Functional expression cloning reveals proapoptotic role for protein phosphatase 4

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Abstract

Functional expression cloning strategies are highly suitable for the analysis of the molecular control of apoptosis. This approach has two critical advantages. Firstly, it eliminates prior assumptions about the properties of the proteins involved, and, secondly, it selectively targets proteins that are causally involved in apoptosis control and which affect the crucial cellular decision between survival and death. The application of this strategy to the isolation of cDNAs conferring resistance to dexamethasone and γ-irradiation resulted in the isolation of a partial cDNA for the catalytic subunit of protein phosphatase 4 (PP4). Cells transfected with this partial cDNA in an expression vector downregulated PP4 and were resistant to both dexamethasone and UV radiation, as demonstrated by both membrane integrity and colony-forming assays. These observations suggest that PP4 plays an important proapoptotic role in T lymphocytes.
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Introduction

The molecular analysis of apoptosis is of considerable biological and medical significance. This results, firstly, from the central role played by this process in many physiological regulatory mechanisms and, secondly, from the serious consequences that can result either from inappropriate apoptosis or from apoptosis failure.

The most profound consequences of apoptosis failure arise when the genetic and biochemical abnormalities occur upstream in the apoptosis pathway, that is, before the cell becomes committed to die. Such abnormalities are already known to play causal roles in the development of cancer, autoimmune and degenerative diseases.[1,2]

The identities of some apoptosis-controlling molecules have already been established and further components are being identified through sequence similarities and physical interactions with known components.[3] In contrast, functional expression cloning offers a complementary approach to the identification of critical genes which does not rely on such prior assumptions and allows direct targeting of the molecules involved in the crucial precommitment stages of the apoptosis pathway.[4–6] This is feasible as this strategy exploits the gene's cell survival-promoting activity itself to allow its isolation.

Many intracellular signalling pathways initiating a wide range of responses include reversible phosphorylation of one or more of their components. This is as true for apoptosis as for the other gene-directed cellular responses of differentiation and proliferation. At the molecular level, therefore, apoptosis is often regulated by the level of phosphorylation of a particular protein, that is, by the balance between opposing kinase and phosphatase activities acting on that protein. The activities of several key apoptosis-controlling molecules are regulated by phosphorylation at specific sites, as well as by other mechanisms. Such phosphorylation-regulated molecules include Bcl-2 family members,[7–10] Forkhead transcription factor FKHRL[11] and Caspase 9.[12] Although the kinases catalysing specific phosphorylation of some of these regulatory proteins have been identified,[13] less has been definitively established for the equally important dephosphorylation by phosphatases.

The PPP family of serine/threonine protein phosphatases plays diverse roles within the cell.[14] The effects of phosphatase inhibitors, with varying degrees of specificity, on intact cells have suggested that these enzymes play important roles in apoptosis control, although the precise phosphatase involved in different situations is often not clearly defined.[15–17]

Selection of apoptosis-deficient cell clones from a population of mouse thymoma cells infected with a retroviral cDNA library derived from FDCP-1 haemopoietic cells resulted in the isolation of a cDNA fragment of the PP4 catalytic subunit. The expression of this cDNA fragment produced resistance to several apoptotic stimuli and an accompanying reduction in

both endogenous protein phosphatase 4 (PP4) mRNA and protein. These studies suggest an important proapoptotic role for PP4, and this was supported by the increase in apoptosis observed in cells overexpressing PP4.

Results

Isolation of cDNAs associated with resistance to apoptosis

W7.2 mouse thymoma cells were used as recipients since they have been used in many apoptosis studies. Dexamethasone-induced apoptosis of W7.2 cells is particularly well characterised.[18–21] W7.2 cells infected with the FDCP1 cDNA retroviral library, as described in Materials and Methods, were induced to undergo apoptosis by 6 days culture with 20 nM dexamethasone, followed by 500 cGy γ-irradiation, and these cultures were then grown in soft agar. From 10^6 retrovirally infected cells present at the start of the treatment, 18 colonies were eventually formed. In total, 10 of the 18 colonies were picked, eight of these grew in liquid culture and four of these contained inserts which demonstrated antiapoptotic activity when subcloned into pRUFneo; two of these four contained sequences antisense to *fau*[22] (to be reported elsewhere) and two W7.2c colonies, DG3.ab1 and DG3.ab4, contained an identical cDNA insert, 4n10, indicating that they may be progeny of the same original retrovirally infected cell. Insert 4n10 corresponded to the 3′ end (945 bases) of the mRNA encoding the mouse protein phosphatase 4 (PP4/PPX) catalytic subunit. This insert contained 604 bases of coding sequence and the whole of the 3′-untranslated region (341 bases) (Figure 1).

In order to exclude rigorously any spontaneous apoptosis-inhibiting mutations, the insert was amplified by PCR with PFU proofreading polymerase, cloned into pCR-Blunt II-TOPO, subcloned into pRUFneo and reinfected into fresh W7.2c cells, referred to as DG3.ab4 2° clones. When cells were challenged with the apoptosis inducers X-radiation or the corticosteroid analogue dexamethasone, clear protection of colony-forming ability was observed (Table 1).

Overexpression of 4n10 suppresses cell death mediated by dexamethasone and UV

To further confirm and analyse the antiapoptotic effect of 4n10, the insert was directionally cloned into the mammalian expression vector pcDNA3.1(−) and stably transfected into W7.2 cells. These cells were then cultured in the absence or the presence of dexamethasone (0.1 μM). After 72 h, cells overexpressing 4n10 were 60% more viable in dexamethasone than control cells (Figure 2a), based on cell counting and nigrosin exclusion. 4n10 transfectant clones were also tested for long-term survival by plating in soft agar after 48 h exposure to dexamethasone. Figure 2b shows that colony-forming ability of 4n10 clones after treatment with dexamethasone was significantly protected compared to those of controls. In addition, 4n10 expression produced a clear protection of cell survival and colony-forming ability in response to UV treatment (Figure 3a, b). W7.2/pcDNA3.1(−) and W7.2/pcDNA3.1(−)-4n10 were irradiated with UV at

254 nm and 20 J/m². Viable cell number and colony-forming assays were performed after 48 h. Exposure of stably transfected W7.2/pcDNA3.1(−)-4n10 to UV resulted in a marked inhibition of cell death relative to control cells (Figure 3a). In the colony-forming assay, UV-treated 4n10-expressing clones produced many more colonies than the UV-treated W7.2 vector-only controls (Figure 3b), indicating that overexpression of 4n10 can also suppress UV-induced apoptosis.

Suppression of apoptosis by 4n10 is associated with downregulation of PP4

In order to investigate whether insert 4n10 influences apoptosis by altering the expression of PP4, the expression of PP4 in W7.2/pcDNA3-4n10 cells was examined at both the RNA and the protein level.

RT-PCR was performed on the transfected cells to confirm the overexpression of 4n10. Using the primers PP4-fwd (333–357), 4n10-fwd (23–43 on 4n10; 481–502 on PP4) and PP4-rev (933–949), bands of 620 and 468 bp were produced in the pcDNA3.1(−)-transfected control clones, representing products corresponding to two sections of the endogenous PP4 mRNA. In the 4n10-transfected clones, the ratio of the two bands was changed, that is, a much stronger 468 bp band (derived from both endogenous message and the 4n10 transcripts) and a less-intense 620 bp band (which is derived from full-length PP4 alone) were obtained, confirming the expression of 4n10 and consistent with the downregulation of PP4 (Figure 4).

Northern blotting analyses were then carried out in order to examine further the effects of 4n10 on PP4 mRNA. Blots were probed using a PP4-specific probe on RNA from W7.2/pcDNA3.1(−) and W7.2/pcDNA3.1(−)-4n10 cells. (Figure 5a). The endogenous PP4 transcript (1.5 kb) was significantly expressed in all cells, whereas a band at about 1 kb, which corresponds to 4n10, was only expressed in 4n10 clones, confirming the expression of the insert in these clones. Normalised PP4 expression was significantly reduced in 4n10 clones by 41% ($\pm$4.4% S.E. $n=22$) compared to the level of expression in vector-only controls (Figure 5c). Interestingly, a possible cleavage product was clearly visible below the 4n10 band in the 4n10-transfected clones only, suggesting that 4n10 may affect the stability of the full-length PP4 mRNA. The sum of this new band (0.87 ± 0.14 (S.E., $n+15$)) together with the remaining original band (1.52 ± 0.20 (S.E., $n=15$)) was 94% of the PP4 band in controls (2.54 ± 0.35 (S.E., $n=13$)), consistent with it being a cleavage product, although other explanations are of course possible.

Analysis of individual 4n10-transfected clones showed a strong correlation between the reduction in endogenous PP4 message and resistance to dexamethasone-induced apoptosis (Figure 5d), indicating that normal levels of PP4 expression conferred sensitivity to apoptosis and that this sensitivity was reduced when PP4 message was downregulated.

To determine whether 4n10 has an effect on PP4 protein levels in W7.2 cells, Western blotting was performed using a polyclonal anti-human PP4 antibody (Figure 6). The results show that transfection of pcDNA3.1(−)-4n10 into W7.2 substantially reduces PP4 protein level compared to vector-only control cells.
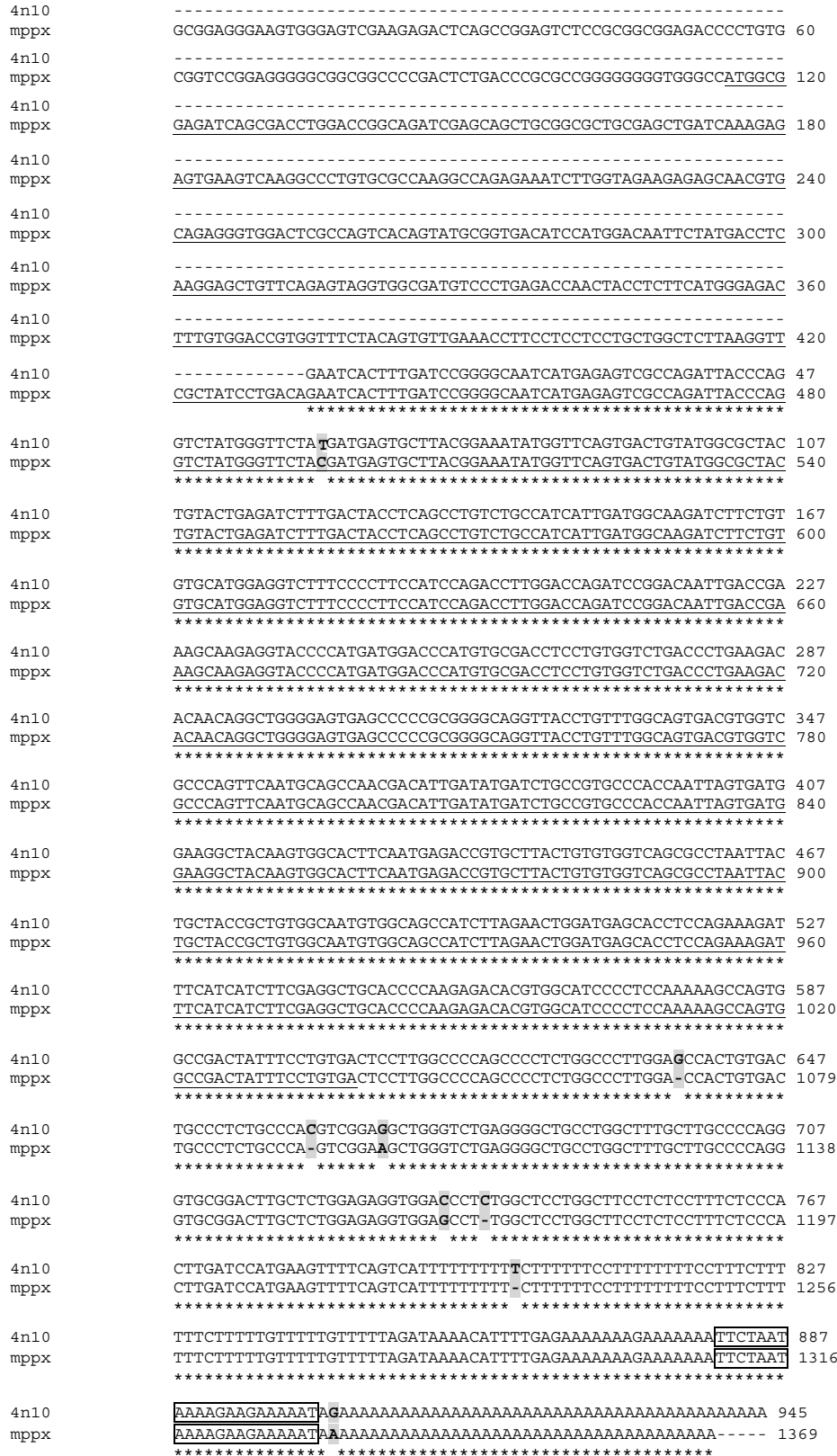
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4n10      ------------------------------------------------------------
mppx      GCGGAGGGAAGTGGGAGTCGAAGAGACTCAGCCGGAGTCTCCGCGGCGGAGACCCCTGTG 60

4n10      ------------------------------------------------------------
mppx      CGGTCCGGAGGGGGCGGCGGCCCCGACTCTGACCCGCGCCGGGGGGGTGGGCCATGGCG 120

4n10      ------------------------------------------------------------
mppx      GAGATCAGCGACCTGGACCGGCAGATCGAGCAGCTGCGGCGCTGCGAGCTGATCAAAGAG 180

4n10      ------------------------------------------------------------
mppx      AGTGAAGTCAAGGCCCTGTGCGCCAAGGCCAGAGAAATCTTGGTAGAAGAGAGCAACGTG 240

4n10      ------------------------------------------------------------
mppx      CAGAGGGTGGACTCGCCAGTCACAGTATGCGGTGACATCCATGGACAATTCTATGACCTC 300

4n10      ------------------------------------------------------------
mppx      AAGGAGCTGTTCAGAGTAGGTGGCGATGTCCCTGAGACCAACTACCTCTTCATGGGAGAC 360

4n10      ------------------------------------------------------------
mppx      TTTGTGGACCGTGGTTTCTACAGTGTTGAAACCTTCCTCCTCCTGCTGGCTCTTAAGGTT 420

4n10      -------------GAATCACTTTGATCCGGGGCAATCATGAGAGTCGCCAGATTACCCAG 47
mppx      CGCTATCCTGACAGAATCACTTTGATCCGGGGCAATCATGAGAGTCGCCAGATTACCCAG 480
                       ********************************************

4n10      GTCTATGGGTTCTATGATGAGTGCTTACGGAAATATGGTTCAGTGACTGTATGGCGCTAC 107
mppx      GTCTATGGGTTCTACGATGAGTGCTTACGGAAATATGGTTCAGTGACTGTATGGCGCTAC 540
          **************  ********************************************

4n10      TGTACTGAGATCTTTGACTACCTCAGCCTGTCTGCCATCATTGATGGCAAGATCTTCTGT 167
mppx      TGTACTGAGATCTTTGACTACCTCAGCCTGTCTGCCATCATTGATGGCAAGATCTTCTGT 600
          ************************************************************

4n10      GTGCATGGAGGTCTTTCCCCTTCCATCCAGACCTTGGACCAGATCCGGACAATTGACCGA 227
mppx      GTGCATGGAGGTCTTTCCCCTTCCATCCAGACCTTGGACCAGATCCGGACAATTGACCGA 660
          ************************************************************

4n10      AAGCAAGAGGTACCCCATGATGGACCCATGTGCGACCTCCTGTGGTCTGACCCTGAAGAC 287
mppx      AAGCAAGAGGTACCCCATGATGGACCCATGTGCGACCTCCTGTGGTCTGACCCTGAAGAC 720
          ************************************************************

4n10      ACAACAGGCTGGGGAGTGAGCCCCCGCGGGGCAGGTTACCTGTTTGGCAGTGACGTGGTC 347
mppx      ACAACAGGCTGGGGAGTGAGCCCCCGCGGGGCAGGTTACCTGTTTGGCAGTGACGTGGTC 780
          ************************************************************

4n10      GCCCAGTTCAATGCAGCCAACGACATTGATATGATCTGCCGTGCCCACCAATTAGTGATG 407
mppx      GCCCAGTTCAATGCAGCCAACGACATTGATATGATCTGCCGTGCCCACCAATTAGTGATG 840
          ************************************************************

4n10      GAAGGCTACAAGTGGCACTTCAATGAGACCGTGCTTACTGTGTGGTCAGCGCCTAATTAC 467
mppx      GAAGGCTACAAGTGGCACTTCAATGAGACCGTGCTTACTGTGTGGTCAGCGCCTAATTAC 900
          ************************************************************

4n10      TGCTACCGCTGTGGCAATGTGGCAGCCATCTTAGAACTGGATGAGCACCTCCAGAAAGAT 527
mppx      TGCTACCGCTGTGGCAATGTGGCAGCCATCTTAGAACTGGATGAGCACCTCCAGAAAGAT 960
          ************************************************************

4n10      TTCATCATCTTCGAGGCTGCACCCCAAGAGACACGTGGCATCCCCTCCAAAAAGCCAGTG 587
mppx      TTCATCATCTTCGAGGCTGCACCCCAAGAGACACGTGGCATCCCCTCCAAAAAGCCAGTG 1020
          ************************************************************

4n10      GCCGACTATTTCCTGTGACTCCTTGGCCCCAGCCCCTCTGGCCCTTGGAGCCACTGTGAC 647
mppx      GCCGACTATTTCCTGTGACTCCTTGGCCCCAGCCCCTCTGGCCCTTGGA-CCACTGTGAC 1079
          *************************************************  *********

4n10      TGCCCTCTGCCCACGTCGGAGGCTGGGTCTGAGGGGCTGCCTGGCTTTGCTTGCCCCAGG 707
mppx      TGCCCTCTGCCCA-GTCGGAAGCTGGGTCTGAGGGGCTGCCTGGCTTTGCTTGCCCCAGG 1138
          ************* ******  **************************************

4n10      GTGCGGACTTGCTCTGGAGAGGTGGACCCTCTGGCTCCTGGCTTCCTCTCCTTTCTCCCA 767
mppx      GTGCGGACTTGCTCTGGAGAGGTGGAGCCT-TGGCTCCTGGCTTCCTCTCCTTTCTCCCA 1197
          ************************** ***  ****************************

4n10      CTTGATCCATGAAGTTTTCAGTCATTTTTTTTTTCTTTTTTCCTTTTTTTTCCTTTCTTT 827
mppx      CTTGATCCATGAAGTTTTCAGTCATTTTTTTTT-CTTTTTTCCTTTTTTTTCCTTTCTTT 1256
          ********************************* **************************

4n10      TTTCTTTTTGTTTTTGTTTTTAGATAAAACATTTTGAGAAAAAAAGAAAAAAATTCTAAT 887
mppx      TTTCTTTTTGTTTTTGTTTTTAGATAAAACATTTTGAGAAAAAAAGAAAAAAATTCTAAT 1316
          ************************************************************

4n10      AAAGAAGAAAAATAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 945
mppx      AAAGAAGAAAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA----- 1369
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Figure 1 ClustalW alignment of the DNA sequences of 4n10 and the catalytic subunit of PP4 (mouse). Sequence gaps and nucleotide mismatches are highlighted. The 21 bp 3′ sequence that is completely conserved between mouse and human sequences is boxed. The open reading frame of the full-length PP4 sequence is underlined

Table 1 Colonies surviving apoptosis inducers

	1000 cGy X-rays[a]	Dexamethasone 30 nM 6 days[b]	Dexamethasone 60 nM 6 days[b]	Dexamethasone 60 nM continuous[c]
pRUFneo I	7	0	0	0
DG3.ab4 2°	44	32	2	3
pRUFneo II	18	0	0	0

Retrovirally infected DG3.ab4 2° W7.2c cells were treated in parallel with two independently derived clones of W7.2c cells infected with the retroviral vector pRUFneo alone. Starting cell numbers were 2×10^4 for X-irradiation, 2×10^5 for dexamethasone 6-day treatments and 1×10^5 for continuous dexamethasone treatment. [a]X-irradiation from a 6-MV cyclotron radiotherapy unit at 200 cGy/min on ice. [b]6 days exposure to dexamethasone prior to cloning in soft agar. [c]Dexamethasone present in continuous liquid culture

Overexpression of human PP4 promotes spontaneous cell death in CEM-C7 cell line

Further experiments were carried out to examine the effect of the PP4 gene on apoptosis in the CEM-C7 human T-cell line. CEM-C7 cells were transfected with pCMV-SPORT6-PP4 (Accession #BG913014) and pcDNA3. Cells were either maintained as polyclonal populations or soft agar cloned to establish stable clones and to assess their colony-forming ability. A total of 15 clones were examined in order to exclude clonal variation. The presence of the recombinant construct was detected by PCR on genomic DNA (results not shown) and expression was confirmed by Western blot analysis (Figure 7a). Overexpression of the EST clone Human PP4 in CEM-C7 cells significantly reduced the colony-forming ability of CEM-C7 cells. The number of colonies in the plates containing CEM-C7/pCMV-SPORT6-PP4 was significantly reduced in comparison to cells transfected with pcMV-SPORT6 only (Figure 7b; 42%' average of three independent experiments). Further experiments using a fluorescent caspase inhibitor FAM-VAD-FMK, which allows the detection of active caspases in live cells, also confirmed an increase in apoptotic cell number in PP4 transfectant cells (Figure 7c).
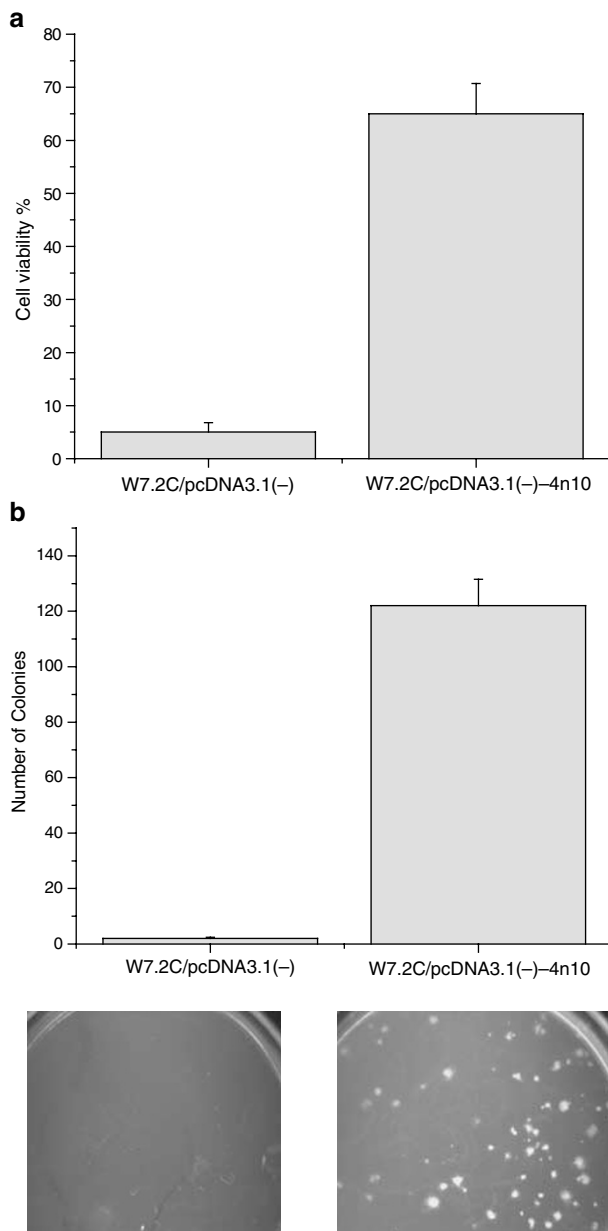
Discussion

Reversible phosphorylation of proteins is a key mechanism in the control of proliferation, differentiation, apoptosis and many other cellular processes. [23,24] Protein activity is determined by the level of phosphorylation, which in turn results from the balance between the relevant protein kinase and phosphatase[14,15] activities, both of which show marked substrate specificity. Although in the past, it is the kinases that have been most intensively studied, considerable attention has

Figure 2 4n10 overexpression inhibits dexamethasone-induced cell death. W7.2c were stably transfected with pcDNA3.1(−) or pcDNA3.1(−)-4nl0. (**a**) Viability of clones W7.2c/pcDNA3.1(−) and W7.2c/pcDNA3.1(−)-4n10 at 72 h, following the addition of 60 nM dexamethasone, as determined by vital dye staining, using 0.2% nigrosin. (**b**) Colony-forming ability of dexamethasone-treated clones. Cells (2×10^5 cells/ml) were cultured in the presence of dexamethasone 48 h prior to plating an equal volume (20 μl) from each culture in soft agar. Representative photographs are shown. Results are expressed as the mean $\pm$ standard error of the mean (S.E.), and are representative of data obtained from 10 independent stable vector-only clones and 15 independent stable 4n10 clones
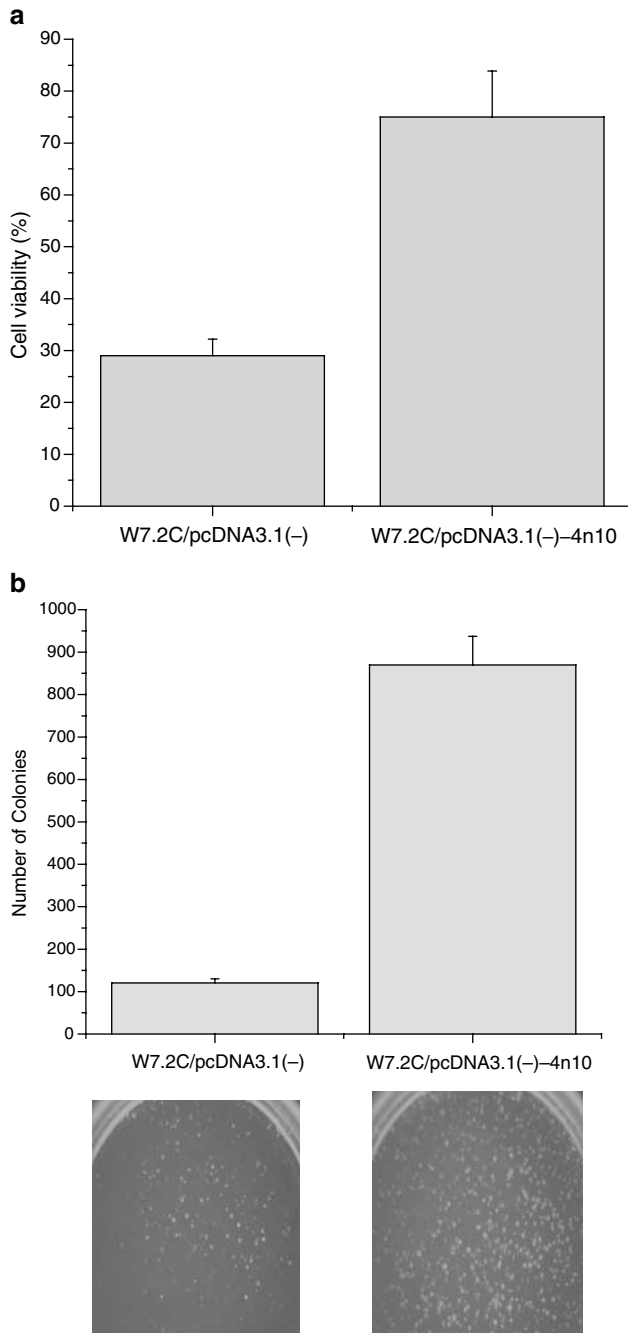
a

b

Figure 3 4n10 protects against UV-induced cell death. W7.2C/pcDNA3.1(−) and W7.2C/pcDNA3.1(−)-4n10 were irradiated with UV at 254 nm and 20 J/m^2. (**a**) Viability of clones after 48 h. (**b**) Colony-forming assay was performed 48 h after UV exposure. Results are expressed as the mean ± S.E., and are representative of data obtained from 10 independent stable vector-only clones and 15 independent stable 4n10 clones

Figure 4 Expression analysis of 4n10 by RT-PCR. PCR was performed on cDNAs from W7.2C/pcDNA3.1(−) (lanes 1–3) and W7.2C/pcDNa3.1(−)-4n10 clones (lanes 4–9) using PP4-fwd , 4n10-fwd and PP4-rev. These results are representative of 15 individual clones expressing 4n10 (the 468 bp fragment)

presented a series of observations that strongly implicate PP4 in the regulation of apoptosis induced either by irradiation or by dexamethasone. Although the effect of PP4 on apoptosis could be mediated through a number of novel or suggested targets,[7–12,18] the reported interaction with c-Rel and other NF-κB proteins is of particular interest.[28] The decrease in PP4 protein observed in the 4n10-expressing cells would be expected to produce a reduction in NF-κB activity,[28] and, since NF-κB activity is crucial to thymocyte apoptosis,[29] this could help to produce the resistance to apoptosis observed.

We have clearly demonstrated, at both RNA and protein levels, that expression of the partial PP4 cDNA 4n10 down-regulates PP4. Although the downregulation of PP4 expression produced by 4n10 is incomplete, it produces an obvious effect on cell survival. The magnitude of this downregulation in individual clones shows a striking correlation with the degree of protection from apoptosis (Figure 5d; $R=0.97$; $P<0.0001$). However, the exact mechanism by which this occurs has not yet been defined. 4n10 does not contain the full coding sequence and its sense orientation makes a direct effect on PP4 mRNA unlikely. However, a growing number of genes have been reported to be subject to regulation mediated through interactions with 3′UTR regions (reviewed by Grzybowska *et al.*).[30] The extreme 3′-untranslated sequence included within 4n10 does contain 21 bases, ttctaataaaagaagaaaat, which are precisely conserved between man and mouse (boxed sequence in Figure 1), potentially indicating a conserved binding site regulating the stability or activity of the mRNA. We are currently investigating the hypothesis that 4n10 expression may compete with the endogenous mRNA for such controlling molecules, producing the reduction in PP4 mRNA and protein levels seen on the Northern and Western blots.

Whatever the exact substrates of PP4, the process of specific dephosphorylation required for apoptosis induced by several signals may suggest novel targets for molecules designed to inhibit apoptosis where it occurs inappropriately in disease.

Materials and Methods

Host cell line W7.2

The W7.2 mouse cell line,[18] originally derived from mouse thymoma line WEHI-105.726, was serially cloned three times in soft agar (method based on Longthorne and Williams)[31] to produce a homogeneous cell population with a minimal background of spontaneous apoptosis-resistant mutants. The apoptosis-sensitive clone W7.2c was selected as the host for these experiments. W7.2c cells were cultured in RPMI-1640 (Sigma, UK) with
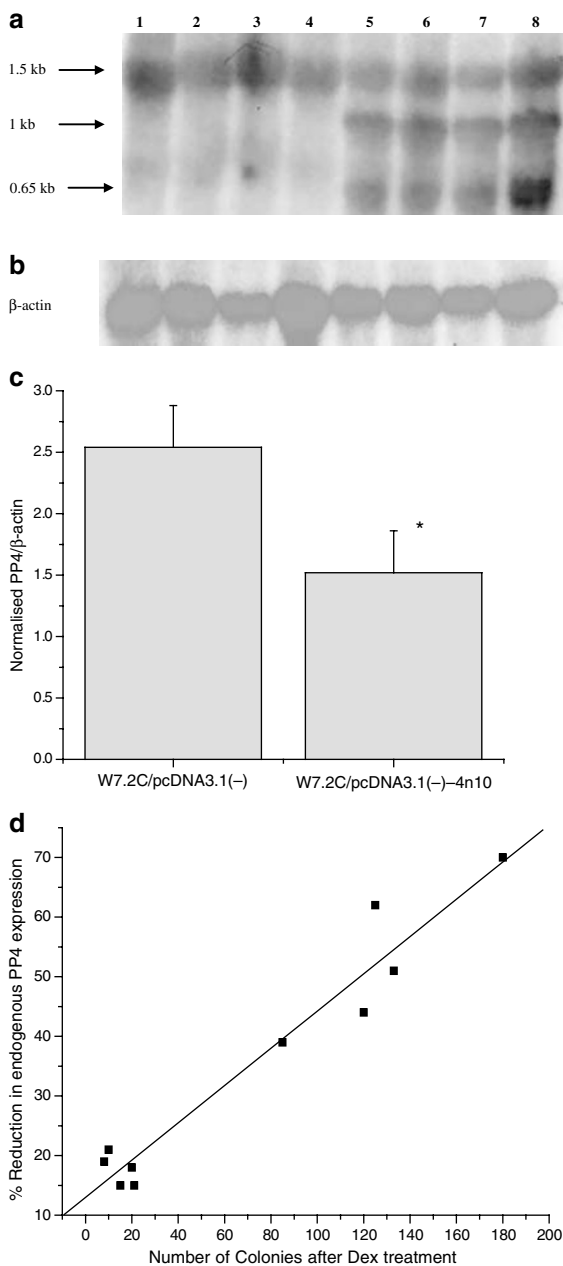
focused recently on the PP2A family of phosphakases that plays a prominent role in several areas of cell regulation.

PP4 (previously known as protein phosphatase X (PPX)), a member of the PP2A family of serine/threonine protein phosphatases,[14,15] has already been implicated in cell regulation, particularly in the centrosome.[25–27] Here, we have
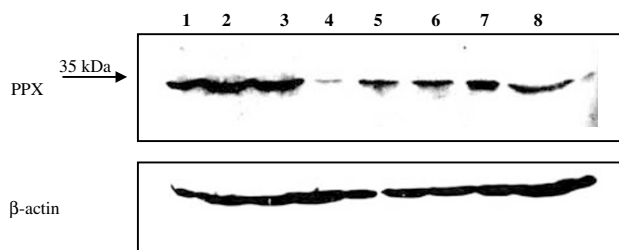
Figure 6 Western blot analysis of PP4 in W7.2C/pcDNA3.1(−) and W7.2C/pcDNA3.1(−)-4n10. Each lane contains 50 μg of whole-cell lysates from W7.2C transfected with vector only (lanes 1–3) or with pcDNA3.1(−)-4n10 (lanes 4–8). Blots were then stripped and reprobed with β-actin antibody (Sigma). These results are representative of 15 individual clones

Infection with the retroviral cDNA library

The pRUFneo retroviral cDNA library was originally prepared from the mouse factor-dependent haemopoietic cell line FDCP1.[32] ψ2 mouse ecotropic packaging cells producing the retroviral library were seeded at 5×10^6 cells/225 cm^2 flask, cultured overnight (to 50–70% confluence), and exposed to 2500 cGy from a CO^{60} γ-radiation source. The supernatant was removed and 25 ml of W7.2c cells at 2×10^5 cells/ml in complete medium with 8 μg/ml polybrene was added for 3 days coculture. The cells in suspension were then pelleted and washed by centrifugation and resuspended in fresh medium prior to freezing in aliquots in liquid nitrogen.

Selection of apoptosis-resistant retrovirally infected W7.2c. clones

Aliquots of W7.2c cells infected with the retroviral cDNA library were thawed and allowed to recover for several days before being incubated for 6 days in 20 nM dexamethasone at a starting density of 5×10^5 cells/ml. The culture was then centrifuged and suspended in fresh medium, exposed to 500 cGy γ-radiation, recentrifuged and resuspended in fresh medium for cloning in soft agar (based on Longthorne and Williams)[31] with 1 mg/ml G418. Single colonies were picked after 16 days at 37°C, expanded and genomic DNA was prepared for analysis by PCR.

Analysis of cDNA inserts

PCR was carried out using vector primers flanking the cloning site.[32] PCR products were sequenced by MWG Biotech. For subcloning, PFU proofreading DNA polymerase was used (Promega), followed by subcloning into pCR-Blunt II-TOPO (Invitrogen). For further analysis of expression, the insert was subcloned into pRUFneo and transfected into Phoenix-E (GP Nolan, Stanford) using FuGene (Boehringer Mannheim) to produce mouse ecotropic retrovirus to infect fresh W7.2c cells.

Establishment of W7.2c cell clones stably transfected with pcDNA3.4n10 expression construct

The 4n10 insert was removed from the pRUFneo vector by digestion with restriction endonucleases BamHI and HindIII and directionally subcloned into the mammalian expression vector pcDNA3.1(−) (Invitrogen). Electroporation of the resulting expression construct or pcDNA3.1(−) was carried out using a gene pulser with a capacitance extender unit (Bio-Rad) in 0.4 cm electrode gap cuvettes. Early logarithmic growth phase
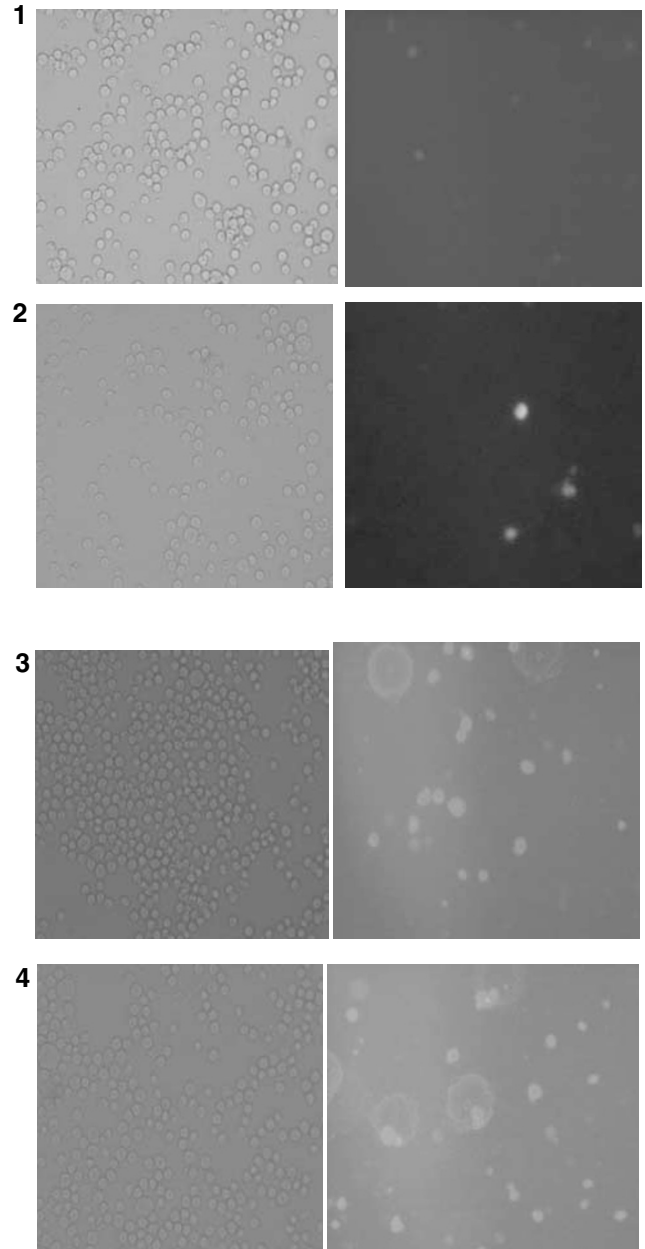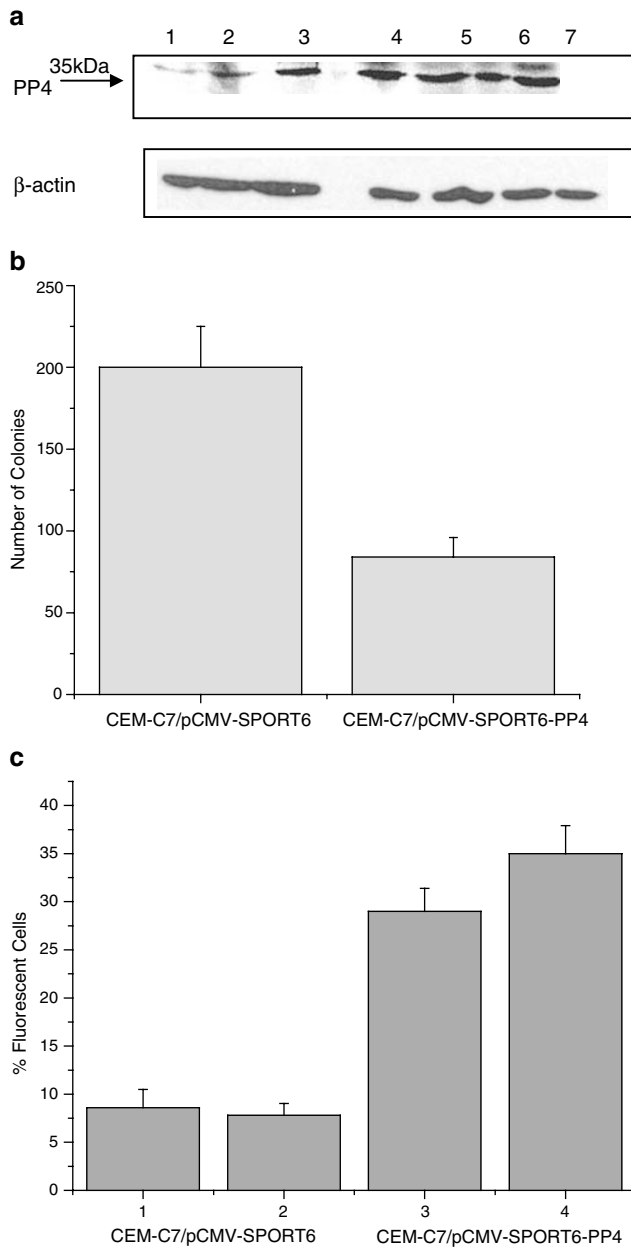

Figure 5 4n10-mediated reduction in endogenous PP4 expression. (**a**) Northern analysis of PP4 after transfection of W7.2C cells with pcDNA3.1(−) (lanes 1–4) or pcDNA3.1(−)-4n10 (lanes 5–8). (**b**) After stripping, the blot was reprobed with β-actin probe as a control. (**c**) Ratios of normalised PP4 to β-actin. Measurement of autoradiographic signals was determined using a phosphorimager. The plotted data were averaged from five independent experiments $\pm$ S.E. *$P<0.01$ compared to vector only. (**d**) Graph of data from individual colonies with the reduction in endogenous PP4 message plotted against the number of colonies surviving dexamethasone treatment. Correlation is highly significant ($R=0.97$; $P<0.0001$)

10% foetal calf serum (FCS; Hyclone, UT, USA) and 200 μg/ml gentamycin (Sigma, UK) at 37°C in a 5% CO_2 humidified incubator. All experiments were carried out on cells in logarithmic growth phase. Cell viability was carried out by vital dye staining using 0.2% nigrosin. Prior to retroviral infection, cells were treated with 90 ng/ml tunicamycin at 3×10^5 cells/ml for 18 h.

Figure 7 PP4 overexpression reduces colony-forming ability and increase apoptosis in CEM-C7 cells. (**a**) Western blot analysis of PP4 expression in CEM-C7/-pcMV-SPORT6 (lanes 1–3) and CEM-C7/pcMV-SPORT6-PP4 (lanes 4–7). Each lane contains 50 μg of whole-cell lysates from CEM-C7 transfected with vector only or with pcMV-SPORT6-PP4. Blots were stripped and reprobed with β-actin antibody. (**b**) Colony-forming ability of CEM-C7/pcMV-SPORT6-PP4 and CEM-C7/pcMV-SPORT6 cultured in the presence of geneticin (G418; 1 mg/ml). Data represent means ± S.E. from three independent experiments, each containing triplicate samples. (**c**) Active caspase detection, as a marker for apoptosis, in CEM-C7/pcMV-SPORT6 and CEM-C7/pcMV-SPORT6-PP4 using Caspa-Tag kit and fluorescence microscopy. Data represent means ± S.E. and are representative of data obtained from two independent vector-only polyclonal populations (1) and three stable vector-only clones (2) and two polyclonal PP4-transfected populations (3) and three independent stable PP4-transfected clones (4). Representative photographs are shown

cells (4×10^6), in 400 μl RPMI medium without serum, were electroporated with 10 μg DNA at 238 V, 1050 μF at room temperature. Following electroporation, cells were resuspended in 10 ml RPMI-1640 containing serum and glutamine. Stable cell lines were established 24 h post-transfection, by soft agar cloning in Iscoves' medium (Sigma) containing 2 mM glutamine and 20% heat-inactivated foetal bovine serum in the presence of 0.5 mg/ml G418 (Sigma) for 2–3 weeks. Individual colonies were picked and expanded in complete medium with 0.5 mg/ml G418.

Stable incorporation of the insert was verified by RT-PCR and Northern blot analysis.

Clonogenic assay

Survival of control and treated W7.2/pcDNA3.1(−) and W7.2/pcDNA3.1(−)-4n10 stable cell lines was assessed by the ability of the cells to form colonies in soft agar, using an equal proportion of culture from

each experimental condition (1/10) (based on Longthorne and Williams).[31] The number of colonies formed was counted following 2–3 weeks incubation at 37°C in 5% CO_2.

RT-PCR

Total RNA from 10^7 W7.2 cells were isolated using Trizol (GIBCO BRL; # 15596-026) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed using Random primer (Promega; # C1181) and Omniscript™ RT Kit (Qiagen; # 205111) according to the manufacturer's guidelines. One-tenth of the reverse transcribed RNA was used in the PCR reaction. The two PP4 transcripts were detected using primers specific to sequences within the PP4 and 4n10 sequences (PP4-fwd: 5′TGTTCAGAGTAGGTGGCGATGTCC-3′, 4n10-fwd: 5′-TCATGAGAGTCGCCAGATTACC-3′ and PP4-rev: 5′-TGCCACATTGC-CACAGC-3′). The two PCR products produced by amplification of the endogenous mRNA, corresponding to different regions of the normal mRNA molecule, were subcloned using TOPO PCR cloning Kit (Invitrogen) and their identities verified by sequence analysis (MWG Biotech).

Northern blot analysis

Total RNA (20 μg) from W7.2 cells was resolved by formaldehyde-containing 1% agarose gel electrophoresis, blotted onto a nylon membrane (Hybond-N, Amersham Pharmacia) and immobilised by UV crosslinking (Bio-Rad, crosslinker). Hybridisation was performed in ULTRAhyb buffer (Ambion) at 42°C with PP4 probe. Primers used to produce PP4 probe were primers 4n10-fwd and PP4-rev, and the radioactive labelling of the probe was carried out by random primer-directed synthesis using the DNA Labelling kit (Amersham Pharmacia) and $[\alpha^{32}P]$ dCTP (3000 Ci/mmol, ICN). Unincorporated $[\alpha^{32}P]$ dCTP was removed from the labelled DNA probe using a NICK column containing Sephadex G-50 (Amersham Pharmacia). Autoradiographic signals were quantitated using a phosphorimager (Bio-Rad). To check the amount of RNA loaded on all lanes, blots were stripped by boiling in 0.5% SDS and rehybridised with a mouse β-actin probe.

Western blot analysis

Whole-cell lysates were prepared from W7.2/pcDNA3.1($-$), W7.2/pcDNA3.1-4n10, CEM-C7/pCMV-SPORT6 and CEM-C7/pCMV-SPORT6-PP4 cells. Briefly, 10^6 cells were washed twice in phosphate-buffered saline (PBS) and resuspended in 50 μl cell lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 1% Nonidet P40, 1 mM EDTA, 1 μM Pepstatin, 10 μg/ml Leupeptin, 1 mM phenylmethylsulphonylfluoride (PMSF). The cell suspension was then incubated on ice for 30 min, before centrifugation at 10 000 × g for 10 min. Protein concentration in the supernatant was determined using the Bio-Rad protein assay kit. Protein (50 μg) was then added to SDS-PAGE sample buffer (10% glycerol, 0.7 M β-ME, 3% SDS, 62 mM Tris-HCL, pH 6.8), boiled for 10 min and loaded onto a 10% polyacrylamide gel alongside a Rainbow molecular weight marker (Sigma). After electrophoresis, the gel was blotted onto a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in blocking buffer (Tris-buffered saline plus Tween 20 (TTBS: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5 plus Tween 20 0.05%; containing 5% nonfat dried milk) for 1 h. After washing with TBS, the membrane was incubated overnight at 4°C in the presence of the primary antibody, goat anti-human PP4 antibody (PPX/PP4 (C-18); Santa Cruz Biotechnology # sc-6118) diluted in blocking buffer (1 : 1000), followed by 1 h incubation in the presence of secondary antibody at room temperature. The secondary

antibody was horseradish peroxidase-conjugated anti-goat IgG (Sigma, A5420) and used at a dilution of 1 : 10 000. Detection was carried out using an ECL chemiluminescence kit (Amersham). To verify equal loading, blots were stripped and reprobed with anti-β-actin antibody (Sigma # A5441).

Establishment of CEM-C7 clones stably transfected with the EST clone Human PP4 (BG913014; IMAGE: 4938570) expression construct

The human leukaemic cell line CEM-C7 was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine and 200 μg/ml gentamycin at 37°C in a 5% CO_2 humidified incubator. CEM-C7 cells were cotransfected with pCMV-SPORT6-PP4 construct (10 μg) and pcDNA3 (1 μg) or with pCMV-SPORT6 and pcDNA3, as described earlier. Following electro-poration, cells were resuspended in 10 ml RPMI-1640 containing serum and glutamine. After 24 h, cells were either soft agar cloned in the presence of 1 mg/ml G418 to establish individual clones or maintained as polyclonal population in RPMI with 1 mg/ml G418. In addition, the number of colonies formed on soft agar cloning was counted following 3 weeks incubation at 37°C in 5% CO_2 and 95% air. Presence of the insert was verified by PCR on the genomic DNA isolated from the stable clones and the polyclonal population using the DNeasy Tissue Kit (Qiagen). The primers used were: hPP4 forward primer: 5′-CCAC-GAAGGCCGGAGAG-3′ (2–18) and hPP4 reverse primer 5′-TTTTTCATTTTTCTTCTTTTATTA-3′ (1322–1345). Expression was con-firmed by Western blotting.

Detection of apoptosis by labelling cells with active caspases

All experiments were carried out using cells in logarithmic growth phase. The CaspaTag TM Fluorescein Caspase Activity Kit (Intergen; # S7300-025), based on the fluorescent caspase inhibitor FAM-VAD-FMK, was used to detect active caspases in the cells as a marker for apoptosis, according to the manufacturer's instructions. Detection was performed using fluorescence microscopy.

Statistical analysis

Statistical analysis for Northern blot analysis was performed by analysis of variance using Origin 6.1. and results were considered significant when $P < 0.01$.

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