Regulation of metabolism by amino acid dependant signal transduction

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Chapter 2

The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway

Abstract

The tumor suppressor PTEN is a dual protein and phosphoinositide phosphatase that negatively controls the phosphatidylinositol (PI) 3-kinase/protein kinase B (Akt/PKB) signalling pathway. Interleukin-13 via the activation of the class I PI 3-kinase has been shown to inhibit the macroautophagic pathway in the human colon cancer HT-29 cells. Here we demonstrate that the wild-type PTEN is expressed in this cell line. Its overexpression directed by an inducible promoter counteracts the interleukin-13 down-regulation of macroautophagy. This effect was dependent upon the phosphoinositide phosphatase activity of PTEN as determined by using the mutant G129E, which has only protein phosphatase activity. The role of Akt/PKB in the signalling control of interleukin-13-dependent macroautophagy was investigated by expressing a constitutively active form of the kinase (myrPKB). Under these conditions a dramatic inhibition of macroautophagy was observed. By contrast a high rate of autophagy was observed in cells expressing a dominant negative form of PKB. These data demonstrate that the signalling control of macroautophagy overlaps with the well known PI 3-kinase/PKB survival pathway and that the loss of PTEN function in cancer cells inhibits a major catabolic pathway.
1. Introduction

Macroautophagy, a multistep process responsible for the degradation of long-lived proteins and organelle renewal, starts with the formation of an autophagosome, which ultimately fuses with the endosomal/lysosomal compartment [1,2]. This pathway is known to be important in the maintenance of cell functions during period of nutrient deprivation [3]. However, recent data have shed light on the importance of autophagy in human pathologies, including some forms of cardiomyopathy (Danon’s disease) [4] and breast cancer [5].

The recent discovery of apg and aut genes in yeast and the identification of orthologous genes in human cells have increased our knowledge of the molecular machinery responsible for the formation of autophagic vacuoles [6,7]. A better understanding of the control of macroautophagy is also dependent upon the identification of signal transduction pathways that control the formation of autophagosomes [8-10].

The drug 3-methyladenine (3-MA), which inhibits the formation of autphagic vacuoles [11], has been shown to target enzymes of the phosphatidylinositol 3-kinase (PI 3-kinase) family [12,13]. Class I and III PI 3-kinases act antagonistically at different steps of autophagy [13]. Class III PI 3-kinase is probably engaged in the control of the formation of autophagic vacuoles by association with other proteins recruited to cytoplasmic membrane as suggested recently for its yeast homolog VPS34 [14]. By contrast, the plasma membrane-associated class I PI 3-kinase would be required to transduce a negative signal for the biogenesis of the autophagic vacuole [13].

The tumor suppressor PTEN is a dual protein/lipid phosphatase mutated in a variety of cancers [15-17], which has been shown to dephosphorylate the 3’ position of the class I PI 3-kinase product phosphatidylinositol (3,4,5)P3 [18] and consequently down-regulates PI 3-kinase/PKB pathway [19]. In the present work we demonstrate that PTEN is expressed in human colon cancer HT-29 cells and negatively regulates IL-13-dependent PI 3-kinase/PKB signalling. Moreover PTEN, via its lipid phosphatase activity, is involved in the signalling control of autophagy, together with the downstream acting Akt/PKB. These results add a new function to the PI 3-kinase/PTEN/PKB pathway and also provide a new link between the control of autophagy and tumor progression.

2. Materials and methods

2.1. Cells and Material

HT-29 cells were cultured as described previously [20]. 3-MA, metrizamide, and other chemical products were from Sigma. Nitrocellulose membranes and the bicinchoninic acid (BCA) protein assay kit were purchased from Schleicher and Schuell (Dassel, Germany) and Pierce, respectively. Enzymes, synthetic oligonucleotides, and cell culture reagents such as Geneticin
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(G418) and hygromycin B were purchased from Life Technologies, Inc. (Eragny, France). The Superfect™ transfection kit was from Qiagen (Courtaboeuf, France). Pfu DNA polymerase, the LacSwitch® II-inducible mammalian expression system, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Stratagene (La Jolla, CA). Monoclonal antibody directed against polyhistidine tag (C terminus), blasticidin and pcDNA6/V5-His A, B, C vectors were from Invitrogen (Carlsbad, CA). Monoclonal antibody against the HA epitope was from Roche Molecular Biochemicals (Mannheim, Germany). Rabbit anti-PTEN, rabbit anti-actin, rabbit anti-phospho-Akt/PKB (Thr308), sheep anti-Akt/PKB, sheep anti-phospho-GSK-3β, and sheep anti-GSK-3β antibodies were from Upstate Biotechnology (Lake Placid, NY). IL-13 and cDNA encoding for the myristoylated and dead forms of HA-tagged Akt/PKB were kindly provided by A. Minty (Sanofi Elf Biorecherche, Labege, France), Dr. T. F. Franke (Columbia University, New York, NY), and Dr. P. N. Tsichlis (Fox Chase Cancer Center, Philadelphia, PA), respectively. L-[U-14C]Valine (specific activity: 288.5 mCi/mmol) and enhanced chemiluminescence detection kit were from Amersham Pharmacia Biotech (Les Ulis, France).

2.2. Expression Plasmids and Transfections

The full-length PTEN cDNA was synthesized from total RNA derived from HT-29 cells by PCR using pfu DNA polymerase and primers that add a 5' BamHI site (5'-GCGGATCCATGACAGCCATCATCAAAGAGATCGTTAGC) and a 3' XbaI site (5'-CGCTCTAGATG*ACTTTTGTAATTTGTGTATGC containing a mutated stop codon (*)). The resulting cDNA was subcloned into pcDNA6/V5-His, and the sequence was verified by automated sequencing using several pairs of primers at position 1-400, 365-800, 770-1000, and 980-1300. Histidine-tagged PTEN cDNA was then amplified by PCR and subcloned into pOPRSVI/MCS operator vector from the LacSwitch® II-inducible mammalian expression system. Histidine-tagged C124S and G129E PTEN mutants were generated by PCR-based site-directed mutagenesis and subcloned into the pOPRSVI/MCS operator vector. In a first set of experiments, HT-29 cells were transfected with 5 μg of Lac-repressor-expressing vector and selected in the presence of 400 μg/ml hygromycin B. In a second set of experiments, stable Lac-repressor-expressing HT-29 cells were transfected with 5 μg of each pOPRSVI/MCS operator vector constructions, including the empty vector, His-tagged wt-PTEN, C124S, and G129E PTEN mutants, and selected in the presence of 800 μg/ml G418 and 200 μg/ml hygromycin B. Under these conditions, expression of inserted cDNA is repressed until inducer (5 mM IPTG) is added to the media for 16 h.

HT-29 cells were transfected with 5 μg each of pCMV6-HA-tagged, myristoylated, and dominant negative Akt/PKB and selected in the presence of 800 μg/ml G418.
2.3. Immunoblotting

Before and after IPTG induction for 16 h, cells were washed twice with ice-cold phosphate-buffered NaCl solution and lysed in cold lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 5 μM phenylmethylsulfonyl fluoride, 5 μg/ml of leupeptin, pepstatin A, and aprotinin, 1 mM Na₃VO₄, 2 mM NaF, and 2 mM Na₄P₂O₇) for 30 min on ice. After centrifugation the protein concentration of cell lysates was determined using the BCA reagent. One-hundred μg of protein were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in TBS T (10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature and then incubated with the appropriate primary antibody for 1 h at room temperature or overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody at 1:3000 dilution for 1 h at room temperature. The polyclonal anti-PTEN, anti-phospho-Akt/PKB (Thr³⁰⁸), anti-Akt/PKB, anti-phospho-GSK-3β, and anti-GSK-3β antibodies were used at 1:1000 dilution. The monoclonal anti-His and anti-HA antibodies were used at 1:2500 and 1:3000 dilution, respectively.

2.4. Measurements of Autophagic Parameters

Autophagic sequestration of LDH and measurement of the degradation of long-lived proteins were determined as reported previously [21]. Cells were cultured in complete medium containing 5 mM IPTG for 16 h and then chased for 4 h in nutrient-free medium (without amino acids and in the absence of fetal calf serum) containing 5 mM IPTG. When used, 3-MA (10 mM) and IL-13 (30 ng/ml) were added to the cells at the beginning of the chase period.

3. Results

3.1. PTEN Controls the IL-13-dependent Activation of PKB in HT-29 Cells

As the PTEN phosphatase tumor suppressor is frequently inactivated in several cancer cells and in a series of related disorders that are characterized by a predisposition to cancer [15-17], we have first determined whether wt-PTEN is expressed in HT-29 cells. As shown in Fig. 1, a rabbit polyclonal antibody raised against a C-terminal peptide of PTEN recognized a Mr 56,000 protein in HT-29 cells. To investigate the presence of mutations of endogenous PTEN, we analyzed its cDNA amplified by RT-PCR. After total sequencing, no mutation was detected on PTEN, including the region corresponding to the common phosphatase P loop motif (HCXXGXXXR located at the position 123-130). To determine whether increased levels of wt-
PTEN or inactive phosphatase mutants had effects on Akt/PKB activation in HT-29 cells, full-length cDNAs were subcloned into an eucaryotic expression pcDNA6/V5/His vector.

**Fig. 1. HT-29 cells express the wild-type form of PTEN.**
Expression of the endogenous PTEN protein in HT-29 cells. Western blot (WB) analysis using a polyclonal anti-PTEN antibody on whole cell lysates of HT-29 cells.

Preliminary attempts to select clones of HT-29 cells overexpressing wt-PTEN were unsuccessful, probably because of the induction of cell death induced by the forced expression of PTEN as reported previously [22]. To circumvent this difficulty, His-tagged constructs expressing either wt-PTEN or an inactive phosphatase mutant (C124S) or the G129E phosphoinositide phosphatase-deficient mutant were transfected after insertion into the IPTG-inducible pOPRSVI/MCS operator vector together with a Lac-repressor-expressing vector. HT-29 cells overexpressing the pOPRSVI/MCS operator vector with no insert were used as a control. G418- and hygromycin B-resistant clones were selected, and PTEN expression was measured at different times after the addition of 5 mM IPTG. After 16 h of induction, expression of PTEN in the selected clones was detected by immunoblotting using a monoclonal antibody directed against the polyhistidine tag (Fig. 2A). Under these conditions no detectable signs of cell death were observed, and a 2.5-fold increase of PTEN proteins was detected using an anti-PTEN antibody (data not shown). IL-13 is known to activate Akt/PKB in HT-29 cells in a class I PI 3-kinase-dependent manner [13,23]. When wt-PTEN-expressing cells were challenged with IL-13 after IPTG addition, we observed a decrease in Akt/PKB phosphorylation when compared with the control cells (Fig. 2B). In contrast, a robust Akt/PKB phosphorylation was observed either in C124S- or G129E-expressing cells after IPTG addition. Whatever the cell population considered no modification of the PI 3-kinase activity was observed in presence or absence of IPTG (data not shown), demonstrating that PTEN functions downstream of PI 3-kinase as expected.
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Fig. 2. Effect of overexpression of PTEN on IL-13-dependent phosphorylation of Akt/PKB in HT-29 cells.

A, induction of PTEN expression in HT-29-Lac-repressor-expressing cells containing IPTG-inducible pOPRSVI/MCS PTEN cDNA expression constructs (His-tagged wt-PTEN, C124S, and G129E PTEN mutants). Expression levels were compared after 16-h culture in the presence (+) or absence (−) of 5 mM IPTG by Western blot using a monoclonal anti-His antibody (top). HT-29-Lac-repressor-expressing cells containing IPTG-inducible pOPRSVI/MCS operator vector with no insert were used as control (vector). Control blot with antibody against actin was performed (bottom). B, effect of PTEN on Akt/PKB phosphorylation. Cell populations were first induced for 16 h with 5 mM IPTG and then cultured in the presence (+) or absence (−) of 30 ng/ml IL-13 for 4 h. Levels of phosphorylated (top) and total (bottom) Akt/PKB protein were measured by Western blot using an anti-phospho-Akt/PKB (Thr308) antibody and a pan-Akt/PKB antibody that recognizes Akt/PKB independent of its phosphorylation status. Note that the phosphorylation of Akt/PKB is dependent on PTEN expression and functional phosphatase activity.

3.2. Up-regulation of Autophagy Is Dependent upon the Lipid Phosphatase Activity of PTEN

We have shown previously that stimulation of class I PI 3-kinase by IL-13 decreased the autophagic capacities of HT-29 cells [13] in a similar way to that observed after treatment with 3-MA, an inhibitor of autophagy [11]. By contrast, in wt-PTEN-overexpressing cells autophagy was no longer sensitive to IL-13 treatment but still sensitive to 3-MA as determined by the degradation of [14C]valine-labeled long-lived proteins (Fig. 3) and the rate of sequestration of the cytosolic enzyme LDH into autophagic vacuoles (Table I). The increase of autophagic capacity observed in wt-PTEN-overexpressing cells after treatment with IL-13 could be due to the reduction of basal level of phosphatidylinositol (3,4,5)P3 in this cell population. As expected autophagy was reduced by IL-13 in C124S PTEN-expressing cells in the presence of IPTG. More importantly IL-13 also decreased the rate of autophagy in cells expressing the G129E mutant, which has only retained the protein phosphatase activity of PTEN. From these results we
concluded that the lipid phosphatase activity of PTEN is required to control autophagy.

Fig. 3. The phosphatidylinositol phosphatase activity of PTEN is required to control autophagy in HT-29 cells. HT-29-Lac-represser-expressing cells containing IPTG-inducible pOPRSVI/MCS PTEN cDNA expression constructs (His-tagged wt-PTEN, C124S, and G129E PTEN mutants) were radiolabeled with 0.2 μCi/ml \[^{14}C\]valine for 16 h in complete medium containing 5 mM IPTG and chased for 4 h in nutrient-free medium containing 5 mM cold valine and 5 mM IPTG. When used 3-MA (10 mM) and IL-13 (30 ng/ml) were present during the chase period. The values reported are the means of three independent experiments ± S.D.

3.3. Activation of Akt/PKB Negatively Regulates the Autophagic Signalling

To demonstrate the role of Akt/PKB in the control of autophagy, HT-29 cells were stably transfected with the HA-tagged constitutively active form of Akt/PKB (MyrPKB) or the HA-tagged dominant negative mutant of Akt/PKB (kdPKB), and expression of both proteins was detected using a monoclonal antibody raised against the HA-tag and a polyclonal anti-Akt/PKB antibody (Fig. 4A). Moreover, the constitutive active form of Akt/PKB was operative as determined by the analysis of the phosphorylation of GSK-3β using a polyclonal phospho-GSK-3β antibody, a direct substrate of Akt/PKB [24]. As shown in Fig. 4B, GSK-3β was phosphorylated in cells transfected with the MyrPKB construct, whereas phosphorylation of GSK-3β was not detected in cells transfected with the kdPKB construct or in control cells. Autophagic parameters (proteolysis, see Fig. 4C, and the rate of LDH sequestration, see Table I) were dramatically decreased in MyrPKB-expressing cells when compared with control cells. By contrast, 3-MA-sensitive autophagy was up-regulated in kdPKB-expressing cells, demonstrating that activation of Akt/PKB negatively regulates the autophagic signalling pathway.
### Table I
Autophagic sequestration of LDH in HT-29 cell populations

<table>
<thead>
<tr>
<th>Transfected construct</th>
<th>Sequestration of LDH&lt;sup&gt;a&lt;/sup&gt; %/b</th>
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<tr>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>Control&lt;sup&gt;b&lt;/sup&gt; + IL-13</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Control&lt;sup&gt;b&lt;/sup&gt; + 3-MA</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>wt-PTEN&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td>wt-PTEN&lt;sup&gt;c&lt;/sup&gt; + IL-13</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>wt-PTEN&lt;sup&gt;c&lt;/sup&gt; + 3-MA</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>C124S PTEN&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>C124S PTEN&lt;sup&gt;c&lt;/sup&gt; + IL-13</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>G129S PTEN&lt;sup&gt;c&lt;/sup&gt; + 3-MA</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>G129E PTEN&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>G129E PTEN&lt;sup&gt;c&lt;/sup&gt; + IL-13</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>G129E PTEN&lt;sup&gt;c&lt;/sup&gt; + 3-MA</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Control&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>Control&lt;sup&gt;d&lt;/sup&gt; + 3-MA</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>MyPKB</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>MyPKB + 3-MA</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>kdPKB</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>kdPKB + 3-MA</td>
<td>1.8 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are the mean ± S.D. (n = 3).
<sup>b</sup> Empty pOPRS VI/MCS operator vector.
<sup>c</sup> Cells are treated with 5 mM IPTG for 16 h before and during LDH sequestration measurement.
<sup>d</sup> Empty pCMV6 vector.
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Fig. 4. Activation of Akt/PKB negatively regulates the autophagic pathway. A, HT-29 cells were transfected with HA-tagged pCMV6 Akt/PKB expression constructs (constitutive active form of Akt/PKB (M^PKB)) or dominant negative mutant of Akt/PKB (^PKB)). HA-tagged pCMV6 with no insert was used as control (vector). Levels of transfected Akt/PKB proteins were measured by Western blot using an anti-HA antibody (top) and pan-Akt/PKB antibody (bottom). Note that this latter antibody recognizes both endogenous and HA-tagged Akt/PKB proteins. B, measurement of Akt/PKB activity. Levels of phospho-GSK-3β and total GSK-3β were measured by Western blot using an anti-phospho-GSK-3β antibody and a pan-GSK-3β antibody. C, measurement of [¹⁴C]valine long-lived protein degradation in cells transfected without (vector) or with Akt/PKB construct in the presence (+) or in absence (−) of 10 nM 3-MA was performed as described in the legend of Fig. 3. Data are the means of three independent experiments ± S.D.

4. Discussion

The results presented here demonstrate that the signalling control of autophagy depends upon the activity of the tumor suppressor PTEN. Somatic mutations or deletion of PTEN are frequently observed in a large variety of cancers either at early or late stages of development [15,25]. Similarly to other colon cancer cell lines, HT-29 cells express wt-PTEN [26]. This result is in line with the observation that PTEN's loss of function is not a common event in colorectal cancers [27,28]. Our data may explain why in contrast to most cancer cells, autophagy is not down-regulated in HT-29 colon cancer cells. However, alterations in PTEN expression and function is not the only cause for the low rate of autophagy in cancer cells. Recently, the protein...
Beclin 1 has been reported to stimulate autophagy and to suppress tumorigenesis in breast cancer cells [5]. Interestingly, Beclin 1 interacts with the class III PI 3-kinase in mammalian cells [29], suggesting that it is probably part of the machinery engaged in the formation of autophagic vacuoles. Together these data show that PTEN and Beclin 1, two proteins with tumor-suppressive properties, control autophagy at different levels, i.e. signalling and autophagosome formation, respectively.

The role of PTEN in controlling autophagy is dependent upon its lipid phosphatase activity, which antagonizes the inhibitory effect of the PI 3-kinase/PKB pathway on the autophagic sequestration. These results point to a molecular connection existing between autophagy and cell death, because it is now well established that cell survival signalling is operative through the activation of Akt/PKB [30]. Conversely, expression of wt-PTEN or its forced expression counteracts the Akt/PKB-dependent cell survival [26,31]. Although the role of autophagy in the execution of a programmed cell death remains to be elucidated, several studies have pointed to its importance in the type II cell death (autophagic cell death) [32] as well as in the modulation of type I cell death (apoptosis) [33]. The recent demonstration that PTEN is essential for embryonic development [34] and that a high expression of PTEN was detected in different tissues during human development [35] give credit to the idea that autophagy is instrumental during development [36] and could utilize some regulatory mechanisms common with those of apoptosis [37].

The role of Akt/PKB in the negative control of autophagy is compatible with the IL-13 signal transduction pathway and the effect of PTEN. Among the known targets of Akt/PKB several lines of evidence indicate that the kinase target of rapamycin (TOR) occupies a central position in the signalling cascade of autophagy in eucaryotic cells [38-40]. However amino acids, which are physiological inhibitors of autophagy (reviewed in Ref. [41]), activate mTOR by an Akt/PKB-independent mechanism in different models including HT-29 cells [42,43]

Further studies are needed to elucidate the mechanism involved in the control of autophagy by the PI 3-kinase/PTEN/PKB pathway. Nevertheless the data reported here point to the molecular connection between the control of a major catabolic route and that of a signalling pathway frequently altered in human cancers.

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References


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