BMP2 exposure results in decreased PTEN protein degradation and increased PTEN levels

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The tumour suppressor gene PTEN encodes a dual-specificity phosphatase that recognizes protein and phosphatidylinositol substrates and modulates cellular functions such as migration and proliferation. Germline mutations of PTEN have been shown to cause Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome and Proteus syndrome. Recently, germline mutations in BMPR1A, the gene encoding the type 1A receptor of bone morphogenetic proteins (BMP) have been found in rare families with Cowden syndrome, suggesting that there may be a link between BMP signaling and PTEN. We thus sought to determine whether BMP2 stimulation alters PTEN protein levels in the breast cancer line, MCF-7. We found that exposure to BMP2 increased PTEN protein levels in a time- and dose-dependent manner. The increase in PTEN protein was rapid and was not due to an increase in new protein synthesis, as cycloheximide treatment did not inhibit BMP2-induced PTEN accumulation, suggesting that BMP2 stimulation inhibited PTEN proteindegradation. Indeed, we found that BMP2 treatment of MCF-7 cells decreased the association of PTEN with two proteins in the degradative pathway, UbCH7 and UbC9. These data indicate that BMP2 exposure can regulate PTEN protein levels by decreasing PTEN’s association with the degradative pathway. This opens up a new mode of regulating PTEN activity to be investigated further and may explain why BMPR1A can act as a minor susceptibility gene for PTEN mutation negative Cowden syndrome.

INTRODUCTION

The tumour suppressor gene PTEN/MMAC1/TEP1 (1), localized to chromosome sub-band 10q23.3, has been implicated in a variety of human cancers, including the inherited hamartoma syndromes, Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome, Proteus syndrome and Proteus-like syndromes (1,2). The protein product of the PTEN gene, PTEN, is a dual-specificity phosphatase, dephosphorylating both lipid and protein substrates (3,4). As a lipid phosphatase, PTEN dephosphorylates the D3 position of phosphatidylinositol, 3,4,5-triphosphate, resulting in decreased activation of the PI3-kinase/Akt pathway. As a protein phosphatase, PTEN has been shown to dephosphorylate focal adhesion kinase (5). PTEN has also been shown to affect proliferation in a protein phosphatase-dependent manner by modulating the mitogen-activated protein kinase pathway (6,7).

While much work has been done on elucidating the activity of PTEN, the regulation of PTEN protein levels via transcription, translation and post-translational mechanisms remains to be precisely elucidated. Recently, activated PPARγ, p53 and EGR1 have been shown to up-regulate PTEN transcription (8–10), however, factors that down-regulate transcription remain to be identified. Several groups have demonstrated that the C-terminal end of PTEN and PTEN post-translational modification by phosphorylation positively regulate PTEN protein stability (11,12). However, the modes of regulation of PTEN protein levels at both the synthesis and degradative stages is not completely understood.

Germline mutations in PTEN have been found to cause the majority of Cowden syndrome, an inherited hamartoma syndrome characterized by a risk of breast and thyroid cancer (13,14), approximately 60% of Bannayan–Riley–Ruvalcaba syndrome (BRRS), a seemingly unrelated congenital disorder characterized by mental retardation, lipomas, hemangiomas and pigmentation of the penile shaft (15–17), and perhaps 20% of Proteus syndrome, commonly referred to as the Elephant Man Disease (18,19). Interestingly, germline mutations in BMPR1A, the gene encoding the type 1A receptor of bone morphogenetic proteins (BMP), has been found in rare families.
with Cowden syndrome (20 and Eng et al., unpublished observations).

BMPs, members of the transforming growth factor-β (TGFβ) super-family, have been shown to play a role in cell proliferation and differentiation in bone (21). While the role of BMP in development and bone formation is being well characterized, little is known about its role in neoplasia. However, BMP2 mRNA has been shown to be expressed in the mammary gland and recently BMP2 has been shown to inhibit MCF-7 proliferation as well as induce the expression of p21 (22). Since PTEN is a tumour suppressor believed to affect p21 expression (Waite et al., unpublished results) and because germline mutations in both PTEN and BMPR1A are associated with Cowden syndrome (14,17,20), an inherited breast cancer syndrome, we sought to determine whether BMP2 alters PTEN protein levels in a breast cancer line.

RESULTS

Exposure to BMP2 results in elevated PTEN protein levels in a time- and dose-dependent manner

The addition of BMP2 to MCF-7 cells has been shown to cause a decrease in proliferation (22) as well as induce the expression of Id-2 (23). Based upon these reported observations and existing data that PTEN over-expression in MCF-7 also causes growth suppression (24,25), we were interested in determining if BMP2 had any effect on the protein level of the tumor suppressor, PTEN. After incubation with 100 ng/ml BMP2, MCF-7 cells experienced a rapid rise in PTEN expression compared with control (Fig. 1). At as little as 1 h post-stimulation, PTEN protein levels were increased (~2-fold by densimetric scanning) in response to BMP2 (Fig. 1). This increase continued to 6 h (~2.2-fold by densimetric scanning), after which point the increase in PTEN protein levels in response to BMP2 decreased and there was little difference between stimulated and non-stimulated cells past 12 h post-stimulation (Fig. 1). In addition, this PTEN response was concentration-dependent; there was a minimal response to stimulation (1–10 ng/ml; Fig. 2). However, PTEN protein levels started to increase when exposed to 30 ng/ml BMP2 and plateaued at 300 ng/ml (~2.5-fold by densimetric scanning). This response was observed at both 1 h (data not shown) and 6 h (Fig. 2) post-stimulation.

BMP exposure does not stimulate new PTEN protein expression

It is now widely accepted that the BMP super-family can regulate the expression of multiple proteins through its regulation of the SMAD pathway (26,27). Thus, it was possible that BMP2 could be stimulating PTEN gene transcription through stimulation of the SMAD pathway. In order to investigate this, we incubated MCF7 cells with cycloheximide, a protein synthesis inhibitor, as described in the Methods. If BMP2 was stimulating PTEN gene transcription, we would expect that cycloheximide treatment would inhibit the effect of BMP2. However, the addition of cycloheximide (CHX) had only a slight effect on PTEN protein levels either alone, or when combined with BMP2 (Fig. 3A). In contrast, cycloheximide addition had a strong effect on p21 protein levels (Fig. 3B), as previously described (28). p21 protein levels were dramatically decreased when cycloheximide was added to the cells, regardless of the presence of BMP2. BMP2 alone had little effect on p21 levels at these early time points (data not shown). The slight decrease in PTEN protein level with CHX may suggest that BMP2 stimulation may have a minimal effect on PTEN transcription, however we found that BMP2 treatment had no effect on PTEN mRNA levels, determined by RT–PCR, at 1 or 6 h post-stimulation (data not shown).

PTEN can be post-translationally modified as a result of protein phosphorylation by casein kinase 2 (CK2) (11). Torres and Pulido demonstrated that protein phosphorylation by CK2 was important for proteasome-mediated PTEN protein degradation (11). Others have also shown that de-phosphorylated PTEN has decreased stability compared to phosphorylated PTEN (11,12,29). Therefore, it is possible that BMP2 stimulation could alter PTEN stability by increasing its phosphorylation. We examined the phosphorylation of PTEN, using phospho-specific PTEN antibodies, and found no change in protein phosphorylation (data not shown). In addition, we found no change in PTEN protein phosphorylation levels by in-vivo labeling with inorganic [32]-P and immunoprecipitation (data not shown). Finally, we found no significant change in CK2 activity in the presence of BMP2 (data not shown).

Together, these data indicate that BMP2 stimulation does not alter the post-translational phosphorylation of PTEN.

BMP2 treatment decreases PTEN association with the protein degradative pathway

If BMP2 does not simulate new protein synthesis, then it may regulate the degradation of PTEN protein. Several studies have shown that PTEN activity is regulated by the stability of the protein. We, therefore, examined whether BMP2 treatment had
an effect on the association of PTEN with the ubiquitin degradative pathway. Tolkacheva and coworkers have demonstrated, in NIH3T3 cells transfected with a vector for HA-tagged ubiquitin, that PTEN can be poly-ubiquinated (30). We were unable to show a PTEN–ubiquitin protein interaction in the MCF-7 cell line which was not transfected. However, we were able to demonstrate that PTEN associates with UbCH7, the ubiquitin-conjugating enzyme that mediates the transfer of activated ubiquitin to substrate proteins (Fig. 4). UbCH7–PTEN protein interaction is decreased in the presence of 300 ng/ml of BMP2 at both 1 and 6 h (Fig. 4A). We also found that the interaction of PTEN with UbC9, another ubiquitin-conjugating protein, was also decreased in the presence of BMP2 (Fig. 4B), although not to the same extent as the PTEN–UbCH7 interaction. Taken together, these data indicate that BMP2 regulates PTEN protein levels by inhibiting its association with the protein degradative pathway.

In sum, therefore, our data demonstrate that BMP2 exposure in MCF-7 cells results in an increase in PTEN protein levels in a time- and dose-dependent manner. This increase in protein is due to increased protein stability of PTEN as evidenced by decreased association of PTEN to components of the ubiquitin pathway.

**DISCUSSION**

As a member of the TGFβ super-family, BMP2 signals through heterotrimeric complexes of type I and type II receptors. These complexes then signal downstream, via either the SMAD pathway or the mitogen-activated protein kinase cascade pathway initiated by TGFβ activated protein kinases, to the nucleus resulting in the regulation of transcription of various genes. The TGFβ pathway is thought to be a tumor suppressor pathway in cancer development. Indeed, inactivation of these pathways has been found in a variety of tumors (20,31–33). Recently, germline BMPRIA mutations have been found in at least two Cowden Syndrome families (20, Eng et al., unpublished), suggesting that there may be a link between the BMP signaling pathway and PTEN activation.

Since the identification of PTEN as a tumor suppressor, much has been elucidated about PTENs role in modulating the PI3 kinase pathway as well as others involved in cellular proliferation. However, little is still known about the regulation of PTEN protein activity. To date, PTEN activity is not regulated by post-translational modification and it appears to be constitutively active. This suggests that the level of PTEN activity might be regulated by other mechanisms that titrate the amount of protein present and is therefore regulated at the transcriptional level and/or at the level of protein degradation. Recent work has begun to elucidate the mechanism of PTEN transcriptional regulation. In addition, two groups have shown that the dephosphorylation of PTEN can have a role in regulating PTEN protein stability (11,12).

We have shown here that PTEN protein levels are also regulated by BMP2. BMP2 treatment of MCF-7 cells, which has been shown to cause a decrease in cellular proliferation, results in elevated levels of PTEN protein due to decreased association with proteins in the ubiquitin degradation pathway. This is not the first time that BMP stimulation has been implicated in regulating the degradation of proteins involved in cellular signaling processes. Activation of the BMP type I receptor has recently been demonstrated to stimulate targeted degradation of Smad1 (34), whereas we demonstrate the inhibition of targeted degradation. Together with this report,
these data suggest that a secondary role from BMP signaling, regulating protein degradation, is emerging. It is likely, that future research will show cross talk between the various BMP pathways to determine if the targeted protein is degraded or not.

The increased PTEN protein pool, due to BMP exposure, does not appear to result in active PTEN as levels of phospho-Akt, which can be used as a measurement of PTEN activity (35,36), remain unchanged in the cells (data not shown). This is in agreement with what Vazquez and co-workers have found (12), i.e. PTEN targeted for degradation is more active than PTEN that is stable. Therefore, BMP-2 stimulation of MCF-7 cells may create a ‘pool’ of PTEN to be utilized at times of need. Immunohistological studies have demonstrated that PTEN expression in the nuclear compartment is higher in normal tissue when compared with counterpart neoplastic tissue (37–39). Recently, work in our laboratory has directly demonstrated that PTEN can be localized to the nuclear compartment in MCF-7 cells, especially during G0–G1 of the cell cycle (40). The significance of these observations remains unclear and is currently under intense investigation. The localization of PTEN to the nucleus could sequester PTEN to an inactive location or nuclear PTEN may be active at the nuclear membrane. It is possible that the inactive pool of PTEN generated by BMP2 stimulation resides in a different subcellular location from active PTEN, however, the mechanisms and roles of PTEN subcellular localization still remain to be elucidated.

Several groups have demonstrated that dephosphorylated PTEN becomes targeted for degradation (11,12). We found, in MCF-7 cells, that BMP2-associated increases in PTEN protein levels does not occur due to changes in PTEN phosphorylation. These data suggest that there are undoubtedly multiple means of regulating PTEN protein levels beyond phosphorylation-dephosphorylation. It remains a possibility that PTEN is altered post-translationally in a manner that has not been identified, and that BMP2 stimulation alters such a modification; however, this remains to be elucidated. In conclusion, we have shown that PTEN interacts with degradative proteins, upon BMP2 exposure, opening up a new mechanism of regulating PTEN protein stability and hence activity, which merits further investigation. Our observations may also explain why BMPRIA can act as a minor (2/80) susceptibility gene for PTEN mutation-negative Cowden syndrome.

**MATERIALS AND METHODS**

**Materials**

DMEM, penicillin/streptomycin and fetal bovine serum (FBS) were from Gibco Life Technologies (Rockville, MD, USA). Antibodies to UbCH7 and UbC9 were obtained from BD Transduction Laboratories (San Diego, CA, USA); anti-p21 was from Santa Cruz (Santa Cruz, CA, USA); all secondary HRP-conjugated antibodies were from Promega (Madison, WI, USA). BCA protein kit, mammalian protein extraction buffer and enhanced chemiluminescence reagents were purchased from Pierce (Rockford, IL, USA). Human recombinant BMP2 was from R&D Systems (Minneapolis, MN, USA). Cycloheximide was purchased from Calbiochem (San Diego, CA, USA). All other reagents were obtained from Sigma (St Louis, MO, USA) or other common commercial sources.

**Cell culture**

The MCF-7 breast cancer cell line was maintained in DMEM plus 10% FBS and 100 units/ml each of penicillin and streptomycin. Cells were plated at 1 × 10^6 cells/60 mm dish in 3 ml of media. After 24 h the media was removed and replaced with serum free media and incubated for an additional 24 h. The media was again removed and replaced with maintenance media and the cells were treated with BMP2 as indicated. In select experiments, 10 μg/ml cycloheximide were added 1 h prior to BMP2 stimulation. Cellular protein was extracted at the indicated times as described below.

**Protein extraction**

At the indicated times, cells were washed twice with ice-cold PBS and scraped into mammalian lysis buffer containing 500 μM PMSF, 5 μg/ml each of leupeptin, aprotinin, and pepstatin, 2 mM sodium orthovanadate, 50 mM NaF and 10 mM β-glycerophosphonate. Insoluble cellular material was removed by centrifugation at 4°C. Protein concentration was determined by the bicinchoninic acid method with bovine serum albumin as a standard (41).

**PTEN immunoprecipitation**

Isolated cellular material (100 μg) was diluted to 500 μl with 50 mM Tris (pH = 7.0) containing 150 mM NaCl, 2 mM EDTA, and 1 mM EGTA as well as the protective agents described above. This was incubated with 75 μl of Protein A-Sepharose beads for 10 min, followed by removal of the beads. Ten microliters of PTEN antibody were then incubated with the precleared cell lysate and incubated overnight at 4°C. Protein A-Sepharose beads were added again and the mixture incubated overnight at 4°C. The beads were pelleted by centrifugation, and washed 3 × with ice-cold PBS. The isolated beads were then resuspended in 20 μl of 50 mM Tris (pH = 7.0) containing 150 mM NaCl, 2 mM EDTA, and 1 mM EGTA plus 20 μl of 2 × Tricine sample buffer (42) and samples were boiled for 10 min. After centrifugation, proteins in the resulting supernatants were separated on a 16% Tris/Tricine peptide gel and then subjected to western blot analysis for UbCH7 and UbC9.

**Western blot**

Unless otherwise indicated, samples were prepared for SDS–PAGE by the Laemmli method (43). Proteins were separated by 10% SDS–PAGE, except for analysis of UbCH7 and UbC9 in which proteins were separated on a 15% Tris/Tricine peptide gel (42), after all electrophoresis, proteins were transferred to nitrocellulose. Equal protein loading was confirmed by staining the nitrocellulose blots with Ponceau S (0.1% Ponceau S in 5% acetic acid) for 10 min. Blots were then washed with dH2O and protein loading was analyzed. When equal loading was
confirmed blots were washed further with TBS-T (10 mM Tris pH = 7.0, 100 mM NaCl and 0.1% Tween 20) to remove residual Ponceau S and then processed as follows. Nitrocellulose blots were blocked by incubation for 1 h in 5% milk in TBS-T. Blots were then incubated for 2 h, at room temperature, with the appropriate antibody. All primary antibodies were diluted 1:1000 in 3% BSA. After the primary incubation, the blots were subjected to six 10 min washes and then incubated for 1 h with the appropriate HRP-conjugated secondary antibody at 1:5000 dilution. Blots were washed again, and visualized by enhanced chemiluminescence according to manufacturer’s recommendations.

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