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ORIGINAL ARTICLE

PBK/TOPK promotes tumour cell proliferation through p38 MAPK activity and regulation of the DNA damage response

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The contribution of the insulin-like growth-factor-I receptor (IGF-IR) to tumour progression is well documented. To identify new mediators of IGF-IR function in cancer, we recently isolated genes differentially expressed in cells overexpressing the IGF-IR. Among these was the serine/threonine kinase PBK/TOPK (PDZ-binding kinase/T-LAK cell-originated protein kinase), previously associated with highly proliferative cells and tissues. Here, we show that PBK is expressed at high levels in tumour cell lines compared with non-transformed cells. IGF-I could induce PBK expression only in transformed cells, whereas epidermal growth factor could induce PBK in non-transformed MCF-10A breast epithelial cells. Suppression of PBK expression using small interfering RNA did not prevent progression through the cell cycle, but caused decreased proliferation over time in culture, and reduced clonogenic growth in soft agarose. PBK knockdown impaired p38 activation after long-term stimulation with different growth factors and reduced DU145 cells motility. Suppressed PBK expression also resulted in an impaired response to DNA damage that was evident by the decreased generation of γ-H2AX, increased DNA damage and decreased cell survival. Taken together, the data indicate that PBK is necessary for appropriate activation and function of the p38 pathway by growth factors. Thus, enhanced expression of PBK may facilitate tumour growth by mediating p38 activation and by helping cells to overcome DNA damage.
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Introduction

The activation of the insulin-like growth-factor-I receptor (IGF-IR) tyrosine kinase by its ligands IGF-I and IGF-II triggers a variety of intracellular signalling cascades, to promote the survival, proliferation and differentiation of normal cells (Vincent and Feldman, 2002). Increased IGF-IR expression and signalling is associated with many tumours and can contribute to the progression of cancer by promoting tumour growth, invasion and metastasis (O'Connor, 2003; Mauro and Surmacz, 2004). Inhibition of IGF-IR expression and signalling using different approaches in tumour cell lines results in suppressed clonogenic growth *in vitro*, as well as inhibition of tumour growth, metastasis and invasion *in vivo* (Surmacz, 2003; Hofmann and Garcia-Echeverria, 2005), indicating that the IGF-IR and its signalling pathway are potentially important molecular targets for new anticancer therapies.

Although it is well established that the IGF-IR activates the PI3-kinase/Akt/mammalian target of rapamycin (mTOR) as well as the mitogen activated protein (MAP) kinase pathways (extracellular signal-related kinase (ERKs), c-jun-N-terminal kinases, and p38) that can promote the survival, growth and motility of tumour cells, there have been few IGF-IR target genes identified that may have a unique role in facilitating tumour growth and the progression of cancer (Dupont *et al*., 2003; Mulligan *et al*., 2002). Proteins encoded by IGF-IR target genes may become new molecular targets in the IGF-IR signalling pathway and also act as valuable biomarkers to assess the activity of the pathway in tumours and normal tissues. To address this, we isolated a series of genes that are differentially expressed in the R+ cell line (mouse embryonic fibroblasts overexpressing the IGF-IR) compared with R− cells (null for IGF-IR) (Sell *et al*., 1993). This screen identified genes that encoded proteins linked to tumour cell growth, proliferation and metastasis and included the protein/discs-large/zonula (PDZ-LIM) domain protein Mystique (Loughran *et al*., 2005a, b). We also isolated the gene encoding the serine/threonine kinase PBK/TOPK.

In normal adult organs, PBK is most abundant in the testis, mainly in proliferating spermatocytes, as well as in several fetal tissues and in proliferating brain neural stem cells (Dougherty *et al*., 2005; Fujibuchi *et al*., 2005). PBK expression has been shown to be upregulated in lymphomas and myelomas and in primary hematological neoplasms (Simons-Evelyn *et al*., 2001; Nandi *et al*., 2004). PBK expression has been correlated with the mitotic phase of the cell cycle where it is thought to be phosphorylated on Thr9 during mitosis by

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cyclinB1/cdk1 complexes (Gaudet *et al*., 2000; Matsumoto *et al*., 2004). PBK has also been reported to function as an MAPKK that can selectively interact with and phosphorylate p38 MAPK *in vitro* (Abe *et al*., 2000). The function of PBK in IGF-I or any growth-factor signalling pathway has not previously been established, although activation of p38 MAP kinase has been associated with IGF-I-mediated cell migration (Bartucci *et al*., 2001; Kiely *et al*., 2005) and with IGF-I-mediated repair from ultraviolet (UV)-induced DNA damage (Heron-Milhavet *et al*., 2001; Heron-Milhavet and LeRoith, 2002).

Here, we investigated the functions of PBK in IGF-IR signalling and in promoting tumour cell proliferation. Although PBK expression correlated with proliferation of cells, PBK silencing did not prevent progression through the cell cycle. However, cells with decreased PBK expression had impaired p38 activation after growth-factor stimulation, which correlated with decreased motility, and after DNA-damaging treatments, together with decreased γ-H2AX generation, increased DNA damage and increased sensitivity towards genotoxic agents. These cells also displayed reduced long-term proliferation and a reduction in anchorage-independent growth. Our data suggest that high PBK expression in tumour cells facilitates tumour growth by contributing to p38-mediated motility and to DNA damage repair responses.

Results

PBK expression is enhanced in many tumour cell lines compared with non-transformed cells and can be induced by IGF-I

The PBK cDNA was isolated in a screen for genes whose expression is enhanced in fibroblasts transformed owing to overexpression of the IGF-IR (R+) compared to fibroblasts lacking the receptor (R−) (Loughran *et al*., 2005b). Previous reports have demonstrated that PBK levels are high in leukaemia and some tumour cell lines (Simons-Evelyn *et al*., 2001; Nandi *et al*., 2004). To confirm and extend these observations, we first analysed PBK protein expression by Western blotting in R+ and R− cells, in a series of cell lines derived from different tumours and in non-transformed cell lines. PBK was detected at higher levels in R+ than R− cells and was also highly expressed in cell lines derived from cancers of different origins (Figure 1a). Interestingly, PBK levels were very low in the non-transformed Rat-1 fibroblasts, H9c2 cardiomyocytes or MCF-10A myoepithelial cell lines. However, PBK expression did not correlate with IGF-IR expression (Figure 1a). The heterogeneity in IGF-IR bands observed may reflect the different origins of the cell lines (human, mouse, rat) and pro-receptors. PBK can be induced upon IL-6 stimulation in murine myeloma cells (Cote *et al*., 2002); hence, we next investigated if IGF-I stimulation could induce PBK expression. Figure 1b shows that PBK levels were increased in R+ cells by 2–4 h stimulation with IGF-I, whereas in MCF-7 cells PBK accumulated by 16–24 h IGF-I stimulation. However, PBK was not induced by IGF-I in Rat-1 or MCF-10A cells.

To investigate the signalling pathways activated by IGF-I necessary for induction of PBK expression, we used pharmacological inhibitors of these pathways in R+ cells. The PI-3 kinase inhibitor LY294002 suppressed IGF-I-mediated induction of PBK protein, as did the mTOR inhibitor Rapamycin and the ERK inhibitor PD89059 after 4 h of IGF-I stimulation (Figure 1c, left). The effectiveness of these inhibitors was demonstrated by showing the reduction in p70S6K and ERK activation after 10 min IGF-I stimulation (Figure 1c, right). Overall, these data indicate that PBK is induced in transformed cell lines, but not in non-transformed cells and this induction requires the phosphatidylinositol 3-kinase (PI3K)/mTOR pathway and the ERK pathway.

PBK is induced by EGF or EGF plus IGF-I in MCF-10A cells, but is not required for progression through the cell cycle

PBK expression has previously been associated with mitosis and it has been proposed to be a target of the mitotic kinase cdk1 (Simons-Evelyn *et al*., 2001; Matsumoto *et al*., 2004). In transformed cells, IGF-I can induce PBK expression, but not in MCF-10A cells (Figure 1b). We therefore asked if epidermal growth factor (EGF), which is a known mitogen for MCF-10A cells, could induce PBK expression. MCF-10A cells were stimulated with IGF-I, EGF or IGF-I + EGF in serum-free medium and assessed for PBK expression, for the activation levels of the Shc/ERK and PI3K/Akt/mTOR pathways, and for cell cycle status. After 24 h, IGF-I alone could not induce PBK expression in MCF-10A cells, whereas EGF did increase PBK levels and IGF-I plus EGF induced even more PBK (Figure 2a). Cell cultures stimulated with EGF or EGF plus IGF-I showed evidence of proliferation with ~40% of the cells in the S phase and G_2/M phases of the cell cycle, whereas IGF-I-stimulated cells could not overcome the G1 arrest induced by the lack of serum (Figure 2b). In these cells, IGF-I did not promote phosphorylation of Shc or ERKs and promoted a modest phosphorylation of Akt and p70S6K, whereas EGF alone or EGF plus IGF-I induced robust phosphorylation of Shc, Akt, ERKs and p70S6K (Figure 2c). These data indicate that PBK expression is induced during progression through the cell cycle in non-transformed cells. The data are also consistent with the requirement for PI3K/mTOR and ERK activation for the induction of PBK by IGF-I in R+ cells.

We next asked if PBK expression is essential for non-transformed cells to progress through the cell cycle. To determine this, we transfected MCF-10A cells with siRNA (small interfering RNA) oligonucleotides, which greatly decreased PBK expression compared with the scrambled oligonucleotide (siScr) (Figure 2d). siRNA-transfected cells were then stimulated with EGF, or IGF-I for 24 h and assessed for the numbers of cells

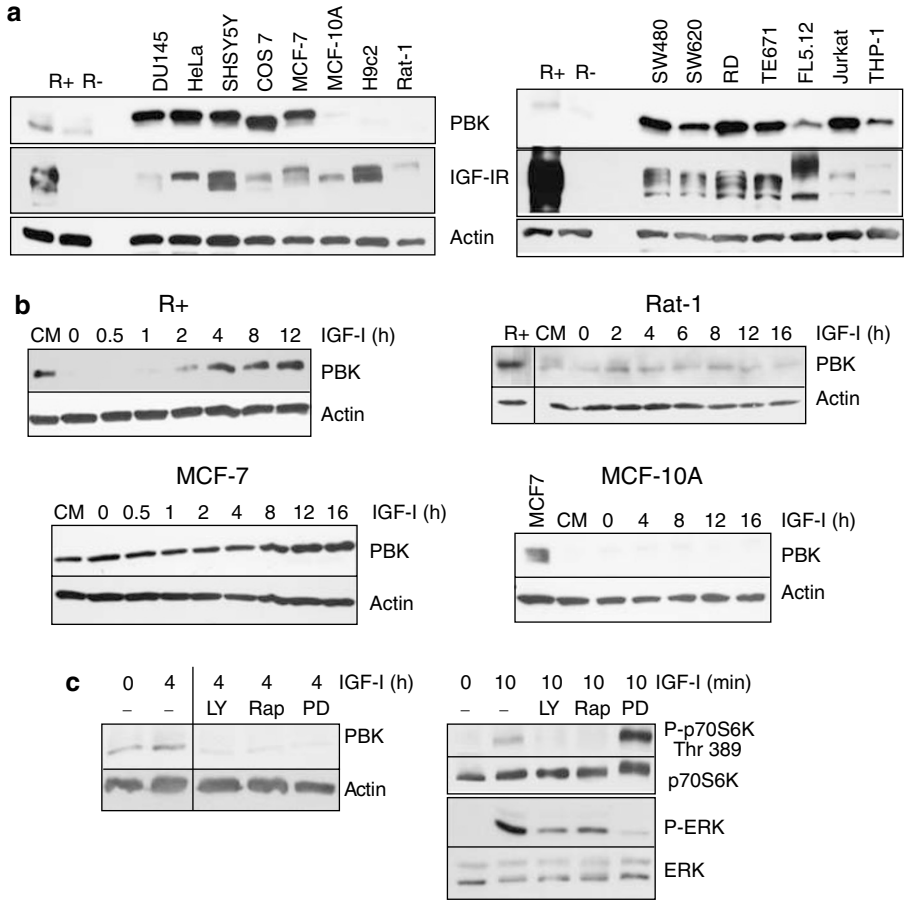

Figure 1 (**a**) Analysis of PBK expression in transformed and non-transformed cell lines. Total lysates of the indicated cell lines were analysed by Western blotting using antibodies against PBK, IGF-IR and actin (loading control). (**b**) Effect of IGF-I stimulation on PBK expression in the transformed cell lines R+ and MCF-7 versus the non-transformed cell lines Rat-1 and MCF-10A. Cells were kept in complete media (CM) or starved for 4 h in serum-free media before stimulation with 100 ng/ml IGF-I for the indicated times. Total lysates were analysed for PBK expression and actin (loading control). R+ and MCF-7 lysates are shown on Rat-1 and MCF-10A panels, respectively, for expression levels comparison. (**c**) Analysis of signalling pathways implicated in IGF-I-regulated PBK expression. R+ cells were pretreated with LY294002 (LY), rapamycin (Rap), PD89059 (PD) or dimethyl sulphoxide (DMSO) (vehicle, −) before IGF-I stimulation. Cells were harvested either after 4 h for analysis of PBK and actin expression, or after 10 min for analysis of activation of the signalling pathways targeted by the inhibitors. Phospho-p70S6K Thr389 is shown as indicator of PI3K and mTOR activation, whereas phospho-ERK levels show ERK activation levels. Total levels are shown as loading controls.

present in each phase of the cell cycle. No significant differences were observed in the cell cycle profiles measured for cells that had PBK suppressed compared with controls (Figure 2d). These data suggest that although PBK expression is high in proliferating cells, this is not essential for cells to progress through the cell cycle after acute stimulation with serum, EGF, or IGF-I plus EGF.

PBK knockdown affects p38 activation after long-term growth-factor stimulation and reduces cell motility

We next turned our attention to the function of elevated PBK expression in tumour cells and investigated the consequences of stably reducing PBK expression in MCF-7 cells. Four pools of MCF-7 cells stably expressing different shRNA constructs targeting PBK were isolated, which showed different degrees of PBK knockdown compared with control shScr cells or parental MCF-7 cells (Figure 3a).

When we examined the signalling responses following IGF-I stimulation, MCF-7/shPBK cells had lower levels of phosphorylated p38 after 18 h of IGF-I stimulation than controls, indicating lower activation of p38. In contrast, IGF-I-mediated activation of Akt was unaltered (Figure 3b). To further test these observations, we suppressed PBK expression in the prostate carcinoma DU145 cells using siRNA transfection. siPBK-transfected cells showed a defect on p38 activation after 18 h stimulation with serum, IGF-I and EGF (Figure 3c). However, short-term activation of signalling pathways, stimulated by IGF-I for up to 30 min (PI3K/Akt, ERKs and p38), was not altered by PBK knockdown in any of these cell lines (data not shown).

As p38 activity has been implicated in growth-factor-induced migration (Huang *et al*., 2004), we tested the

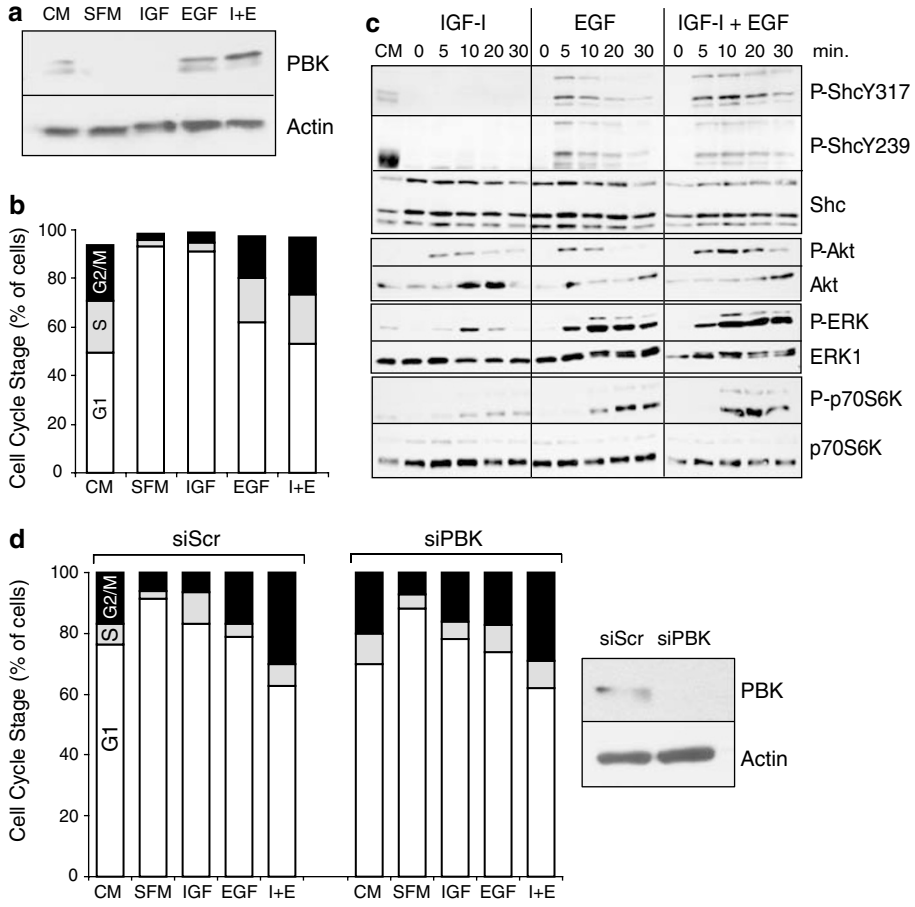

Figure 2 (**a**) PBK induction in MCF-10A cells after stimulation with different growth factors. MCF-10A cells were cultured for 24 h either in CM, serum-free media (SFM), IGF-I-, EGF- or IGF-I plus EGF-supplemented media (I + E). Total lysates were analysed by immunoblotting with PBK and actin antibodies. (**b**) Cell cycle distribution of MCF-10A cells cultured in different conditions. MCF-10A cells were treated as in (**a**) and the DNA content of the cells was analysed by FACS using propidium iodide staining. The graph shows the percentage of cells in G1 (white), S (grey) and G2/M phases (black) in all the conditions analysed. (**c**) Signalling pathways activated by different growth factors in MCF-10A cells. Cells were serum-starved for 4 h and then stimulated with IGF-I, EGF or IGF-I + EGF for the indicated times. The activation status of the signalling pathways was analysed using antibodies recognizing the phosphorylated (activated) forms of Shc, Akt, ERK and p70S6K. Total protein levels are shown as loading controls. (**d**) Effect of PBK knockdown on the cell cycle distribution of MCF-10A cells. Cells were transfected with siPBK or siScr oligos, and 48 h post-transfection the cells were kept for another 24 h in culture as described in (**a**) and the cell cycle profile analysed and plotted as in (**b**). The effectiveness of the siRNA in knocking down PBK expression is shown in the right panel.

effects of siRNA-mediated PBK suppression on cell motility in DU145 cells using the wound-healing assay. PBK knockdown reduced the ability of the cells to close the wound by ~30% in the presence of FBS and by ~50% in the presence of EGF (Figure 3d and e). Taken together, these observations confirm the proposed role for PBK as a MAPKK-like protein that activates p38 (Abe *et al*., 2000) and support a role for PBK in p38-mediated cell motility.

PBK knockdown affects the DNA damage response of the cells and enhances the sensitivity of cells to genotoxic agents

We sought to investigate if PBK is important for p38 activity in other cellular contexts. p38 is activated after treatment with different genotoxic stresses (Mikhailov *et al*., 2005) and contributes to IGF-I-mediated rescue of UV-damaged cells (Heron-Milhavet *et al*., 2001). The IGF-IR has also been shown to activate DNA repair pathways via the ataxia telangiectasia mutated (ATM) kinase (Macaulay *et al*., 2001; Trojanek *et al*., 2003; Yang *et al*., 2005). Hence, we next asked if PBK could have a role in the DNA damage response pathways. UV radiation and doxorubicin treatment activated p38 in shScr cells, but in shPBK1B cells p38 activation was reduced ~30% after 1 h of UV radiation and ~50% after 4 h of doxorubicin treatment, as estimated by quantification of the phospho-p38 levels in these conditions (Figure 4a).

To further assess the DNA damage response in shPBK cells, we analysed if the generation of the phosphorylated form of histone H2AX (γ-H2AX) was altered. This histone isoform is phosphorylated at the sites of double strand breaks (DSB) and is necessary for the recruitment of repair proteins into repair foci (Paull

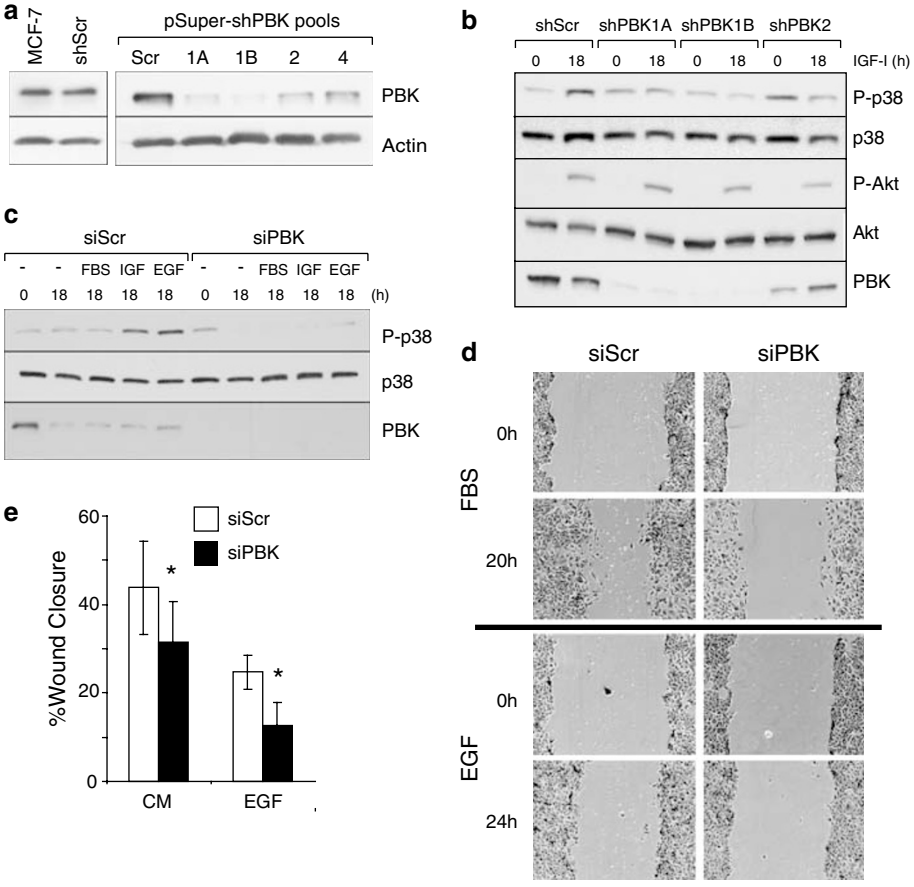

Figure 3 (**a**) PBK expression levels in MCF-7/pSuper-shPBK pools. Total lysates from parental MCF-7 and MCF-7 pools stably expressing different pSuper-shPBK constructs were immunoblotted using antibodies against PBK and actin (loading control). (**b**) p38 activation after long-term IGF-I stimulation in MCF-7/pSuper-shPBK pools. shScr, shPBK1A, shPBK1B and shPBK2 pools were starved for 4 h in SFM ($t=0$) before IGF-I stimulation for 18 h. Total lysates of the cells were analysed for p38 and Akt activation (P-p38 and P-Akt), together with total protein and PBK levels. (**c**) p38 activation after long-term stimulation with different growth factors in siPBK-transfected DU145 cells. siScr or siPBK-transfected DU145 cells were starved for 4 h ($t=0$) and then kept in culture for 18 h in the presence of SFM (−), 10% serum (FBS), IGF-I or EGF. Total lysates of the cells were analysed for P-p38, together with total p38 and PBK levels. (**d**) Effect of PBK knockdown on DU145 cells migration. siScr or siPBK-transfected DU145 were starved for 4 h, scored and put back in culture for 20–24 h to close the wound in the presence of serum (FBS) or EGF. The pictures show representative fields of duplicate experiments. (**e**) Quantification of the wound closure rates of siScr and siPBK DU145 cells. A total of 40–50 measurements of the wound gap present in duplicate experiments were taken, and the average rate of wound closure with respect to time 0 h was calculated (*$P<0.0001$, Student's t-test).

et al., 2000; Stucki *et al.*, 2005). As can be seen in Figure 4b, the number of γ-H2AX-positive cells quantified by laser scanner cytometry after UV, doxorubicin and bleomycin treatment were 40–50% lower in shPBK1B cultures than in control shScr. The basal level in untreated cells was also ~50% lower in shPBK1B cells.

Levels of UV-induced DNA damage were assessed in MCF-7/shPBK cells using Comet assays. shPBK1B cultures displayed a comet moment with twice the value of shScr cells immediately after UV irradiation (time 0), which indicates higher levels of DNA damage compared with controls (Figure 4c). The comet moment remained higher over time after irradiation, which indicates that DNA repair efficiency is diminished when PBK expression is reduced.

The next question to be addressed was whether the defects in the DNA damage repair response associated with decreased PBK expression sensitized cells to DNA-damaging agents. Low-density cultures (250 cells/well) were either irradiated with different UV doses or treated with bleomycin and allowed to grow for 10–12 days. Figure 4d shows that shPBK1B cells survive by 20% less than shScr cells after low doses of UV and bleomycin.

Taken together, these data indicate that PBK contributes to the appropriate activation of the DNA damage response, including p38 activation and H2AX phosphorylation, and that the defects in DNA damage sensing and repair in cells that have reduced PBK expression result in increased sensitivity of the cells to DNA-damaging agents.

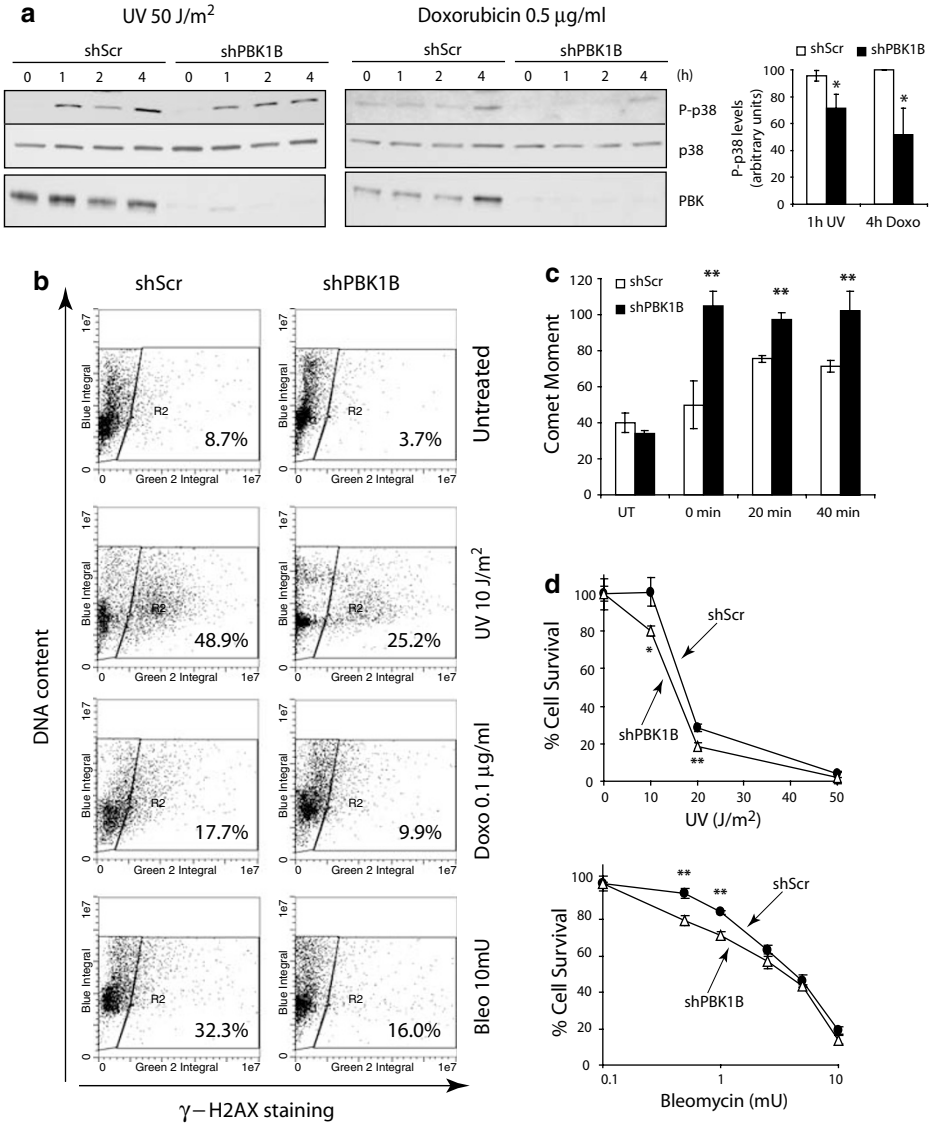

Figure 4 (**a**) Effect of PBK knockdown on p38 activation after DNA damage. shScr and shPBK1B cells were UV-irradiated or treated with doxorubicin for the indicated time points. Total cell lysates were analysed for P-p38, total p38 and PBK levels (left panel). Phospho-p38 levels were quantified by densitometry relative to the loading control of total p38 levels. The average amount of phospho-p38 levels in shScr and shPBK1B cells from three different experiments after 1 h UV radiation and after 4 h doxorubicin treatment is shown in the graph as arbitrary units (right panel) (*$P \leqslant 0.05$, Student's *t*-test) (**b**) Effect of PBK knockdown on γ-H2AX generation. shScr and shPBK1B cells were UV irradiated, doxorubicin- or bleomycin-treated and stained with anti-γ-H2AX antibody and Hoetsch. The amount of γ-H2AX-positive cells (green fluorescence, right region, R2) was estimated by scanning 4000 cells from each treatment using a laser scanning cytometer. The data shown are the example from a representative experiment. (**c**) Effect of PBK knockdown on DNA repair. shScr and shPBK1B cells were either left untreated or UV-irradiated with 200 J/m^2 and put back in culture for the time points indicated. The amount of DNA damage present in the cells was assessed using the comet assay (see Materials and methods). The graph shows the average comet tail moment present in four groups of 200 shScr (white bars) and shPBK1B (black bars) cells (**$P < 0.01$, Student's *t*-test). (**d**) Analysis of long-term survival of MCF-7/pSuper-shPBK pools after UV radiation and bleomycin treatment. Low-density cultures (250 cells/well) of shScr (filled circles) and shPBK1B (open triangles) were treated with different UV (top graph) or bleomycin (bottom graph) doses, and cultured for 14 days to develop colonies. The data are the average of triplicate wells of a representative experiment (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

PBK suppression retards long-term growth and reduces clonogenicity of MCF-7 cells

We next analysed if the defects in migration and DNA damage response observed in shPBK cells had an effect on long-term growth of the cells. MCF-7/shPBK1B or siPBK-transfected MCF-7 cells exhibited similar initial growth rates in culture compared with controls. However, by days 4 and 5 the cultures reached a cell density that was 20–25% lower than that achieved in the control cultures (Figure 5a).

As IGF-IR signalling facilitates anchorage-independent growth, we asked whether suppression of PBK expression would affect MCF-7 cells clonogenic growth. In soft agarose assays, MCF-7/shPBK cell pools with the lowest PBK expression exhibited a decrease of 40–45% in the number of colonies formed compared

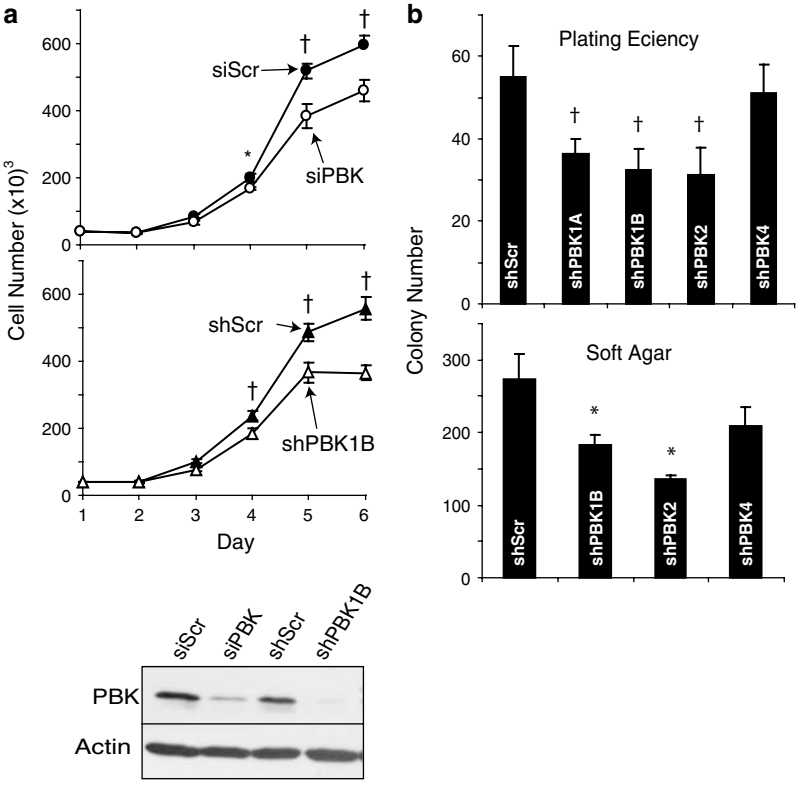

Figure 5 Growth rates of MCF-7/pSuper-shPBK pools and siPBK-transfected MCF-7 cells. (**a**) siScr- (filled circles) and siPBK-transfected (open circles) cells, or shScr (filled triangles) and shPBK1B (open triangles), were seeded at an initial concentration of 4×10^4 cells/well and the growth of the cells monitored every day until day 6 by cell counting. The data presented are the average of quadruplicate wells of a representative experiment (*$P<0.05$, †$P<0.005$, Student's *t*-test). PBK levels are shown in the bottom panel. (**b**) Growth of MCF-7/pSuper-shPBK pools in plating efficiency or soft agar assays. To assess plating efficiency, low-density monolayer cultures of cells were left to develop colonies for 14 days (top graph). For anchorage-independent growth, cells were cultured in soft agarose for 14–18 days and the colonies stained and counted (bottom graph). The graphs show the average colony number of triplicate wells of representative experiments (*$P<0.05$, †$P<0.005$, Student's *t*-test). For PBK expression levels refer to Figure 3a.

with control shScr cells (Figure 5b, bottom). A similar reduction in the number of colonies was observed in plating efficiency assays (Figure 5b, top).

These data indicate that lack of PBK does not alter proliferation upon initial cell division in response to stimulation with growth factors, but causes retarded proliferation after several cycles of division. The data also suggest that PBK contributes to anchorage-independent growth of tumour cells.

Discussion

The data presented here show that PBK is a growth-factor-regulated kinase whose expression is constitutively high in tumour cells and is also induced in normal cycling cells, but its ablation does not affect normal cell cycle progression. We uncovered a new function for PBK as mediator of growth-factor activation of p38 with a role in motility and as part of the DNA damage sensing machinery and necessary for the phosphorylation of H2AX. Thus, the effects of PBK on motility may contribute to the anchorage-independent growth of tumour cells, whereas its function in activating the DNA damage repair machinery may provide tumour cells with a more efficient repair response that would facilitate tumour growth.

PBK is generally constitutively highly expressed in transformed cells compared with non-transformed cells, but it can also be induced by IGF-I in transformed cells via the activity of the PI3K and ERK pathways. A recent report has shown that PBK transcription is regulated by E2F and cAMP-response element binding protein (CREB) (Nandi and Rapoport, 2006). CREB is activated by IGF-I through the PI3K and ERK pathways (Maldonado *et al*., 2005); whereas IGF-I can also induce E2F expression (Shen *et al*., 2004). It is likely that E2F and CREB could be the transcription factors responsible for IGF-I-induced PBK expression.

Interestingly, PBK expression is not induced by IGF-I in the non-transformed cell lines MCF-10A and Rat-1. This indicates that a mitogenic signal is required, which in MCF-10A cells can be provided by EGF. These observations, combined with reports of PBK expression in proliferating neural stem cells and testis (Dougherty *et al*., 2005; Fujibuchi *et al*., 2005), indicate that PBK has an important function in the proliferation of normal cells that appears to be de-regulated in tumour cells. However, suppression of PBK expression did not impair

the short-term growth-factor-stimulated proliferation of cells, indicating that PBK is not an essential part of the cell cycle machinery. We found that growth-factor-induced p38 activation was greatly affected when PBK was suppressed, and this defect correlated with a decrease in cell motility. p38 is an important mediator of cell migration in transformed cells and non-transformed (Huang *et al*., 2004) and it also regulates invasion (Kim *et al*., 2003). Based on this, the effect of PBK knockdown in reducing long-term cell growth and anchorage-independent growth could be explained in part as a consequence of the decrease in p38-mediated motility and maybe a reduction in the invasive potential of the cells.

Our data strongly suggest that PBK also functions in DNA damage sensing and repair. shPBK cells had increased susceptibility to DNA damage and impaired generation of γ-H2AX. γ-H2AX is produced at the sites of DSB by phosphorylation on Ser139 by the PIKK family (ATM, ATR and DNA-PK) (Fernandez-Capetillo *et al*., 2004; Stiff *et al*., 2004). However, in the case of UV radiation, γ-H2AX is produced by a wortmannin-insensitive kinase (Marti *et al*., 2006), which is consistent with our results showing that PBK contributes to γ-H2AX generation. γ-H2AX is responsible for the recruitment of DNA damage response (DDR) proteins to the sites of damage (Paull *et al*., 2000; Lisby and Rothstein, 2004). Ser139 phosphorylation of H2AX is required for MDC1 binding and this interaction promotes the accumulation of DDR proteins (Stucki *et al*., 2005). This suggests that the decrease in phosphorylated H2AX levels observed in shPBK cells, both in normal culture and after genotoxic stress, may cause increased DNA damage owing to a failure in recruiting the required DDR proteins, which could eventually contribute to a defect in long-term cell growth and survival, such as we observed in shPBK cells.

Mutations in DNA repair proteins have been linked to increased susceptibility to develop tumours, such as mutations in the nucleotide excision repair in the xeroderma pigmentosum syndrome and mutated MUTYH in colorectal carcinomas (Risinger and Groden, 2004; Cleaver, 2005). There is also much evidence to indicate that an effective DNA repair machinery is necessary for cell viability and may also be considered a mechanism of tumour cells resistance to therapy. As a consequence, inhibition of DNA repair pathways is emerging as a possibility to enhance the effectiveness of DNA-damaging drugs (Madhusudan and Hickson, 2005). Several groups have reported that disruption of DNA-repair-related proteins such as Chk1 and Mus81 increases the sensitivity of the cells towards DNA-damaging agents (Dendouga *et al*., 2005; Sorensen *et al*., 2005). With this perspective in mind, it is not surprising that the activation of growth-factor receptors associated with cancer progression, such as IGF-IR and EGFR, has a role in promoting DNA repair and in controlling the transcription of DNA repair genes (Lee-Kwon *et al*., 1998; Trojanek *et al*., 2003; Yacoub *et al*., 2003; Yang *et al*., 2005). Several reports have shown that inhibition of the EGFR increases radiosensitivity (Friedmann *et al*., 2004; Sartor, 2004). IGF-IR silencing increases radiosensitivity of mouse melanoma cells and impairs the activation of ATM (Macaulay *et al*., 2001), while it also enhances the sensitivity towards several DNA-damaging agents in human prostate cancer lines (Rochester *et al*., 2005). PBK expression is regulated by both IGF-I and EGF, and participates in the DNA damage repair pathways. Thus, PBK is potentially a new therapeutic target to be used in combination with DNA-damaging drugs or drugs targeting the IGF-IR or EGFR.

Materials and methods

Cell culture and cell synchronization

R+, R−, MCF-7, DU145 and Rat-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM L-Glu and 5 mg/ml penicillin/streptomycin. MCF-10A cells were cultured in a 1:1 mixture DMEM:F-12 media supplemented with 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone and 2 mM L-Glu. All IGF-I (100 ng/ml) or EGF (20 ng/ml) (PeproTech, Rocky Hill, NJ, USA) stimulations were performed in cells that had been serum-starved for 4 h in serum-free media. Pharmacological inhibitors used (LY294002 20 μM, rapamycin 20 nM, PD89059 30 μM, all from Calbiochem, La Jolla, CA, USA) were added 30 min before stimulation.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.5, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethyleneglycol tetraacetate (EGTA), 1 mM NaF, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 μM pepstatin, 1.5 μg/ml Aprotinin). Protein concentration was estimated using Bradford reagent (BioRad, Munich, Germany). Protein lysates were subjected to electrophoresis on 4–15% gradient sodium dodecyl sulphate (SDS)–polyacrylamide gels, transferred onto a nitrocellulose membrane (Protran, Dassel, Germany) and blocked with 5% non-fat dried milk diluted in Tris-buffered saline/0.05% Tween 20 (Sigma, St Louis, MO, USA) (TBS/T). The membrane was probed overnight with the following primary antibodies: anti-PBK (Transduction Laboratories/BD, Heidelberg, Germany), anti-IGF-IR (Santa Cruz, CA, USA), anti-β-actin (Sigma), anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-phospho-p42/p44 (Thr202/Tyr204), anti-p42/p44, anti phospho-p70S6K (Thr389), anti-p70S6K, anti-Akt, (Cell Signalling Technology, Beverly, MA, USA), anti-phospho-Akt (Ser473) (Biosource, CA, USA). Next day, the membrane was incubated for 1 h with horse radish peroxidase (HRP)-conjugated secondary antibody (Dako, Glostrup, Denmark) and developed using enhanced chemiluminescence (ECL) (Amersham Biosciences, Buckinghamshire, UK). Densitometric quantification was performed using the program Gene Tools (Syngene, Cambridge, UK).

Cell cycle analysis and flow cytometry

For cell cycle analysis, cells were trypsinized and centrifuged for 5 min, 1000 r.p.m. The pellet was resuspended in cold phosphate-buffered saline (PBS) containing 200 μg/ml RNAse A (Sigma), and kept on ice. Five minutes before analysis by fluorescence-activated cell sorting (FACS), NP-40 and propidium iodide (Sigma) were added at a final concentration of 0.1% and 50 μg/ml, respectively. DNA content was measured in the FL2 channel of a FACS station using CellQuest software, (BD).

PBK siRNA transfection and generation of shRNA expressing cell lines

siPBK and siScr (negative control no. 1) oligos were purchased from Ambion Ltd (Cambridgeshire, UK). Oligos were transfected (25 nM) using Oligofectamine (Invitrogen, CA, USA) following the manufacturer's recommendations.

Five different 19-nucleotide long sequences targeting human PBK/TOPK coding sequence and a scrambled sequence were selected using the web-based tool from Genscript (www.genscript.com). Each pair of complementary oligos cloned into the pSuper.Neo Vector (Oligoengine, Seattle, WA, USA) contained the sense and the antisense sequences separated by a nine-base pair nucleotide loop with the *Eco*RV site, together with a BamHI overhang at the 5′-end and a *Xho*I overhang at the 3′-end. The annealed oligos were ligated into *Bgl*II/*Xho*I-digested pSuper.Neo vector. The presence of the insert was confirmed by DNA sequencing.

MCF-7 cells were transfected with the six different pSuper constructs (shScr and shPBK1–5) using Lipofectamine (Invitrogen) following the manufacturer's protocol. Cells were cultured in the presence of 1 mg/ml G418 (Calbiochem) for 2 weeks and assessed for PBK expression by Western blot analysis. Four pools were selected for further studies (shPBK1A, shPBK1B, shPBK2 and shPBK4) because they showed different degrees of PBK knockdown compared with shScr and parental cells.

Wound healing assay

siPBK- and siScr-transfected DU145 were plated in six-well plates to near confluence. After 24 h, the cells were serum-starved for 4 h and then the monolayer was scratched using a sterile tip (time 0 h). The cells were left to migrate into the wound for 20–24 h in the presence of 10% serum or EGF (20 ng/ml) and were then fixed and stained with Giemsa. Ten pictures were randomly taken from duplicate wells using the $\times 10$ objective, and 4–5 measurements of wound closure were taken from each picture to quantify cell migration with respect to time 0 h.

DNA-damaging treatments

UV irradiation was performed using a UV Stratalinker 1800 (Stratagene, La Jolla, CA, USA). Adherent cells were washed once with PBS, the PBS removed until only a thin layer of liquid was left, irradiated and returned to culture at 37°C in complete media.

Bleomycin (Calbiochem) and doxorubicin (Sigma) were diluted in culture media and added to the cells for the indicated time points.

Foci staining and laser scanning cytometry

MCF-7/shPBK cells were grown in coverslips for 24 h and then treated as indicated in the text. After 1 h, cells were fixed with 4% paraformaldehyde in PBS, blocked with 1% bovine serum albumin (BSA)/PBS and incubated for 1 h at 37°C using anti-γ-H2AX antibody (Upstate Biotech, Charlottesville, VA, USA) diluted in blocking buffer. Cells were washed in PBS before incubation with anti-mouse Cy2 secondary antibody (Jackson Laboratories, West Grove, PA, USA) and Hoechst (nuclear counterstain) for 30 min at 37°C. After extensive washing, the coverslips were mounted into slides.

The amount of γ-H2AX-positive cells present was quantified by laser scanning cytometry (LSC) (Compucyte, Cambridge, MA, USA) using nuclear staining to identify single cells, following the protocol described in Compucyte web page (www.compucyte.com/published.htm). Four thousand cells were scanned for each sample.

Comet assays

Comet assays (Trevigen, Gaithersburg, MD, USA) were used to quantify DNA damage in the cells (Speit and Hartmann, 1999). The comet assays were performed following the manufacturer's instructions using the 'Neutral Electrophoresis' protocol, which detects single-stranded DNA breaks, double-stranded DNA breaks, and some apurinic and apyrimidinic sites. Three to four groups of 200 cells were scored per time point and the comet tail moment in each group was quantified using the classification described in Wang *et al*. (2005).

Proliferation, colony formation in soft agarose and plating efficiency assays

MCF-7/shScr and MCF-7/shPBK1 cells, or siScr and siPBK transfected cells, were cultured in complete medium at 4×10^4 cells per well in a 24-well plate. To monitor cell growth at intervals, attached cells were removed from quadruplicate wells using trypsin-ethylenediaminetetra acetic acid (EDTA) and the viable cells were counted in a haemocytometer by Trypan blue exclusion.

Anchorage-independent growth was determined by assaying colony formation in soft agarose. MCF-7/pSuper-shPBK cells (10^3/well) were resuspended in 0.33% low-melting point agarose (Sigma) in DMEM/10% FBS and plated in triplicate onto 35-mm dishes containing a 2-ml base agarose layer (0.6%). After 14 days, colonies were stained with 0.01% crystal violet and counted.

To assess plating efficiency, triplicate low-density cultures (500 cells/well in six-well plates) were left to grow in the presence of complete media for 14 days. After this time, the colonies were fixed and stained with crystal violet. Colonies bigger than 0.5 mm were counted.

For UV and bleomycin sensitivity assays, cells were plated in triplicate at 250 cells/well in six-well plates in complete medium. Next day, the cells were washed with PBS, irradiated with different UV doses or kept in the presence of different concentrations of bleomycin for 24 h. After the treatments, the media was replenished and the cells were cultured for 10–12 days and at this time colonies were stained using crystal violet. Survival is plotted as the percentage of colonies present after treatment versus untreated cells.

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