosphere in 75-cm² flasks. For transfection, pBK-CMV vector alone as control, or AtF_ApBK-CMV vector as antisense construct as described above was introduced into cells by DOTAP. Briefly, 10 μ g vector mixed with DOTAP was incubated with 1 \times 10⁶ cells in serum-free medium at 37°C for 6 h. The transfected cells were then seeded in 96-well plate (1 \times 10⁴ cells per well) with complete medium containing 400 μ g ml⁻¹ geneticin (G418) for selection of recombinant clones expressing G418 resistance. After 4 weeks, individual clones surviving in the presence of G418 were further expanded to mass culture.

Cell extract preparation

For cell extract preparation, 2.5 \times 10⁶ cells washed twice with ice-cold phosphate-buffered saline (PBS) were suspended in 500 μ l

solution A (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 1 mM EGTA, 50 mM sodium chloride (NaCl), 1% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 0.5 μ g ml⁻¹ aprotinin, 50 mM NaF, 20 mM sodium pyrophosphate and 1 mM sodium orthovanadate) and then homogenized on ice by Sonic Dismembrator (model 150, Fisher) for 3 \times 10 s at 40% power output. Cell lysates were then ultracentrifuged at 100 000 g for 30 min at 4°C and the supernatants were used as the cell extracts. Protein concentration of the cell extract was determined by BCA protein assay and adjusted to 1 mg ml⁻¹ with solution A.

Immunoprecipitation and PDPK F_A activity assay in the immunoprecipitate

Cell extract (300 μ g cell protein) was incubated with 0.5 μ l affinity-purified anti-PDPK F_A antibody (1.5 μ g pure IgG) at 4°C for 1.5 h and then with 100 μ l protein A-Sepharose CL-4B (20% v/v in solution A) for another 1.5 h with shaking. The immunoprecipitate was collected by centrifugation, washed twice with solution B (20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM benzamidine, and 0.1 μ M aprotinin) containing 0.5 M LiCl, once with 1 ml solution B, and then resuspended in 200 μ l solution B. For PDPK F_A activity assay in the immunoprecipitate, 10 μ l immunoprecipitate at appropriate dilution were incubated with 15 μ l mixture containing 20 mM Tris-HCl at pH 7.0, 0.5 mM DTT, 10 μ g heparin, 0.2 mM [γ -³²P]ATP (~1000 cpm pmol⁻¹), 20 mM magnesium chloride (MgCl₂) and 60 μ M phospho GS-2 (YRRAVPPSPSLSRHSSPHQ-pSEDEEE) at 30°C for 10 min. ³²P-incorporation was measured by spotting 20 μ l reaction mixture on phosphocellulose paper (1 \times 3 cm²), washing 3 times with 75 mM phosphoric acid (H₃PO₄) and counting in liquid scintillation counter. A unit of PDPK F_A is that amount of enzyme that incorporates 1 pmol of phosphate per min into the peptide substrate.

Immunoblot analysis

For immunoblot analysis, the cell extract containing 10 μ g cell protein was subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to PVDF membrane and then immunoblotted with 50 ng ml⁻¹ primary antibodies as indicated and then with goat anti-rabbit or anti-mouse IgG antibody conjugated with peroxidase (1:3000) essentially as described in previous reports (Yang et al, 1994, 1998; Yu and Yang, 1994). Immunoblot was developed with the ECL system using peroxidase substrate at 25°C for chemiluminescence detection (Gillespie and Hudspeth, 1991). The luminescent light emission was recorded on X-ray film and quantified by computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

Clonogenic growth in soft agar

Antisense and control-transfected clones of K562 cells were plated in soft agar for clonogenicity as described by Jeha et al (1996). Briefly, a feeder layer consisting of medium supplemented with 20% serum and 0.5% agarose was plated in a 60-mm tissue culture dish. The feeder layer was overlaid with a top layer that included 1 \times 10⁴ cells with 20% serum and 0.35% agarose. After 10 days, colonies were stained with 2 ml of 1 mg ml⁻¹ MTT solution at 37°C for 12 h and then counted and photographed.

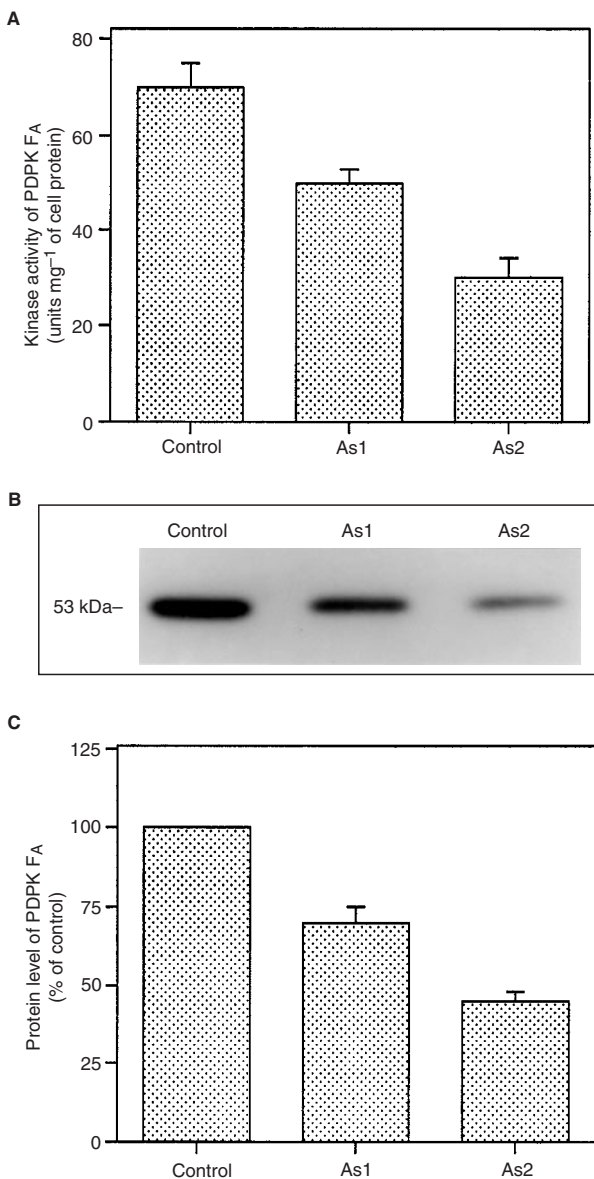


Figure 1 Suppression of PDPK F_A protein expression in antisense clones of K562 cells. The cell extracts (300 µg cell protein) of PDPK F_A antisense (As1 and As2) and control-transfected (control) clones as indicated were immunoprecipitated by using 1.5 µg anti-PDPK F_A antibody. The PDPK F_A activity in the immunoprecipitate was measured using phospho GS-2 as substrate (A). The same cell extracts containing 10 µg cell protein were subjected to 10% SDS-PAGE, electrotransferred to PVDF membrane and immunoblotted with 50 ng ml⁻¹ of anti-PDPK F_A antibody (B) followed by computing densitometric quantification of the relative amount of PDPK F_A (% of control) on the immunoblot (C). Data were taken from the representative results of three independent experiments and expressed as means ± s.d. As1/As2: the two antisense clones of K562 cells stably transfected with PDPK F_A antisense expression vector

RESULTS

Establishment and characterization of antisense clones that stably induced a decrease of PDPK F_A

Transfection of K562 cells was performed with AtF_ApBK-CMV vector as the antisense construct or with pBK-CMV vector as control following G418 selection as described in Materials and

Table 1 Biological properties of PDPK F_A antisense clones of K562 cells

Cell clones ^a	Protein level of PDPK F _A ^b (% of control)	Doubling time ^c (H)	Soft agar colonies ^d (% Colonies/dish)
Control	100	18.2 ± 0.6	100
As1	70	20.1 ± 0.4	12.2 ± 0.5
As2	45	24.3 ± 0.3	2.4 ± 0.8

^aControl, control-transfected clone; As1/As2, the two PDPK F_A antisense clones. ^bThe relative amount of PDPK F_A determined by immunoblot analysis. ^cAntisense or control clones (2.5 × 10⁴ cells ml⁻¹) were cultured at 37°C in medium supplemented with 10% serum and counted every 24 h by trypan blue exclusion method. The doubling time was calculated during the exponential growth phase. Results are the averages of four independent experiments and expressed as means ± s.d. ^dAntisense or control clones (1 × 10⁴ cells/dish) in a top layer consisting of medium supplemented with 20% serum and 0.35% agarose were overlaid on the feeder layer consisting of medium supplemented with 20% serum and 0.5% agarose in 60-mm culture dish. After 10 days at 37°C, colonies were stained with 2 ml mg⁻¹ ml⁻¹ MTT solution at 37°C for 12 h. Data were taken from the averages of three independent experiments and expressed as % of control.

Methods. Several G418-resistant clones were successfully subcloned and expression levels of PDPK F_A were determined by immunoprecipitate kinase assay for cellular activity and by immunoblot analysis for protein expression. Similar protein and activity levels (70 ± 5 units mg⁻¹ cell protein) of PDPK F_A were found to exist in both untransfected parental and control-transfected clones (data not further illustrated). The results indicate that neither transfection nor G418 treatment could affect the expression of PDPK F_A in K562 cells. In contrast, two antisense clones were obtained in which the cellular activities of PDPK F_A were decreased to the levels of 50 ± 3 units mg⁻¹ cell protein (As1) and 30 ± 4 units mg⁻¹ cell protein (As2) respectively (Figure 1A). Immunoblot analysis revealed that the protein levels of PDPK F_A in these two antisense clones were also suppressed in a similar manner (Figure 1B). Computing densitometric analysis further revealed that the protein levels of As1 and As2 were suppressed to 70% and 45% of control level respectively (Figure 1C). The suppressed activity (Figure 1A) and protein (Figure 1 B, C) levels of PDPK F_A in the antisense clones appeared to be very similar, demonstrating that inhibition of PDPK F_A activity in antisense clones is due to suppression of the protein expression. Moreover, although the DNA sequence of PDPK F_A has 85% identity with glycogen synthase kinase-3β (GSK-3β) (Woodgett, 1990), the PDPK F_A antisense expression vector constructed here appeared to have no significant effect on the protein expression of GSK-3β in K562 cells (not shown). We also determined another well-established PDPK member, namely the mitogen-activated protein kinases (MAPKs), and again no significant effect on the expression of Erk-1/-2 in these two antisense clones could be observed (not shown). The results demonstrate that the antisense expression vector constructed here specifically suppressed PDPK F_A in K562 cells and had no effect on the expression of the other DNA sequence-homologous PDPK members such as GSK-3β and MAPKs.

Suppression of PDPK F_A expression in antisense clones resulted in blocking the growth of K562 cells

To study the role of PDPK F_A in controlling the growth of K562 cells, the biological properties of antisense expressing K562 cells

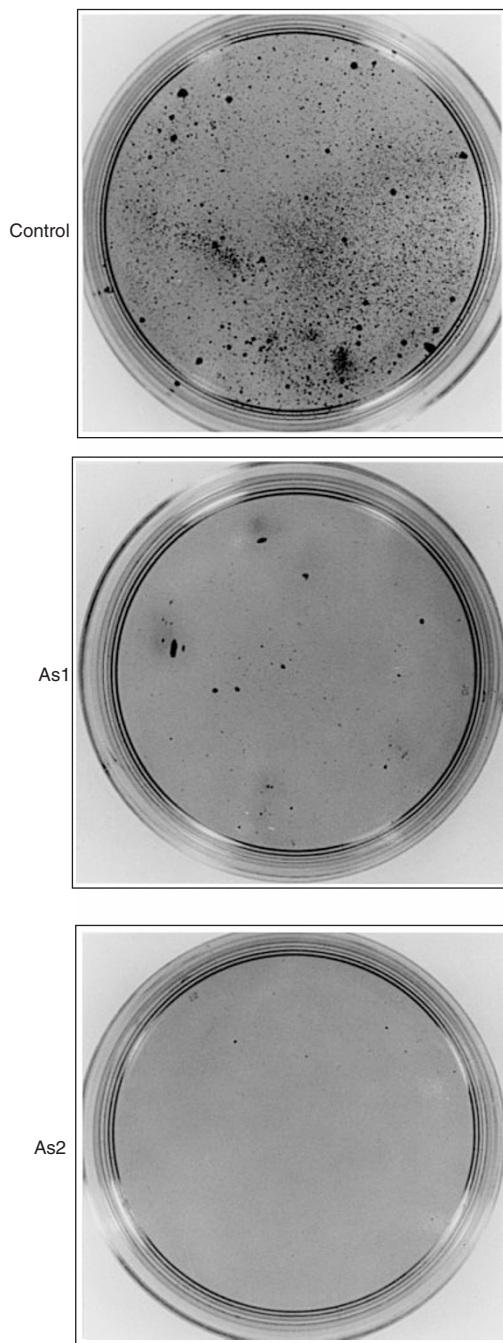


Figure 2 Assessment of clonogenicity of PDPK F_A antisense clones in soft agar. Clonogenicity of PDPK F_A antisense (As1 and As2) and control-transfected (control) clones was assessed by growing 1×10^4 cells in 0.35% agarose to form colonies as described in Materials and Methods. After 10 days, the colonies stained with MTT solution at 37°C for 12 h were photographed. Data were taken from the representative results of three independent experiments

were examined. In studying the growth property of the antisense clones, we found that the two antisense clones proportionally had lower growth rates compared with control-transfected cells. Doubling time, calculated during the exponential growth phase, was significantly and proportionally increased from 18.2 h of control up to 24.3 h as summarized in Table 1. Since clonogenic growth in soft agar was established as an important parameter that

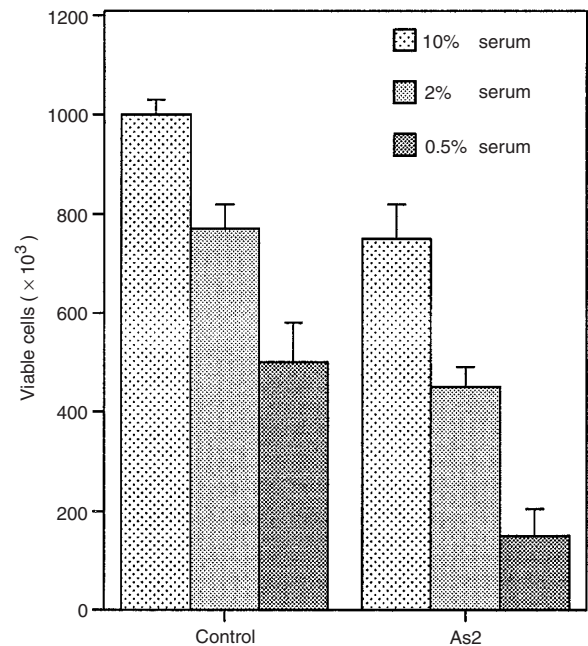


Figure 3 Serum-dependent growth in PDPK F_A antisense clone. PDPK F_A antisense (As2) and control-transfected (control) clones (2.5×10^5 cells) were cultured in different concentrations of serum as indicated at 37°C for four days and the viable cells were counted by trypan blue exclusion method. Results are the averages of four independent experiments and expressed as means \pm s.d

has the best correlation with *in vivo* tumorigenicity (Freedman and Shin, 1974; Shin et al, 1975; Jeha et al, 1996), we next subjected all the cell clones to the analysis of clonogenic growth in soft agar. The most inhibited antisense clone (As2) had almost no ability of forming colonies in soft agar and intermediate phenotype was observed in the clone expressing intermediate level of PDPK F_A (As1) as shown in Figure 2 and summarized in Table 1. The results demonstrate that specific suppression of PDPK F_A expression is sufficient to inhibit proliferation and to diminish clonogenic potential of K562 cells in soft agar. Moreover, since non-transformed cells have a greater degree of dependence on serum for their growth than their transformed counterparts (Holley and Kiernan, 1968; Todaro and DeLarco, 1978; Jeha et al, 1996), we also determined the serum-dependent growth change in the antisense clones. K562 cells like most transformed cells, were able to grow well even in 0.5% serum (Figure 3). In sharp contrast, the antisense clone with low level of PDPK F_A could not grow at all in 0.5% serum (Figure 3). Therefore, by reducing expression of PDPK F_A in the antisense-transfected cells, a more normal phenotype, that of serum-dependent growth was restored. All the results taken together demonstrate that specific suppression of PDPK F_A expression is sufficient to interfere with the growth properties of K562 cells.

DISCUSSION

In this study, cloning of PDPK F_A cDNA and its antisense expression vector and specific antibody were successfully established. The antisense expression vector could be stably introduced into human chronic myeloid leukaemia cells K562. Two stable antisense clones were subcloned, which constitutively suppressed ~30% and ~55% of the total PDPK F_A activity in the untransfected

parental cells respectively. Data presented here first demonstrate that endogenous PDPK F_A expression in leukaemia cells can be suppressed by genetic engineering. Although the DNA sequence homology between PDPK F_A and GSK-3 β is ~85% (Woodgett, 1990), the constructed antisense vector presented here displayed little effect on the expression of GSK-3 β in K562 cells. We also tested the effect of this PDPK F_A -specific antisense vector on the well-established PDPK member, namely Erk-1/-2, and again no suppression of Erk-1/-2 expression could be observed. The results taken together demonstrate that the antisense expression vector constructed here can potently and specifically suppress the endogenous PDPK F_A expression in K562 cells.

By using the PDPK F_A -specific antisense expression vector developed in the present study, we further demonstrate that specific suppression of PDPK F_A expression in the stable transfected antisense clones proportionally and potentially leads to a modification of the leukaemia malignant phenotypes. These included prolonged doubling time, poor clonogenic growth in soft agar and increased serum dependence. The decreased serum requirement of transfected cells has been attributed to autocrine growth stimulation by growth factor-like molecules that are produced by the cancer cells themselves (Holley and Kiernan, 1968; Todaro and DeLarco, 1978; Jeha et al, 1996). The increased serum requirement of antisense clones presented here provides evidence that PDPK F_A may play an important role in autocrine growth stimulation process of chronic myeloid leukaemia. Moreover, since clonogenic growth in soft agar was established to have the best correlation with in vivo tumorigenicity (Freedman and Shin, 1974; Shin et al, 1975; Jeha et al, 1996), the diminished clonogenicity in antisense clones presented here strongly implicates that PDPK F_A may play an essential role in regulating human chronic myeloid leukaemia cell growth. PDPK F_A may, therefore, represent a potential new target for anticancer research. Whether suppression of PDPK F_A activity using PDPK F_A blockers such as genistein, kaempferol, apigenin and quercetin (Yang et al, 1998; Lee et al, 1998) may provide a useful clinic target for therapeutic intervention inhibiting the malignant behaviour of chronic myeloid leukaemia obviously presents an intriguing issue deserving further investigation. Nevertheless, the present study clearly demonstrates that specific suppression of proline-directed protein kinase F_A is sufficient to interfere with the growth of human chronic myeloid leukaemia cells, providing an initial evidence for a critical role of this PDPK in leukaemia cell growth.

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