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SHORT COMMUNICATION

Functional inhibition of PI3K by the βGBP molecule suppresses Ras–MAPK signalling to block cell proliferation

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The mechanisms of signal transduction from cell surface receptors to the interior of the cell are fundamental to the understanding of the role that positive and negative growth factors play in cell physiology and in human diseases. Here, we show that a functional link between phosphatidylinositol-3-OH kinase (PI3K) and Ras is suppressed by the *β*-galactoside binding protein (*β*GBP) molecule, a cytokine and a negative cell-cycle regulator. Ras–mitogen-activated protein kinase (MAPK) signalling is blocked by *β*GBP owing to its ability to inhibit the p110 catalytic subunit of PI3K, whose basal activity is required for Ras activation. Functional inhibition of p110 by *β*GBP results in downregulation of PI3K activity, suppression of Ras-GTP loading, consequent loss of MAPK activation and block of cell proliferation. This study sheds light on the molecular mechanisms whereby *β*GBP can control cell proliferation and, by extension, may potentially control tumorigenesis by controlling PI3K.
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The Ras–mitogen-activated protein kinase (MAPK) signalling pathway plays a key role in the activation dynamics of transcriptional events required for mammalian cells to enter the cell cycle. Activation of Ras requires growth factor/receptor tyrosine kinase (RTK) interaction, RTK cross-phosphorylation leading to the formation of high-affinity binding sites for SH2 domains of the Grb2 and Shc adapter proteins, the binding of the nucleotide exchange factor SOS to the SH3 domains of Grb2 and the conversion by SOS of GDP-Ras into active GTP-Ras. The ensuing signalling cascade leads to the phosphorylation of MAPK and the activation of genes programmed to initiate cell proliferation (Downward, 2003; Mitin *et al*., 2005). Previously, we have shown that growth factor-induced cell proliferation in mammalian cells is counteracted by events triggered at the cell surface by the *β*-galactoside binding protein (*β*GBP) molecule (Wells and Mallucci, 1991), but the mechanistic events involved in this process have remained unresolved until now. Endogenously released by somatic cells (Wells and Mallucci, 1991) and secreted by $CD4^+$- and $CD8^+$-activated T cells (Blaser *et al*., 1998), *β*GBP binds with high affinity (K_d 1.5×10^{-10} M) to $\sim 5 \times 10^4$ receptors/cell to control cell-cycle entry and S/G_2 traverse in normal cells (Wells and Mallucci, 1991, 1992). As a cytostatic factor, *β*GBP induces reversible S/G_2 arrest in normal cells while activating programmed cell death in cancer cells (Wells and Mallucci, 1991; Mallucci *et al*, 2003). We now report that in murine embryonic fibroblasts (MEF), chosen as a paradigm of normal cells, *β*GBP inhibits phosphatidylinositol-3-OH kinase (PI3K) activity, also that PI3K activity is necessary for growth factor-induced activation of Ras and that basal levels are sufficient. Comparative studies with PI3K inhibitors indicate that the p110 catalytic subunit of PI3K is a prime target of *β*GBP-induced signalling.

To determine the mode by which *β*GBP inhibits cell proliferation, we have investigated mechanistic events within the signalling context that lead to the phosphorylation and activation of transcription factors involved in cell-cycle entry and cell proliferation. We found (data not shown) that in MEF from secondary culture, held in G_0 by 20 nM Hu-r-*β*GBP and stimulated with either 10 ng/ml platelet derived growth factor (PDGF), 10 ng/ml epidermal growth factor (EGF), 5 ng/ml fibroblast growth factor (FGF) or 10% fetal calf serum, there was no evidence of c-fos, c-jun nor, predictably, of c-myc mRNA expression indicating that no activating signals had reached the promoter elements of these genes either via Ras-dependent or Ras-independent pathways. Consistent with this conclusion is the evidence of Figure 1a showing near absence of MAPK phosphorylation, as indicated by the lack of decreased migration of the MAPK protein in band shift assays in cells treated with *β*GBP and challenged with either growth factors or serum, in contrast with the degree of MAPK phosphorylation evident where *β*GBP was absent. To determine whether treatment with *β*GBP was interfering with the process of RTK activation, we assessed PDGF receptor phosphorylation at tyrosine 751, a docking site for PI3K (Panayotou *et al*., 1992), using a specific anti-phospho-

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monoclonal antibody and assessed EGF receptor phosphorylation using an anti-phospho-tyrosine monoclonal antibody after immunoprecipitation of the receptor and electrophoretic migration. In both instances, we found no evidence that RTK phosphorylation had been impaired by the presence of βGBP (Figures 1b and c).

Since Ras plays a key role in the activation of MAPK, we investigated whether the inhibition of MAPK activation in cells treated with βGBP and stimulated with PDGF related to loss of Ras activity. For this, we assessed the ability of p21[ras] to bind the binding domain of Raf-1 (Finney *et al*., 1993) and measured the phosphorylation state of p42 and p44 MAPK by antibody recognition of the dual-phosphorylated protein (Kharitonevkov *et al*., 1997) as a function of time from the addition of 20 nM Hu-r-βGBP. We found that exposure to βGBP inhibited Ras-GTP (guanosine triphosphate) loading and MAPK phosphorylation to virtually undetectable levels within 2–3 h from the addition of βGBP (Figures 2a and b, lower bands) with no changes in total Ras and MAPK proteins (Figures 2a and b, upper bands). Demonstration that βGBP had impeded the activation of Ras and that absence of MAPK phosphorylation related to the abrogation of Ras activation rather than to an effect downstream of Ras was obtained bypassing Ras by the addition of 20 nM 12-*O*-tetradecanol phorbol-13-acetate (TPA), a phorbol ester that activates MAPK signalling via protein kinase C to MEK (Schonwasser *et al*., 1998). Under these conditions, Ras-GTP loading was prevented (Figure 2c, lower bands) while MAPK remained active (Figure 2d, lower bands), in contrast to the absence of active Ras and active MAPK in the cells not treated with TPA (Figures 2c and d, upper bands).

While the inhibition of MAPK activation by treatment with βGBP relates to the abrogation of active Ras, Ras need not be the prime target of βGBP-induced signalling, as a cohort of events operates downstream of phosphorylated RTKs, which are not affected by βGBP (Figures 1b and c), and upstream of Ras (Downward, 2003; Mitin *et al*., 2005). However, our analysis of events involved in the activation of Ras (data not shown) demonstrated that, while Ras-GTP loading was being inhibited in the presence of βGBP, Grb2 recruitment to the receptor and Grb2-SOS association had not been affected, that the ability of SOS to activate Ras *in vitro* was maintained and that Ras-GAP activity was not altered. We therefore asked whether the association of GTP-Ras with the catalytic subunit (p110α) of PI3K class IA (Rodriguez-Viciana *et al*., 1994) had a bearing on the negation by βGBP of Ras activation, whether PI3K activity was required for Ras activation and whether βGBP would affect PI3K activity. To answer these questions, we have assessed directly the catalytic ability of the endogenous PI3K enzyme, as PI3K activity based on the detection of 3-phosphoinositides (Vanhaesebroeck *et al*., 1997, 2001), the result of PI3K and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) activity, is not representative of the active state of the endogenous enzyme which physically interacts with Ras and whose functional mode may be relevant in Ras regulation.

To determine whether the inhibition of Ras activation by βGBP was mediated by a prime effect on PI3K, we

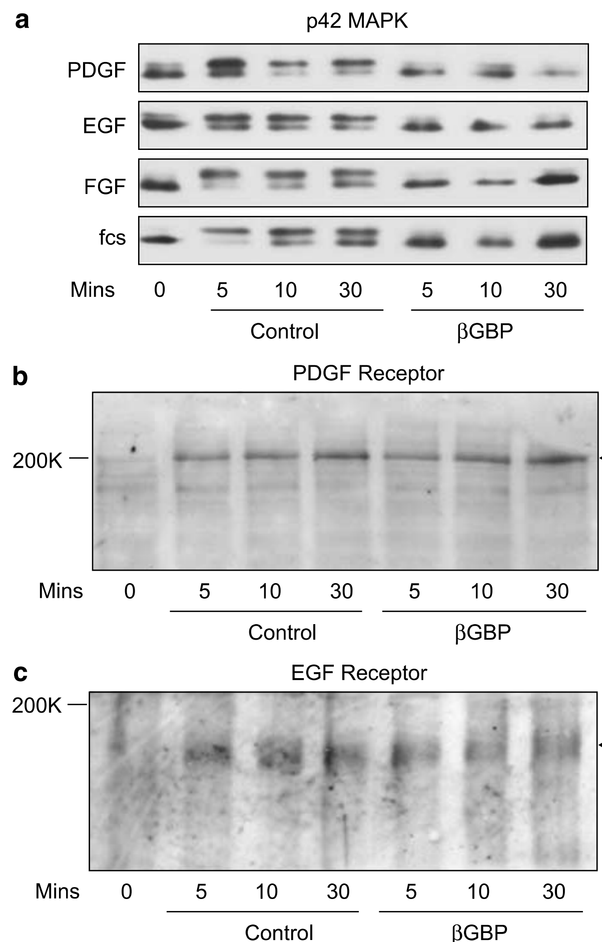

Figure 1 Hu-r-βGBP inhibits MAP kinase activation but does not affect tyrosine kinase receptor phosphorylation. Secondary embryonic fibroblasts from C57 Bl/6 mice were prepared as described previously (Wells and Mallucci, 1991). Cells were treated with 20 nM Hu-r-βGBP for 3 h before stimulation with growth factors. (**a**) Cells were stimulated with 10 ng/ml PDGF, 10 ng/ml EGF, 5 ng/ml FGF or 10% fetal calf serum for 5, 10 and 30 min. Western blots were probed with antibodies to total p44/p42 MAPK (Santa Cruz, Santa Cruz, CA, USA) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare, Amersham, UK). MAPK phosphorylation was visualized by mobility shift. (**b**) The PDGF receptor was visualized in western blots (arrowhead) of cultures stimulated with 10 ng/ml PDGF, probed with anti-phospho-PDGF receptorβ (Tyr 751) monoclonal antibody (Cell Signaling Technology, Boston, MA, USA) followed by HRP-conjugated secondary antibody. (**c**) Cultures stimulated with 10 ng/ml EGF were lysed and the EGF receptor was immunoprecipitated using an anti-EGF receptor antibody (Transduction Labs, San Jose, CA, USA) followed by anti-protein G sepharose. The immunoprecipitated EGF receptor was visualized in western blots (arrowhead) using specific anti-phosphotyrosine/anti-protein-phosphotyrosine antibodies (Transduction Labs). Hu-r-βGBP was expressed in *Escherichia coli* BL21(DE3) using hGal-1 cDNA in PET21a and purified by lactose–agarose affinity chromatography (Hirabayashi *et al*., 1989). Purity was assessed by MALDI-TOF.

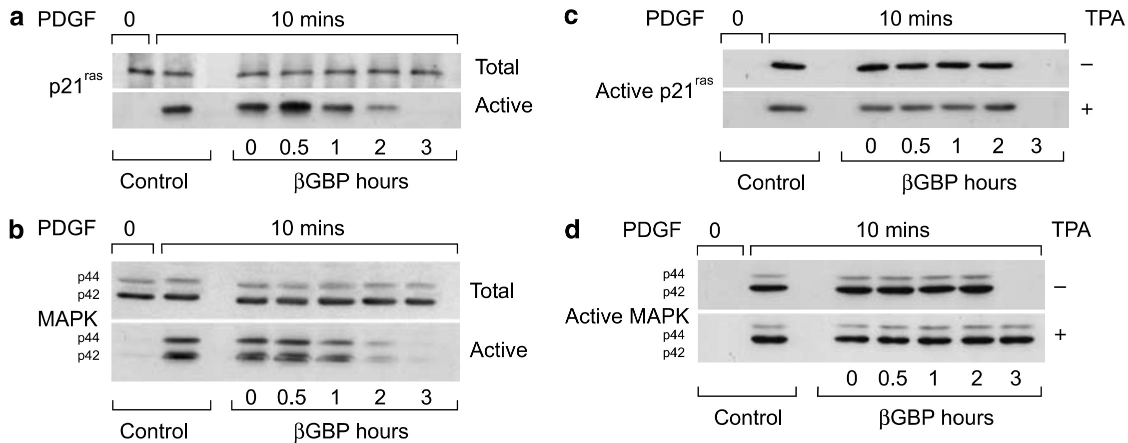

Figure 2 Inhibition of p21^{Ras} and MAPK activation by Hu-r-βGBP. Cells treated with 20 nM Hu-r-βGBP for the times indicated before stimulation with 10 ng/ml PDGF were lysed after 10 min. (**a**) Lysates were incubated with the Ras binding domain of Raf-1 coupled to GST agarose beads to precipitate active p21^{Ras} and western blots of active p21^{Ras} were probed with anti-panRas antibody (Transduction Labs). Total p21^{Ras} was assessed using the same anti-panRas antibody on western blots from total cell lysates. (**b**) Western blots were probed with anti-phospho-p44/p42 MAPK (Thr202/Tyr204) monoclonal antibody (Cell Signalling Technology) for assessment of active MAPK levels and with anti-ERK2 antibody (Santa Cruz) for assessment of total MAPK. (**c**) Cells were treated with 20 nM TPA for 30 min before PDGF stimulation, lysed and p21^{Ras} analysed as described in (**a**). (**d**) Cells were treated with 20 nM TPA for 30 min, lysed and MAPK was assessed as described in (**b**). $\pm$ indicate presence or absence of TPA.

isolated class IA PI3K by immunoprecipitation using a monoclonal antibody to the p85α adapter/regulatory subunit and assessed the ability of p110 to convert a standard phosphatidylinositol(4,5)P2 (PIP2) to phosphatidylinositol(3,4,5)P3 (PIP3) in a 3 h kinase reaction by measuring the PIP3 generated in a competitive enzyme-linked immunosorbent assay (ELISA). Timescale experiments from the addition of βGBP were carried out in parallel with wortmannin (Powis *et al*., 1994) and LY294002 (Vlahos *et al*., 1994), both pharmacological inhibitors of the p110 catalytic subunit, used at concentrations that would produce an effect similar to that of the βGBP molecule. We found that in all cases the response to PDGF stimulation, in terms of PIP3 generated, was reduced with a similar pattern and within a comparable time to values lower than basal PIP3 levels (Figure 3a, dark grey histograms). Notably, the inhibitory effect was not a response to the activation of PI3K by PDGF as βGBP could bring down basal PI3K activity to similar values and within a similar time scale as in the case of PDGF stimulation (Figure 3a, light grey histograms). The virtual absence of changes in the levels of p110α and p110β shown in Figure 3b (p110δ not detectable) indicates that, similar to wortmannin and LY294002, βGBP had affected PI3K function and that, similarly to the two inhibitors, it may act by inducing conformational changes that reduce the functional ability of the regulatory pocket site, that is, the catalytic domain of p110 (Walker *et al*., 2000).

We next examined whether, as in the case of βGBP, wortmannin and LY294002 would also inhibit Ras activation and whether there would be correspondence between the inhibition of PI3K and the inhibition of Ras. Figure 3c shows that, similar to βGBP, wortmannin and LY 294002 abrogated the activation of Ras and MAPK, and that this effect coincided with the time when active PI3K was below basal levels. These results indicate that the p110 catalytic unit of class IA PI3K is a prime target of βGBP-induced signalling, that PI3K activity is a necessary requirement to permit Ras activation and that basal levels of PI3K activity are sufficient. Notably, such a permissive role for PI3K has also been observed in immortalized COS-7 cells where basal levels of PI3K activity were required for the activation of Ras by low but mitogenic concentrations of EGF (Wennstrom and Downward, 1999), a condition conceivably close to that of a physiological mitogenic input.

Further evidence to validate the effect of βGBP on the p110 subunit of PI3K was sought using lysophosphatidic acid (LPA) to activate class IB PI3K (Koch *et al*., 1994) where the p110γ catalytic subunit is complexed with either a p101 (Vanhaesebroeck *et al*., 2001) or a p84 (Suire *et al*., 2005) adapter protein, which are activated by G$\beta\gamma$ (Leopoldt *et al*., 1998; Suire *et al*., 2005). We found that βGBP was effective in inhibiting LPA stimulation of class IB PI3K (Figure 4a). We then immunoprecipitated class IB PI3K using antibodies to the p101 adapter subunit of class IB PI3K and found no changes in p110γ levels, again indicating that βGBP had affected PI3K function. However, when probing the same precipitate with an antibody to p110α, we found, to our surprise, that p110α was also present (Figure 4b), although, seemingly, not at equal ratio as longer exposure was required for visualization. This result indicates that, further to association with p110α (Rodriguez-Viciana *et al*., 1994), Ras can co-associate with p110γ, bridging class IA and IB PI3K, which co-precipitate. This conclusion was validated by the evidence of Figure 4c showing that in the immunoprecipitates obtained using an anti-p85 monoclonal antibody, in addition to p110α, p110γ was also present. We

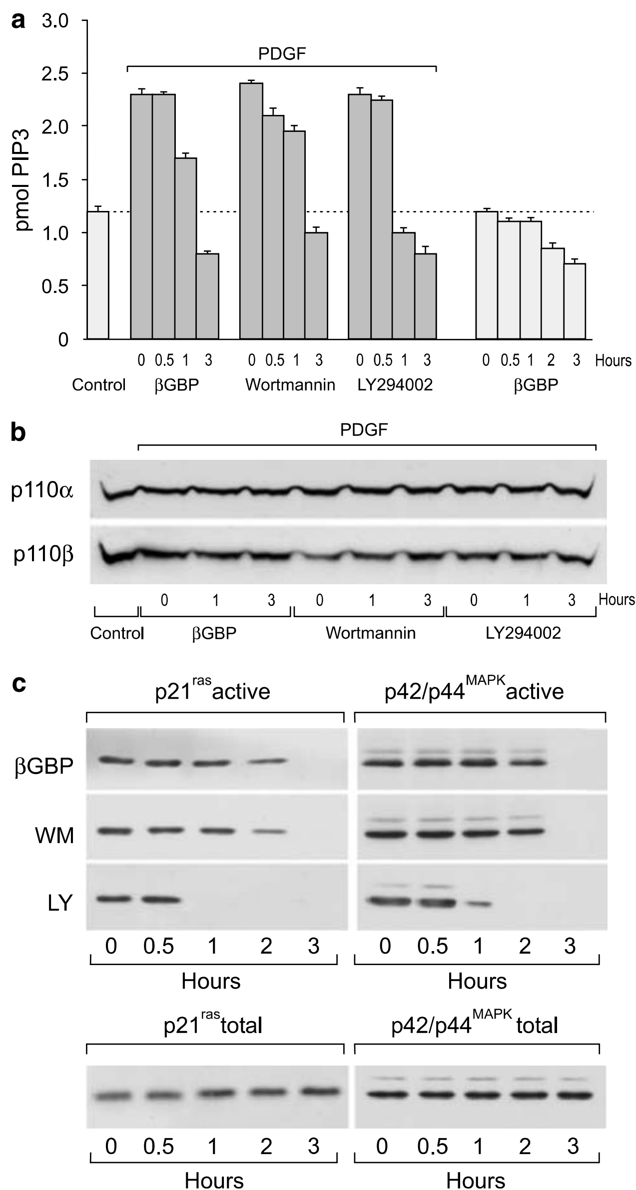

Figure 3 Inhibition of class IA PI3K activity in cells treated with Hu-r-βGBP. PI3K was immunoprecipitated from cell lysates using an anti-PI3K p85 antibody (Upstate Biotechnology, Lake Placid, NY, USA) followed by protein A agarose. The immunoprecipitated PI3K was incubated in a kinase reaction for 3 h with 40 pmol PI(4,5)P2 substrate and the PI(3,4,5)P3 generated was assayed in a competitive ELISA (Echelon Biosciences, Salt Lake City, UT, USA). (**a**) Assessment of class IA PI3K activity. Cells were treated with 20 nM Hu-r-βGBP, 10 μM wortmannin or 20 μM LY294002 for the times indicated, before stimulation with 10 ng/ml PDGF for 10 min or only treated with βGBP. (**b**) Assessment of p110α and p110β levels in the immunoprecipitates (**a**) by western blotting using anti-p110α monoclonal antibody (BD Bioscience, San Jose, CA, USA) and anti-p110β polyclonal antibodies (Santa Cruz); exposure time was 5 min. (**c**) Correlation between inhibition of class IA PI3K and abrogation of Ras and MAPK activation. Cells were treated with 20 nM Hu-r-βGBP, 10 μM wortmannin or 20 μM LY294002 for the times indicated. Active and total $p21^{Ras}$ and active and total p42/p44 MAPK were assessed as described in Figure 2.

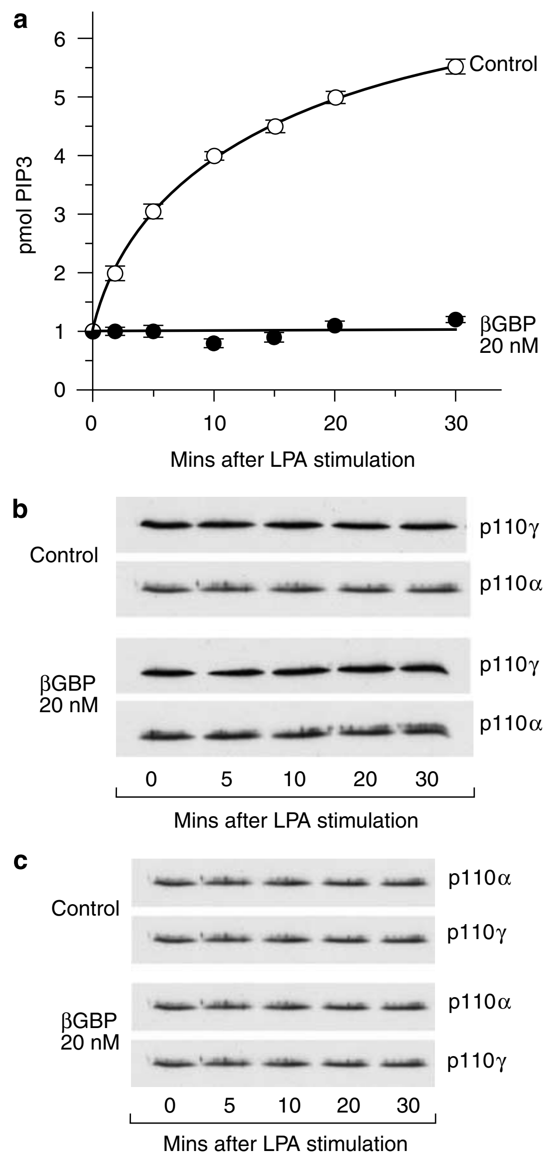

Figure 4 Inhibition of class IB PI3K activity in cells treated with Hu-r-βGBP. (**a**) Control cells and cells treated for 3 h with 20 nM Hu-r-βGBP were stimulated with 10 μM LPA and PI3K activity was assessed as described in Figure 3. Data plotted are means of triplicate experiments ± s.e.m. (**b**) Assessment by western blotting of p110γ and p110α in the immunoprecipitates obtained using anti-p101 rabbit polyclonal antibodies (Upstate Biotechnology), using rabbit polyclonal anti-p110γ antibodies (gift from Len Stephens; exposure time 5 min) and anti-p110α monoclonal antibody (BD Bioscience; exposure time 60 min). (**c**) Assessment by western blotting of p110α and p110γ in the immunoprecipitates obtained using anti-p85 antibody (Upstate Biotechnology) using anti-p110α monoclonal antibody (BD Bioscience) (exposure time 5 min) and rabbit polyclonal anti-p110γ (gift from Len Stephens; exposure time 60 min).

believe this is the first evidence for class IA and IB PI3K physical interlinking. Association of class IA and IB PI3K is in accordance with previous evidence for functional dependence of class IA and IB PI3K where p110γ stimulation is affected by loss of p85 function (Hawes *et al*., 1996).

This report is the first study on the molecular mechanisms whereby βGBP counteracts growth factor-induced cell proliferation by controlling a functional link between PI3K and Ras, where PI3K is the upstream target on which βGBP acts. Functional inhibition of p110 by βGBP results in downregulation of PI3K activity, suppression of Ras-GTP loading, consequent loss of MAPK activation and block of cell proliferation. It is of interest to notice, within the context of βGBP as a downregulator of oncogene signalling, that by mapping on human chromosome 22q12–q13.1 (Baldini *et al*., 1993), a region translocated or deleted in a number of human tumours (Aurias *et al*, 1984; Bridge *et al*., 1990; Rey *et al*., 1993; Turc-Carel *et al*., 1998), the gene encoding βGBP (Chiariotti *et al*., 1991) is brought to attention as a prospective tumour suppressor gene.

Significantly, the ability of βGBP to downregulate PI3K activity can extend beyond its regulatory function in normal cells. By controlling cellular processes, which include cell proliferation, survival and migratory ability (Cantley, 2002; Vivanco and Sawyers, 2002; Garcia *et al*., 2006), PI3K has become an elective target for therapeutic intervention (Hennessy *et al*., 2005 and references within). The evidence that βGBP exerts its cytostatic effect by targeting PI3K and suppressing Ras-MAPK signalling may help explain its anticancer effect in a variety of tumour cells, including cells which have developed drug resistance (Mallucci *et al*, 2003; Ravatn *et al*., 2005), and provide a better understanding of the role of βGBP in the control of tumorigenesis.

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