Synergy in tumor suppression by direct interaction of Neutral Endopeptidase with PTEN

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Summary

We show in this study that endogenous NEP and PTEN associate in cells directly through electrostatic interactions between a highly basic residue stretch in the intracellular domain of NEP and the major phosphorylation site in PTEN's tail. NEP binds and engages in higher order complexes both phosphorylated and unphosphorylated PTEN. NEP recruits PTEN to the plasma membrane and enhances its stability and phosphatase activity. As a result, an enzymatically inactive NEP mutant preserves the ability to bind PTEN, inactivates the Akt/PKB kinase, and partially suppresses the growth of PC cells. This study demonstrates a molecular cooperation between NEP and PTEN tumor suppressors in which NEP constitutively recruits and activates PTEN to inhibit the PI3K/Akt oncogenic pathway.

Introduction

Alterations in neuropeptide regulation contribute to the development and progression of prostate cancer (PC) (Hansson and Abrahamsson, 2001; Nelson and Carducci, 2000). Neuropeptides such as neurotensin, bombesin, and endothelin-1 (ET-1) bind to G protein-coupled receptors (GPCR) and initiate signaling pathways that stimulate cell growth (Rozengurt, 1998). Neutral endopeptidase 24.11 (NEP, neprilysin, CD10) is a 90–110 kDa zinc-dependent metallopeptidase that cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates multiple physiologically active peptides, including bombesin and ET-1 (Kenny, 1993; Shipp et al., 1991). NEP is a type II integral membrane protein, containing an inverted membrane orientation and possessing an extracellular carboxyl terminus that contains an active catalytic domain (Kenny, 1993). It is a member of the M13 family of zinc peptidases, which also includes endothelin converting enzymes (ECE-1 and ECE-2), KELL, and PEX (see Turner et al., 2001, for review). A variety of tissues normally express NEP, including prostatic epithelial cells.

We have been investigating the role of NEP in prostate cancer (PC), first reporting decreased NEP expression in androgen-independent PC cells but not androgen-dependent LNCaP cells and in tumor cells of metastatic PC tissue specimens from patients with androgen-independent but not androgen-dependent PC (Papandreou et al., 1998). Lack of NEP expression has also been observed in primary PCs (Chu and Arber, 2000; Freedland et al., 2003). Together, these studies suggest that NEP loss may contribute to the development and progression of PC.

The biological and regulatory effects of NEP had been presumed to result entirely from the catalytic inactivation of neuropeptide substrates (Shipp and Look, 1993). Recently, however, we have shown that NEP can regulate cell migration via mechanisms both dependent and independent of its catalytic function (Sumitomo et al., 2001). We also showed that catalytically active NEP inhibits neuropeptide-mediated activation of the insulin growth factor-1 receptor (IGF-1R) and the resulting downstream activation of the PI-3 kinase and Akt/PKB kinase (Sumitomo et al., 2000). PI-3 kinase generates phosphatidylinositol-3,4,5-

SIGNIFICANCE

Neutral endopeptidase (NEP, CD10) is a cell surface enzyme expressed by prostatic epithelial cells that cleaves and inactivates neuropeptides implicated in the growth of androgen-independent prostate cancer (PC). PTEN tumor suppressor, a lipid phosphatase that antagonizes phosphatidylinositol 3-OH kinase (PI3K), is frequently inactivated in PC. Our study provides the first evidence of the cooperation between NEP and PTEN tumor suppressors in the synergistic inhibition of the PI3K/Akt pathway in PC and opens the perspective toward an altered regulation of wild-type PTEN that may be present in a large number of cancers. The novel plasma membrane recruitment mechanism presented here demonstrates a long sought link between PTEN and its lipid substrates and may provide the basis for a targeted cancer therapy.

triphosphate (PIP_3) by phosphorylating phosphatidylinositol-4,5-biphosphate (PIP_2) (see Cantley and Neel, 1999, for review). PIP_3 is a second messenger that activates multiple downstream effectors including Akt/PKB kinase, p70S6-kinase, Tec-family tyrosine kinases, and the small G protein Rac. The Akt/PKB serine threonine kinase is a pivotal molecule for cancer progression that promotes cell growth, cell survival, and cell invasiveness (for review see Hill and Hemmings, 2002).

PTEN phosphatase antagonizes the activity of PI-3 kinase by dephosphorylating PIP_3 to PIP_2 (Maehama and Dixon, 1998). PTEN is a tumor suppressor frequently inactivated in a number of cancers including prostate, brain, and uterine cancer (Cairns et al., 1997; Li et al., 1997; Steck et al., 1997; Tashiro et al., 1997). Its inactivation in tumors results in increased activity of Akt/PKB (Haas-Kogan et al., 1998). PTEN is a 403-residue protein structured in an amino (N)-terminal phosphatase domain and a carboxy (C)-terminal C2 domain that binds phospholipid membranes (Lee et al., 1999). The unstructured C-terminal 50-residue region (tail) contains two PEST sequences involved in the stability of the molecule (Georgescu et al., 1999) and a PDZ (PSD95, Dlg, and ZO1) binding motif. The MAGUK adaptor-type PDZ-domain proteins MAGI2 (Wu et al., 2000a), MAGI3 (Wu et al., 2000b), and hDLG (Huang et al., 2000) were reported to interact via the C-terminal PDZ motif with PTEN. A major phosphorylation site has been mapped to residues S380, T382, and T383 in PTEN tail and shown to modulate the stability and activity of PTEN (Vazquez et al., 2000). If the function of PTEN as phosphoinositide phosphatase is well documented (see Maehama et al., 2001, for review), the regulation of its activity is still poorly understood.

We report here that in addition to the catalytically dependent mechanism of PI-3 kinase inactivation by cleavage of neuropeptides, NEP utilizes a catalytically independent mechanism that involves the direct binding of NEP with the PTEN tumor suppressor. Mutagenesis studies mapped the site of interaction between the two molecules to a positively charged amino acid stretch in the intracellular domain of NEP and the major phosphorylation site in PTEN's tail, thus defining a new modality of interaction with PTEN. NEP recruits endogenous PTEN to the membrane, leading to prolonged PTEN protein stability and increased PTEN phosphatase activity. This results in a constitutive downregulation of Akt/PKB activity and partial suppression of tumor growth for a catalytically inactive NEP mutant that preserves the ability to bind PTEN. The importance of the cooperation between PTEN and NEP for growth suppression was further confirmed in a panel of PC cell lines that consistently showed disruption of the NEP-PTEN pathway.

Results

NEP directly associates with the PTEN tumor suppressor

We previously reported that NEP indirectly inactivates Akt/PKB kinase by cleaving the neuropeptides bombesin and ET-1 (Sumitomo et al., 2001). To determine whether NEP has a direct effect on Akt/PKB, we investigated whether there is a link between NEP and the PTEN tumor suppressor that converges to inactivate Akt/PKB. We performed co-immunoprecipitation experiments in cancer cell lines expressing different combinations of the two proteins (Figure 1A, top panel). Western blot analysis with a PTEN antibody of proteins immunoprecipitated with an NEP antibody showed that PTEN co-immunoprecipitated with NEP in MeWo melanoma cells that express both PTEN and NEP proteins (Figure 1A, bottom panel). There was no complex formation in cells lacking either one of the two proteins, such as LNCaP that do not express PTEN or TSU-Pr1 that lack NEP. The association between PTEN and NEP was confirmed by co-immunoprecipitating NEP with a PTEN antibody (Figure 1A, middle panel). These experiments showed that NEP and PTEN interact in vivo at endogenous protein expression levels.

We next determined whether the catalytic activity of NEP is necessary for the association with PTEN. NEP-deficient TSU-Pr1 PC cells were engineered to inducibly express upon tetracycline removal wild-type NEP (WT-5 cells), catalytically inactive NEP mutant (M-22 cells), truncated NEP mutant lacking its cytoplasmic domain (CM-2 cells), or vector control (TN-12 cells). PTEN co-immunoprecipitated with both wild-type and catalytically inactive NEP, indicating that the interaction is independent of NEP's peptidase activity (Figure 1B). PTEN did not associate with the CM-2 NEP mutant lacking the cytoplasmic domain, implying that the cytoplasmic tail of NEP is necessary for binding PTEN.

Because NEP co-immunoprecipitates with other molecules (Ganju et al., 1996; Sumitomo et al., 2001), we examined whether the interaction between NEP and PTEN is direct. NEP was immunoprecipitated from cells and subjected to Far Western blot analysis with a GST-PTEN probe (Figure 1C). GST-PTEN directly bound filter-immobilized NEP. This direct interaction took place only when the cytoplasmic domain of NEP was intact, confirming that the interaction site with PTEN maps to the cytoplasmic domain of NEP.

The major carboxy-terminal phosphorylation site in PTEN binds to a basic-residue stretch in the cytoplasmic domain of NEP

To identify the region within PTEN that associated with the intracellular domain of NEP, we co-expressed NEP with a series of FLAG-tagged PTEN deletion mutants in 293T cells. Proteins from cell lysates were immunoprecipitated with NEP antibody and immunoblotted with M2 antibody that recognizes FLAG-PTEN proteins (Figure 2A). NEP associated with full-length PTEN, with a truncation mutant lacking the amino terminus (C-term) and with the deletion mutants PTEN-385 and PTEN-398. NEP did not associate with PTEN-379 or with deletion mutants lacking carboxy-terminal residues upstream of 379, indicating that the site of interaction between PTEN and NEP maps to amino acids comprised between 379 to 385 in the carboxy-terminal region of PTEN. Surprisingly, this 6-residue sequence contains a serine/threonine cluster (Ser380, Thr382, Thr383, and Ser385) (Figure 2B) that represents the major phosphorylation site in PTEN (Vazquez et al., 2000). To analyze whether the interaction between PTEN and NEP depends on the phosphorylation status of PTEN, we immunoprecipitated NEP from cell lysates treated with the serine/threonine phosphatase PP2A (Figure 2C). A lower amount of PTEN associated with NEP in cell lysates treated with PP2A than in cell lysates left untreated, suggesting that NEP associates with both phosphorylated and unphosphorylated PTEN. To further assess the importance of the phosphorylation sites for the interaction between NEP and PTEN, a triple PTEN-3A mutant, in which

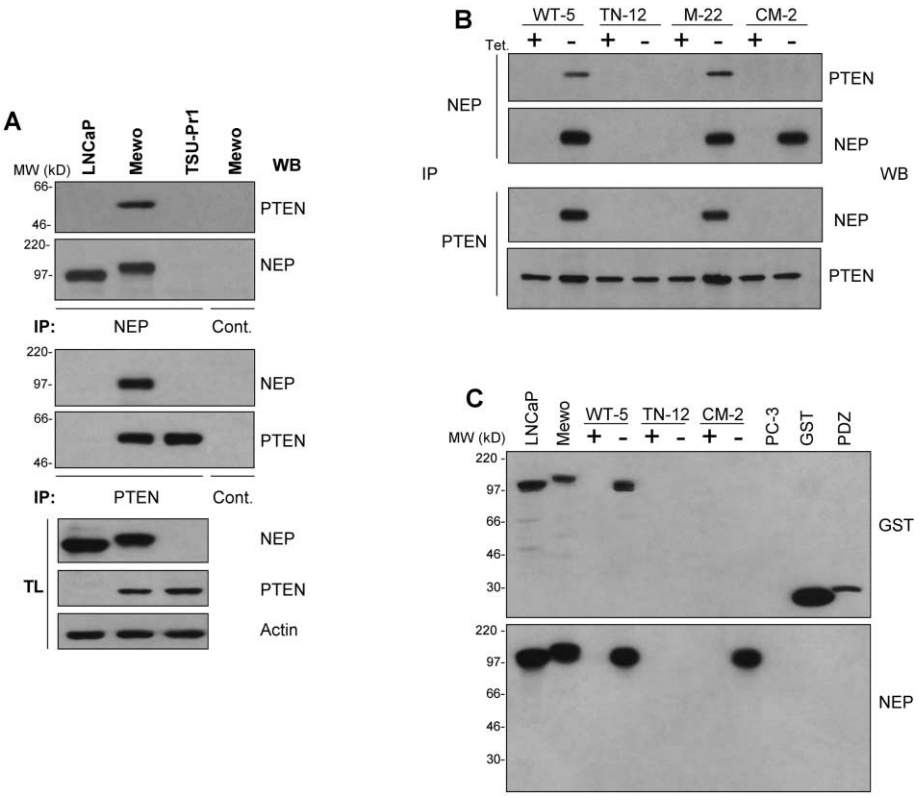

Figure 1. NEP protein co-immunoprecipitates with PTEN protein

A: NEP (upper panel) and PTEN (middle panel) immunoprecipitated with NEP and PTEN mAbs were analyzed by Western blotting with NEP mAb and PTEN pAb. Note NEP and PTEN co-immunoprecipitate in Mewo melanoma cells that express endogenous NEP and PTEN. Mouse IgG was used as negative control (Cont.) for immunoprecipitation. Lower panel shows Western blot of total cell lysates (TL) illustrating endogenous protein levels of NEP and PTEN, with actin control.

B: WT-5, TN-12, M-22, and CM-2 tet-off inducible cells were cultured with (+) and without (−) 1 μg/ml tetracycline. Cell lysates were analyzed as described in **A**. Note PTEN co-immunoprecipitates with both wild-type (WT-5) and catalytically inactive (M-22) NEP, but not with the NEP mutant lacking the cytoplasmic domain (CM-2). TN-12 represents control cells containing identical vectors lacking NEP cDNA.

C: WT-5, TN-12, and CM-2 cells were cultured with (+) and without (−) tetracycline. NEP immunoprecipitated with pAb were Far Western blotted using GST-PTEN probe and anti-GST mAb (upper panel). GST-PTEN bound to NEP-expressing LNCaP and Mewo, and wild-type NEP (WT-5) but not cytoplasmic deleted NEP protein (CM-2). Recombinant GST protein (0.1 μg) was used as a positive control for anti-GST antibody, and the PDZ domain from a protein isolated in yeast two-hybrid screen (M.-M.G., unpublished data) as positive control for GST-PTEN. Lower panel represents the same blot shown in upper panel stripped and reprobed with anti-NEP mAb.

Ser380, Thr382, and Thr383 were changed to alanines (Figure 2B), was co-expressed with NEP (Figure 2C). In this case, there was no association between NEP and the phosphorylation-deficient PTEN-3A mutant. NEP's ability to bind dephosphorylated PTEN but not the PTEN-3A mutant indicates that this binding is indeed restricted to the short sequence of the major phosphorylation site whose integrity in terms of hydrophilicity and negative charge is necessary for NEP binding.

The 6-residue stretch of PTEN responsible for the association with NEP is a highly acidic sequence especially when phosphorylated (Figure 2B). The examination of the NEP cytoplasmic tail that is highly conserved across species revealed a motif of positively charged amino acids near the transmembrane domain (Figure 2D). Mutagenesis of a cluster of three positively charged lysines within the cytoplasmic domain of NEP to glutamine, asparagine, and isoleucine (QNI) blocked NEP's association to PTEN (Figure 2E). These experiments indicated that there is a direct association between PTEN and NEP based on electrostatic interactions between a negatively charged phosphorylation site in PTEN carboxyl terminus and a highly positively charged site located in the cytoplasmic tail of NEP.

NEP associates with both phosphorylated and unphosphorylated forms of PTEN

A previous study using gel filtration fractionation showed that the unphosphorylated form of PTEN is present in rat liver cells in a high molecular weight complex while the phosphorylated form is monomeric (Vazquez et al., 2001). To determine the form of the endogenous PTEN that associates with NEP, we performed gel filtration over a Sephacryl S-300 column with whole-cell lysates from TSU-WT-5 cells cultured in the presence or in the absence of tetracycline (Figure 3A). Similarly to the rat liver cells, which have undetectable expression of NEP protein (Ronco et al., 1988), in TSU-WT-5 cells not expressing NEP, PTEN eluted in two peaks: one at high molecular mass (669 kDa) and one corresponding to the monomeric form (apparent molecular mass 67 kDa). When NEP was expressed in these cells by tetracycline withdrawal, the 67 kDa form of PTEN shifted into a higher molecular weight complex of approximately 200 kDa apparent molecular mass. The apparent molecular mass of this complex corresponds roughly to the sum of the molecular masses of PTEN and NEP, suggesting that the monomeric phosphorylated form of PTEN associated with NEP in cells. Interestingly, the PTEN form present in high molecular weight complexes was also shifted upon NEP expression, suggestive of NEP participation in this complex also. Induced NEP eluted in a plateau covering the monomeric to low order complexed forms and a strong peak corresponding to the high order complex (more than 669 kDa) in which dephosphorylated PTEN cofractionated. Although induced NEP levels in TSU-WT-5 cells are comparable to endogenous levels, we also confirmed that the induced NEP elution pattern was identical to the endogenous NEP pattern from LNCaP cells (not shown).

To confirm that the data found by gel filtration experiments indicating that both phosphorylated and unphosphorylated forms of endogenous PTEN associate with NEP, we quantified

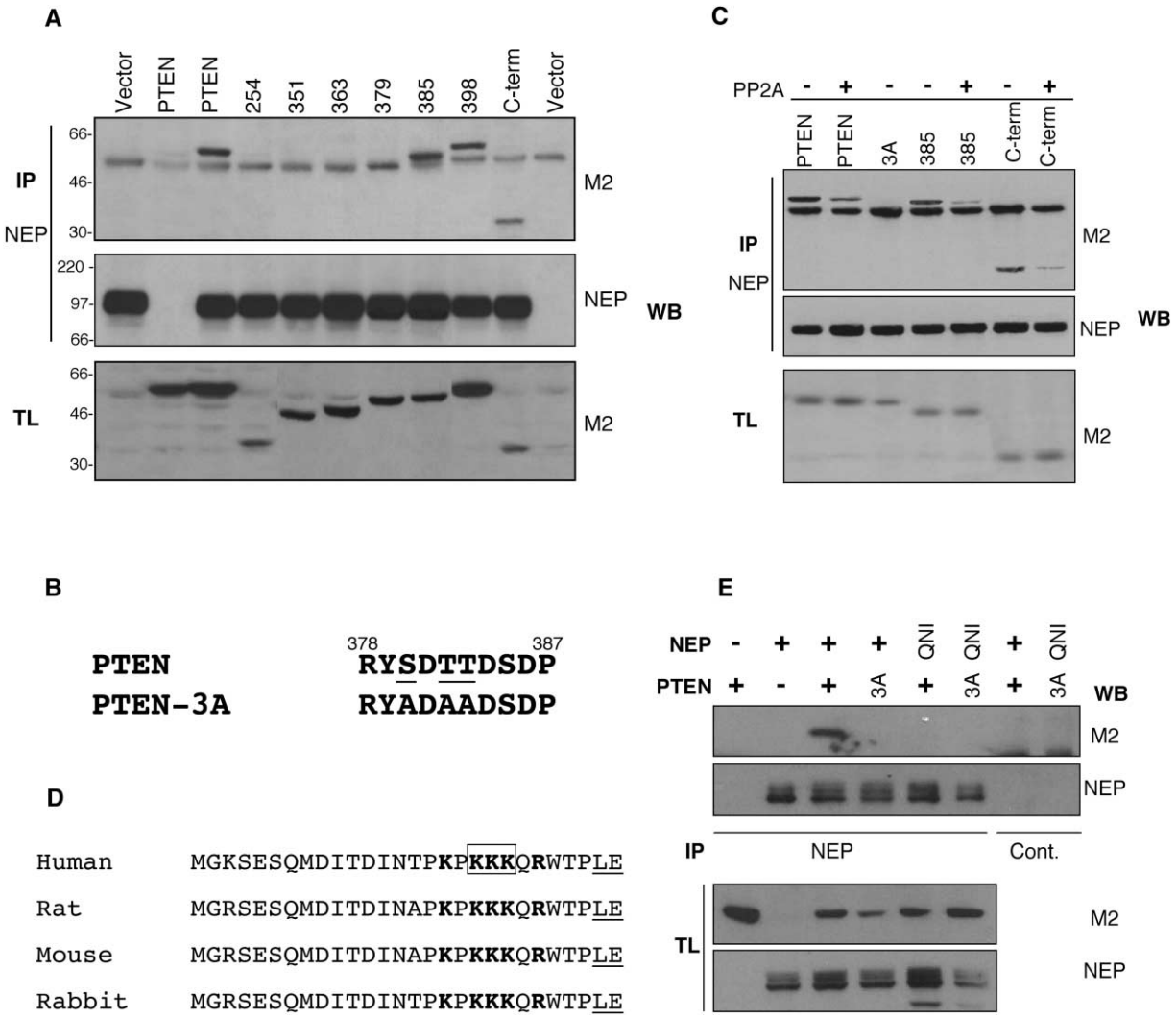

Figure 2. A basic motif in NEP cytoplasmic domain binds to major phosphorylation site in PTEN's tail

A: Cell lysates from 293T cells transiently co-expressing FLAG-tagged PTEN wild-type and mutants together with wild-type NEP protein were immunoprecipitated with NEP mAb and immunoblotted with the M2 antibody recognizing FLAG-tagged proteins (upper panel) and NEP mAb (middle panel). Note that PTEN and NEP co-immunoprecipitate only in cells expressing full-length PTEN protein or PTEN containing carboxy-terminal residues upstream of amino acid 379. The lower panel shows the expression level of the PTEN mutants in total lysates (TL) (50 μg of protein) from transiently transfected 293T cells analyzed by immunoblotting with the M2 antibody.

B: The PTEN sequence containing a serine/threonine cluster (Ser380, Thr382, Thr383, and Ser385) representing the major phosphorylation site in PTEN. Underlined residues were changed to alanine in PTEN-3A mutant.

C: Identical experiment as in **A** using selected PTEN constructs and PTEN-3A mutant. Cell lysates were incubated with (+) or without (−) the serine/threonine protein phosphatase PP2A. Note the lack of association between NEP and PTEN-3A mutant and the decreased association between NEP and PTEN proteins from lysates treated with PP2A.

D: NEP cytoplasmic tail containing a highly conserved positively charged amino acid motif (in bold) near the transmembrane domain (underlined). The lysine triplet mutated to QNI in the NEP-QNI mutant is boxed.

E: Cell lysates from 293T cells transiently transfected with vectors expressing wild-type (+) NEP or PTEN, and mutated PTEN-3A and NEP-QNI were immunoprecipitated with anti-NEP mAb and Western blotted with M2 recognizing FLAG-tagged PTEN and with NEP mAb. Negative control (Cont.) mouse IgG was used for immunoprecipitation. The expression level from total cell lysates (TL) of the transfected proteins is shown in the lower panels. Note NEP and PTEN only associate in cells expressing wild-type NEP and PTEN proteins.

the amount of phosphorylated PTEN that associated with immunoprecipitated NEP relative to the amount of the respective endogenous PTEN forms from the total cell lysates of TSU-WT-5 cells (Figure 3B). To this purpose, we probed the same blot with an antibody specifically recognizing phosphorylated PTEN on residues Ser380, Thr382, and Thr383, followed by an antibody recognizing total (both phosphorylated and unphosphorylated) PTEN. A lower amount of phosphorylated PTEN (approximately 10% of phosphorylated PTEN input) than of total PTEN (approximately 40% of total PTEN input) co-immunoprecipitated with NEP, indicating that both phosphorylated and unphosphorylated forms of PTEN associate with NEP.

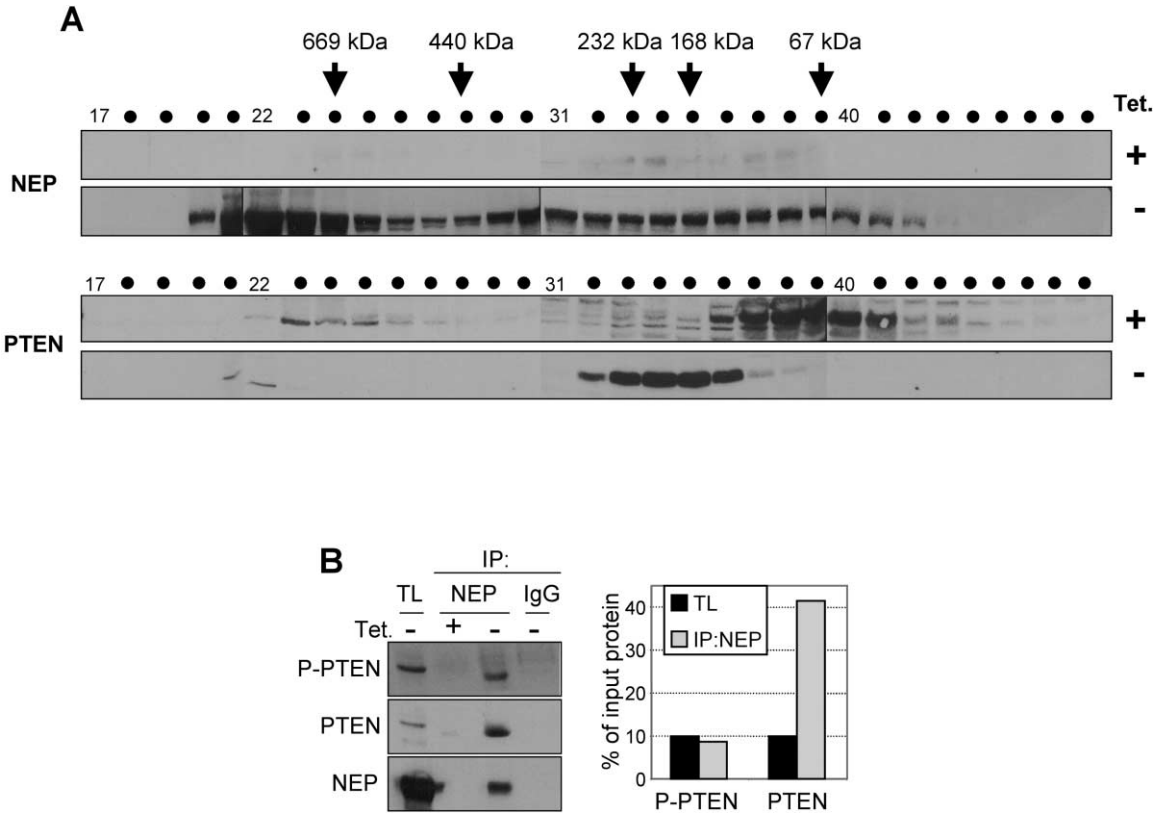

Figure 3. Both phosphorylated and dephosphorylated forms of PTEN interact with NEP

A: TSU-WT-5 cells cultured with (+) and without (−) tetracycline were fractionated on an S-300 gel filtration column and immunoblotted with anti-NEP antibody, stripped, and reprobed with anti-PTEN antibody. Note the shift with the apparent molecular mass of NEP of both PTEN peaks, monomeric phosphorylated and complexed dephosphorylated, in the presence of NEP.

B: Total cell lysates (TL) representing 10% of the input protein used for immunoprecipitation and NEP immunoprecipitates (IP) from TSU-WT-5 cells treated as in **A** were analyzed by Western blotting with phospho(Ser380/Thr382/Thr383)-PTEN (P-PTEN), total PTEN, and NEP antibodies. Mouse IgG was used as negative control for immunoprecipitation. The graph shows the quantification of the interaction between NEP and phosphorylated PTEN or total PTEN, relative to the 10% input from total cell lysates.

NEP recruits PTEN to the plasma membrane and increases its stability and phosphatase activity

NEP is a transmembrane molecule, localized at the plasma membrane. To examine the consequence of NEP-PTEN association on PTEN intracellular distribution, WT-5 and CM-2 cells that inducibly express full-length NEP or the cytoplasmic-deleted NEP mutant, respectively, were labeled for immunofluorescent analysis (Figure 4). In cells that did not express NEP or that expressed the cytoplasmic-deleted NEP mutant that did not interact with PTEN, PTEN had a perinuclear intracellular localization. Following expression of NEP, a fraction of PTEN was recruited at the plasma membrane, where it colocalized with NEP (Figure 4).

The carboxy-terminal region of PTEN has been implicated in the stability of the molecule (Georgescu et al., 1999). To examine the possible impact of the NEP-PTEN association on PTEN stability, we analyzed the protein expression level of PTEN in cell lysates by using the inducible system of NEP expression (Figure 5A). In WT-5 or M-22 cells that express NEP proteins able to interact with PTEN, the expression levels of PTEN were increased. In CM-2 cells that express the cytoplasmic-deleted NEP mutant that does not interact with PTEN, PTEN levels were unchanged. To determine if the increased stability of PTEN in NEP-expressing cells is a consequence of reduced degradation, we performed pulse-chase assays in WT-5 cells in the presence or in the absence of tetracycline (Figure 5B). The half-life of PTEN was longer in induced NEP-positive WT-5 cells ($T_{1/2} \geq 6$ hr) compared with noninduced NEP-negative WT-5 cells ($T_{1/2} = 3$ hr). These results suggested that in cells where PTEN associates with NEP, PTEN levels are increased by reduced degradation of the protein.

To confirm that NEP controls the levels of PTEN in cells that endogenously express both proteins, we depleted NEP by introducing small interfering RNA (siRNA) for NEP into MEWO melanoma cells (Figure 5C). NEP-specific enzyme activity consistently decreased by 25% in MEWO NEP-siRNA-transfected cells as compared to controls (data not shown). Western blot analysis of MEWO cell lysates also showed an approximately 25% decrease in NEP protein levels in cells receiving siRNA (Figure 5C). In these cells, PTEN protein levels decreased more than 30% as compared to controls, while there was no effect on the amount of the β-actin protein. These data indicated that the loss of NEP in MEWO cells has a significant impact on PTEN levels.

Finally, to determine whether the stabilization and plasma membrane recruitment modified the enzymatic activity of PTEN,

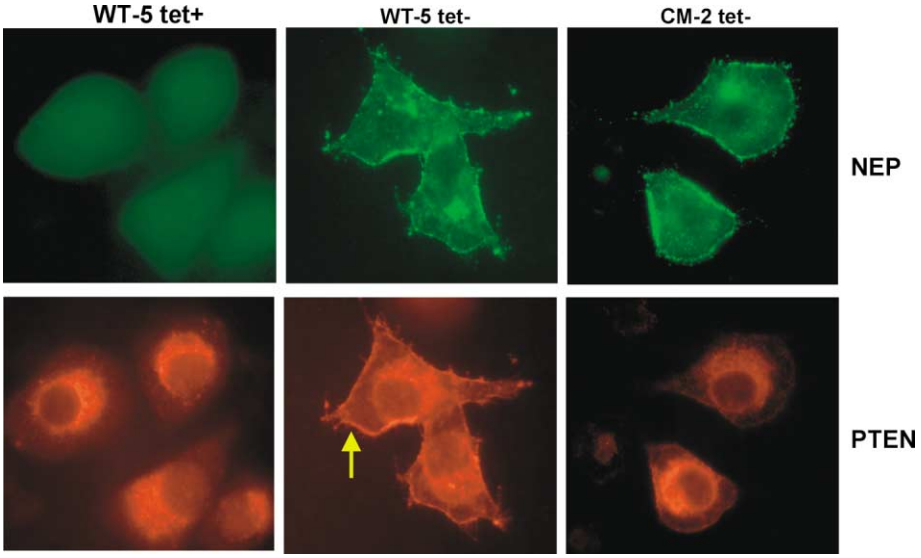

Figure 4. NEP recruits PTEN to the plasma membrane

WT-5 cells stably expressing wild-type NEP and CM-2 cells expressing cytoplasmic-deleted mutant of NEP were cultured with (+) and without (−) tetracycline and stained with anti-NEP antibody (upper panel) and anti-PTEN antibody (lower panel). Note perinuclear intracellular localization of PTEN in cells lacking NEP (left) or full-length NEP (right) in contrast to cells expressing NEP (center) in which a fraction of PTEN was recruited at the plasma membrane, where it colocalized with NEP (arrow).

an in vitro phosphatase assay was performed on PTEN immunoprecipitated from cells expressing full-length or cytoplasmic-deleted NEP mutant (Figure 5D). The lipid phosphatase activity of PTEN toward PIP_3 was significantly increased in the presence of full-length active or inactive NEP (WT-5 and M-22 cells), but not in the presence of the cytoplasmic-deleted NEP mutant (CM-2 cells) or in control TN-12 cells. These results showed that in the presence of NEP, the phosphatase activity of PTEN is increased.

The NEP-PTEN cooperation is necessary for full suppression of Akt/PKB activation and cell growth

PTEN inactivates Akt/PKB by dephosphorylating PIP_3 to PIP_2 (Stambolic et al., 1998). Because the phosphatase activity of PTEN was increased in the presence of both catalytically active and inactive NEP, we expected that catalytically inactive NEP would also inactivate Akt/PKB through its direct interaction with PTEN. To test this, we explored Akt/PKB phosphorylation by Western blot analysis in cells induced to express wild-type or catalytically inactive NEP (Figure 6A). WT-5, TN-12, and M-22 cells were cultured in serum-free media overnight, resulting in minimal levels of phosphorylated Akt/PKB (not shown) (Sumitomo et al., 2000). Cells were then treated with 10 nM ET-1 for 10 min, which induced Akt/PKB phosphorylation in cells lacking NEP expression (lanes 1, 2, 5, 6, and 7). Wild-type NEP inhibited Akt/PKB phosphorylation (lane 3). In the same conditions, the catalytically inactive NEP mutant M-22 also inhibited Akt/PKB phosphorylation, although not completely (lane 8). Similarly, treatment of cells expressing wild-type NEP with CGS24592 NEP-specific peptidase inhibitor failed to restore the complete activation of Akt/PKB (lane 4). To assess the influence of NEP on Akt/PKB activity in the presence of a PI-3 kinase agonist that is not susceptible to degradation by NEP, we performed similar experiments using IGF-1 (1 ng/ml). Expression of NEP in WT-5 and M-22 cells similarly inhibited Akt/PKB phosphorylation, whereas no effect was seen in CM-2 cells that express NEP lacking the cytoplasmic domain (Figure 6B). These experiments indicated that NEP is able to inactivate Akt/PKB through both indirect catalytically dependent and direct catalytically independent mechanisms. The last mechanism most likely relies on the direct interaction between NEP and PTEN, resulting in enhancement of PTEN's phosphatase activity.

We further investigated the molecular requirements for NEP growth suppression by testing the ability to suppress cell growth of wild-type, catalytically inactive, and cytoplasmic domain mutant NEP (Figure 6C). Both the catalytically inactive and the cytoplasmic domain NEP mutants conferred intermediate growth suppression to cells between that of wild-type NEP and of vector control. These data were in correlation with the Akt/PKB inactivation data and indicated that the growth suppression by NEP required both an intact extracellular peptidase activity and the intracellular domain of NEP that recruits PTEN to the plasma membrane.

To confirm that the cooperation between NEP and PTEN is important for PC cell growth, we analyzed the expression of NEP and PTEN as well as the downstream activity of Akt/PKB in four PC cancer cell lines (Figure 7). The melanoma cell line Mewo that expresses both NEP and PTEN was taken for comparison. None of the PC cell lines expressed simultaneously PTEN and NEP, suggesting that these molecules belong to the same pathway. One cell line, PC-3, was deficient in both proteins. Akt/PKB was phosphorylated and thus activated in all the PC cell lines, in spite of the presence of wild-type PTEN in two of them, TSU-Pr1 and DU145. However, the highest levels of Akt/PKB phosphorylation were observed in PC-3 cells that lacked both molecules and the lowest levels in Mewo cells that expressed both molecules. These observations are highly suggestive for synergy between NEP and PTEN for the full suppression of Akt/PKB activity in cancer cells.

Discussion

PTEN is an important tumor suppressor whose inactivation frequency in cancers is ranked second after p53 tumor suppressor inactivation (Cantley and Neel, 1999). Increasing evidence suggests that NEP peptidase can function as a tumor suppressor. Loss of NEP expression has been reported in a variety of solid tumors (Nanus et al., 1997). More specifically, NEP expression is

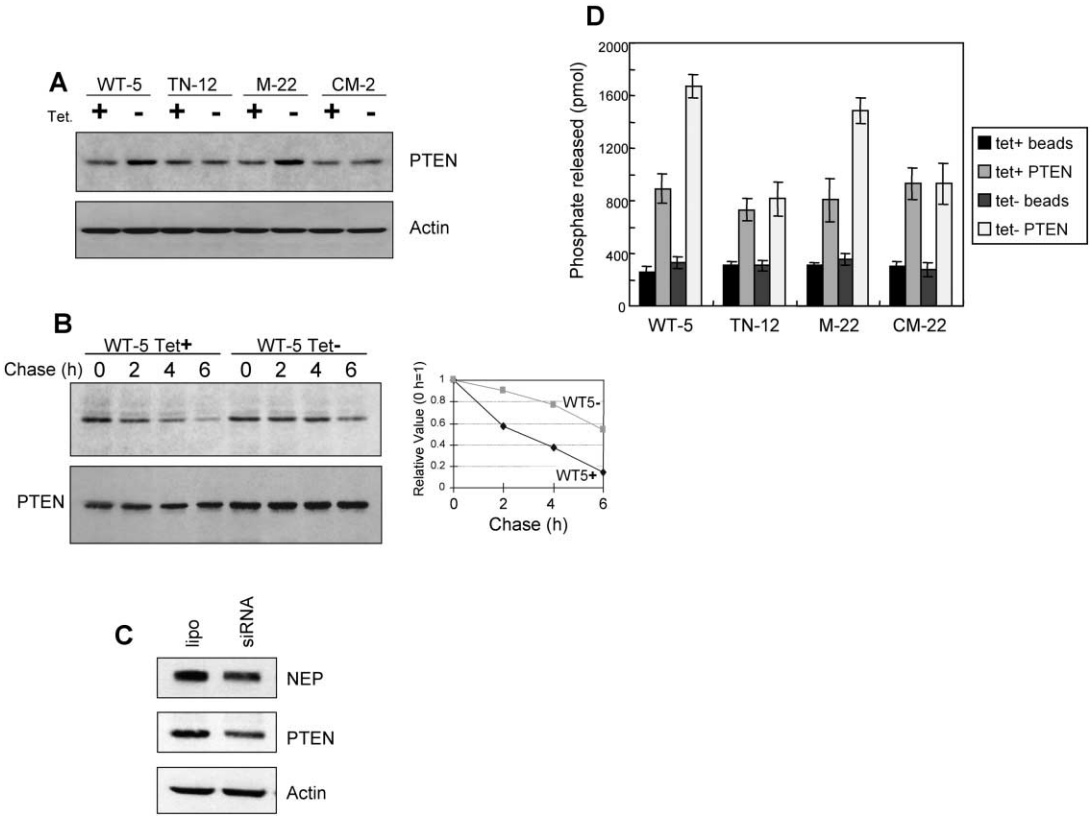

Figure 5. NEP stabilizes PTEN protein expression and increases PTEN lipid phosphatase activity

A: PTEN protein expression levels were determined by Western analysis using a PTEN mAb in cell lysates from WT-5, TN-12, M-22, and CM-2 cells in the presence (+) or absence (−) of tetracycline. Note increased PTEN protein in cells expressing NEP protein containing intact cytoplasmic domain in contrast to empty-vector control cells (TN-12) or to cells expressing the cytoplasmic-deleted NEP mutant (CM-2).

B: The turnover of PTEN was evaluated by pulse-chase assay as described in Experimental Procedures. The half-life of PTEN was prolonged in NEP-positive WT-5 cells under tetracycline-free (Tet−) medium compared with WT-5 cells cultured in the presence of tetracycline (Tet+) that suppresses NEP expression. The relative intensity of each band obtained by autoradiography was measured by ImageJ software and represented in the graph.

C: Partial NEP gene silencing analysis shows decrease in endogenous PTEN protein levels in MEWO cells treated with lipofectamine and NEP siRNA in comparison with control cells treated with lipofectamine alone (lipo). No effect was noted on β-actin protein. These data are representative of multiple independent experiments.

D: In vitro phosphatase assay on PTEN immunoprecipitated from WT-5, TN-12, M-22, and CM-2 cells in the presence and absence of tetracycline (tet). Bars represent standard deviations from triplicate measurements. Note significant increase in PTEN lipid phosphatase activity in cells expressing intact NEP cytoplasmic domain following tetracycline removal compared to control TN-12 or cytoplasmic-deleted NEP mutant CM-2.

diminished in a significant percentage of primary and metastatic PCs (Chu and Arber, 2000; Papandreou et al., 1998; and our unpublished data), most likely by reduced transcription (Dhanasekaran et al., 2001). Reduction in NEP activity in lung cancer cells has been directly implicated in the development of lung cancer (Bunn et al., 1998; Cohen et al., 1996; Shipp et al., 1991). The mechanism by which the loss of NEP contributed to tumor progression was believed to rely solely on the inability of the cell to inactivate mitogenic peptides (Shipp and Look, 1993). Our recent work including the data from this study demonstrated that both the catalytic function and the ability of the intracellular domain of NEP to participate to protein complexes are required for tumor growth suppression (Figure 8). We previously identified two distinct mechanisms by which NEP exerts a tumor-suppressive effect. A first mechanism consists of the catalytic inactivation of neuropeptide substrates that signal through G protein-coupled receptors to activate Src kinase (Sumitomo et al., 2000). Src directly activates FAK, which regulates cell migration (Sumitomo et al., 2000), or induces ligand-independent activation of IGF-1R and subsequent Akt/PKB activation that regulates cell growth (Sumitomo et al., 2001). A second mechanism consists of the direct association between NEP and Lyn kinase, which recruits the p85 regulatory subunit of PI-3 kinase and competitively blocks the interaction of the PI-3 kinase with FAK, leading to decreased cell migration (Sumitomo et al., 2000). In the current study, we have identified a third mechanism of NEP antitumor action by its direct association with the PTEN tumor suppressor. NEP recruits PTEN to the plasma membrane and increases its stability and phosphatase activity. Functionally, this results in the inactivation of Akt/PKB and growth suppression by a catalytically inactive mutant of NEP that preserves its ability to bind PTEN.

The association between NEP and PTEN opens new insights for PTEN regulation, an area where little is known. PTEN function is to inactivate the PI-3 kinase pathway. This pathway is active, as measured by Akt/PKB activity and/or phosphorylation, in

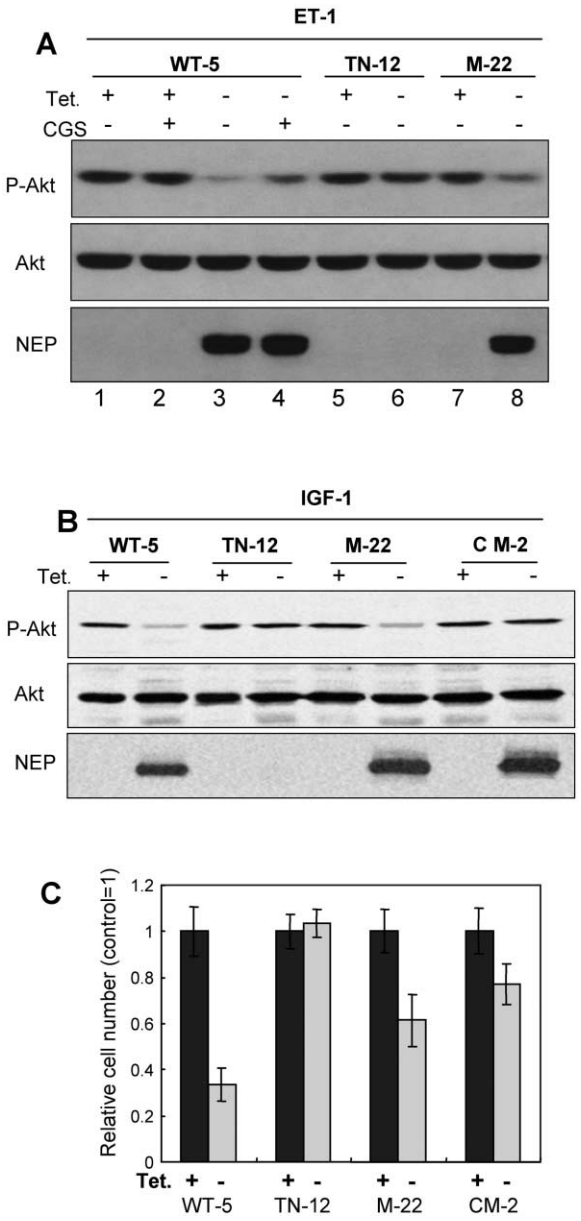

Figure 6. Catalytically inactive NEP partially inactivates Akt/PKB and tumor growth

A: WT-5, TN-12, and M-22 cells were cultured overnight in serum-free media in the presence (+) or absence (−) of tetracycline. 10 nM ET-1 was added for 10 min prior to cell lysis. Cell lysates were Western blotted with pAbs to phospho-Akt (P-Akt) and total Akt. Where indicated, cells were also treated with 10 nM of the NEP enzymatic inhibitor CGS24592 for 2 hr prior to treatment with ET-1. Note the decrease in phospho-Akt in both M-22 cells expressing catalytically inactive full-length NEP and in WT-5 cells expressing wild-type NEP that had its catalytic activity inhibited by CGS24592.

B: WT-5, TN-12, M-22, and CM-2 cells were cultured as indicated in **A** and treated with 1 ng/ml of IGF-1, which is not a substrate for NEP. Note the comparable decrease in phospho-Akt in both WT-5 and M-22 cells expressing full-length wild-type or catalytically inactive NEP, respectively, but not in CM-2 cells expressing NEP lacking the cytoplasmic domain.

C: Equal numbers of WT-5, TN-12, M-22, and CM-2 Tet-off inducible cells were plated and grown in medium supplemented with 10% FCS with (+) or without (−) tetracycline (Tet.). After 5 days, the number of cells was counted. All experiments were performed in triplicate and error bars represent standard errors. p values estimate differences between cells grown with and without tetracycline: WT-5 cells, $p = 0.0062$; M-22 cells, $p = 0.0193$; CM-2 cells, $p = 0.0421$. The presented data are representative of two independent experiments with similar results.

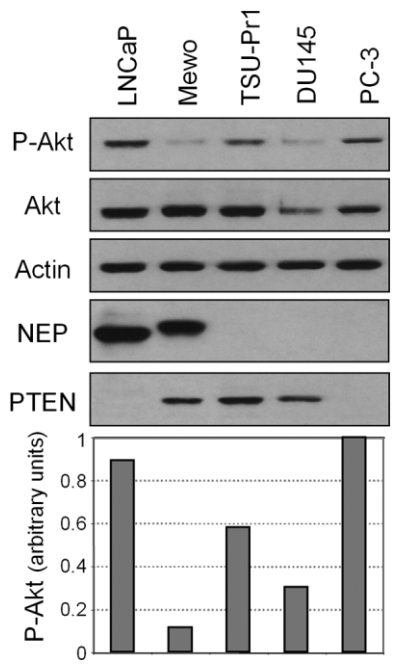

Figure 7. Synergy between NEP and PTEN for Akt/PKB inactivation in PC cell lines

Equal numbers of PC cells grown in medium supplemented with 10% FCS were lysed, and 50 μg proteins from total lysates were analyzed by Western blot with the indicated antibodies. The graph shows the level of phosphorylated Akt/PKB normalized to the total Akt/PKB level. Note highest or lowest levels of phospho-Akt in cells that lack or express both NEP and PTEN molecules, respectively.

tumors or cancer cell lines lacking PTEN (Haas-Kogan et al., 1998). However, we observed in this study that there is moderate Akt/PKB activation in some cancer cell lines that expressed wild-type PTEN but lacked NEP. Our interpretation is that in these cancer cell lines that lack NEP, the regulation of PTEN is impaired. This assumption is based on the data showing clear alteration of PTEN expression levels after partial gene silencing of NEP by siRNA in cells expressing both proteins. Moreover, the induced expression of NEP in one of the NEP-deficient cell lines regulated PTEN's activity by multiple mechanisms, resulting in a constitutive activation of PTEN that increased significantly the threshold for Akt/PKB activation by neuropeptides and growth factors. The mobilization of monomeric PTEN in complexes, as shown by gel filtration experiments, may have increased the stability of PTEN. The recruitment of PTEN to the cell membrane from a perinuclear pool most likely played an important role in PTEN's activation, and we have previously shown that the artificial targeting of PTEN to the membrane increases its tumor suppressor function (Georgescu et al., 2000). Recent studies have shown by density gradient centrifugation that both NEP and PTEN partly cofractionated with caveolin-1-enriched membrane microdomains or rafts (Caselli et al., 2002; Riemann et al., 2001). Moreover, both caveolin, the main structural protein of rafts, and cholesterol that is enriched in rafts were functionally associated with PC progression (Tahir et al., 2001; Zhuang et al., 2002). As the membrane distribution of PTEN appears crucial for PTEN's regulation, we are currently investigating whether caveolin-1 is present in the NEP-PTEN

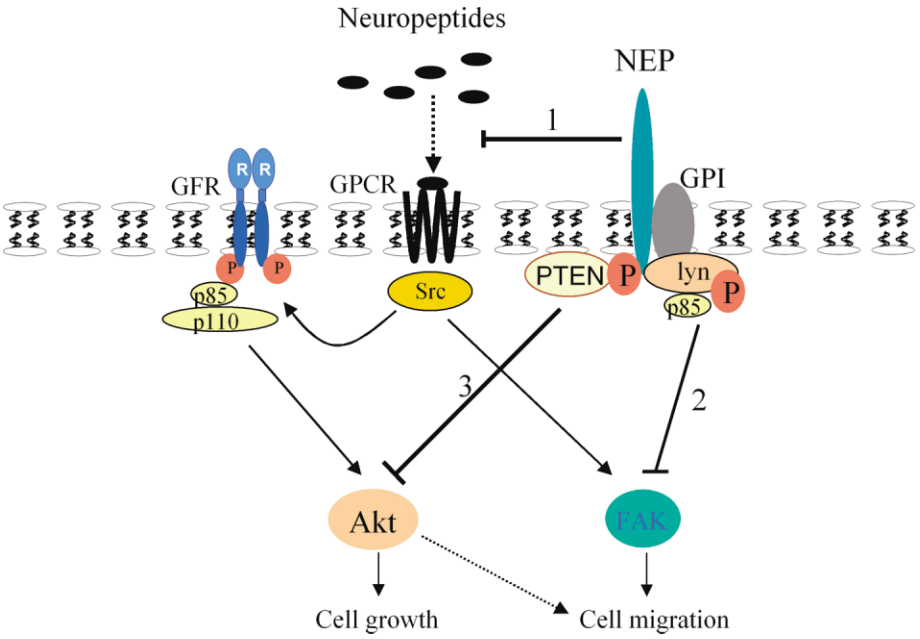

Figure 8. Mechanistic model of NEP's inhibition of cell growth and migration

NEP has at least three independent regulatory functions that can affect PC cells: (1) NEP catalytically inactivates a variety of neuropeptide substrates such as bombesin and ET-1, thereby blocking neuropeptide actions, including Src phosphorylation and association with FAK, normally resulting in cell migration, and neuropeptide-mediated ligand-independent activation of growth factor (GF) receptors such as IGF-IR; (2) NEP indirectly associates with p85 regulatory subunit of the PI-3 kinase through Lyn kinase, thereby inhibiting the interaction of PI-3 kinase with FAK; and (3) NEP directly associates with and stabilizes PTEN tumor suppressor, leading to dephosphorylation of PIP3 to its less active form, and resulting in negative regulation of downstream cell growth and cell survival pathways such as those regulated by Akt/PKB kinase.

gel filtration peaks in order to define what form of PTEN colocalized with NEP at the plasma membrane.

A very remarkable finding was the critical localization of the binding sites on PTEN and NEP. These sites likely mediate an electrostatic interaction between the two molecules and consist of well-defined motifs limited to very short amino acid sequences. We could disrupt the reciprocal interaction by changing as few as three residues within each motif. This finding has prompted us to perform database searches for detecting these motifs in other proteins that may associate with either NEP or PTEN and potentially modulate the NEP-PTEN interaction.

Most interestingly, the NEP-interacting motif on PTEN superposes with the major phosphorylation site in PTEN (illustrated in Figure 2B). PTEN's phosphorylation at this site increases the stability but decreases PTEN's activity (Vazquez et al., 2000) and the interaction of the neighboring PDZ binding motif with proteins from the MAGUK family (Tolkacheva et al., 2001; Vazquez et al., 2001). We showed that the phosphorylation regulates the interaction between PTEN and NEP as well, but in a different manner compared with the MAGUK proteins. As long as the major phosphorylation site was intact, both phosphorylated and unphosphorylated forms of PTEN bound to NEP. It thus appears that the phosphorylated form of PTEN may not necessarily be the inactive PTEN form but, in certain circumstances, it may be physiologically active. Recent studies indicate that protein kinase CK2 (formerly casein kinase II) catalyzes phosphorylation of PTEN (Miller et al., 2002; Torres and Pulido, 2001). The NEP cytoplasmic tail contains two consensus recognition sequences for CK2, and similar to PTEN, NEP is phosphorylated by CK2 (Ganju et al., 1996). While phosphorylation of PTEN by CK2 is believed to inhibit PTEN function (Miller et al., 2002; Torres and Pulido, 2001), these studies on PTEN have been performed in cell lines lacking NEP expression such as U87-MG glioblastoma cells. Our studies suggest that NEP also associates with the phosphorylated PTEN when expressed within the same cell. The effects of CK2 phosphorylation of NEP on NEP function are unknown. CK2 is elevated in proliferating and neoplastic tissues (Pinna, 1997), and conceivably CK2 may regulate the interaction of PTEN and NEP through phosphorylation.

We have shown here that NEP and PTEN are on the same pathway for PC growth suppression. However, PTEN has been implicated in a large array of cancers and noticeably, tumors in which PTEN is frequently inactivated arise in tissues with highest expression of NEP (Ronco et al., 1988). In addition to PC, a striking example where both NEP and PTEN are inactivated in a high percentage of cases is small cell lung cancer (Shipp et al., 1991; Yokomizo et al., 1998), and it would be of considerable importance to characterize the NEP-PTEN pathway in this setting as well. Our study provides the first evidence of the cooperation between NEP and PTEN tumor suppressors in PC and opens the perspective toward an altered regulation of wild-type PTEN that may be present in a large number of cancers.

Experimental procedures

Cell culture

LNCaP, TSU-Pr1, DU145, PC-3 PC, and 293T cells were maintained as described (Georgescu et al., 1999; Sumitomo et al., 2000). Mewo melanoma cells were maintained in modified Eagle's medium containing 10% FCS. TSUGK27 is a TSU-Pr1 cell line stably transfected with pGK hygro and PUHD 15-1 containing the coding sequence for the tet repressor adjacent to the coding sequence for the C-terminal domain of VP16 (named the tet-responsible transactivator or tTA), downstream of the hCMV promoter (Gschwend et al., 1997). This cell line was cultured in media containing 150 μg/ml of hygromycin and 1 μg/ml of tetracycline. Reagents used include protein phosphatase $2A_1$ (PP2A) (Calbiochem-Novabiochem Ltd., La Jolla, CA) and CGS24592 NEP competitive inhibitor (Novartis Pharmaceutical). The following antibodies were used: mouse monoclonals (mAbs) to NEP (J5, Beckman Coulter, Fullerton, CA; NCL-CD10-270, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK; FR4D11, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), to PTEN (A2B1, Chemicon, International, Inc., Temecula, CA), and to FLAG-tag (M2, Sigma-Aldrich, St. Louis, MO); rabbit polyclonals (pAbs) to NEP (5B5, Arris Pharmaceutical Corp., San Francisco, CA), to PTEN (FL-403, Santa Cruz; Anti-PTEN, Upstate Biotechnology, Inc., Lake

Placid, NY), to Akt (H-136, Santa Cruz), to phospho(Ser380/Thr382/Thr383)-PTEN and phospho(Ser473)-Akt (Cell Signaling Technology, Inc., Beverly, MA), and to actin (Chemicon).

Plasmid construction and gene transfer

NEP wild-type TSU-GK27-NEP (WT-5), control TSU-GK27-Neo (TN-12), and catalytically inactive NEP TSU-GK27-mNEP (M-22) cells were generated and maintained as previously described (Dai et al., 2001; Sumitomo et al., 2000). NEP cytoplasmic domain deletion mutant (pTRE/NEPdcyto) was constructed by site-directed mutagenesis (QuickChange; Strategene, La Jolla, CA) and used to generate inducible TSU-GK27-CM cells (CM-2). The $K^{12}KK^{14}$ to QNI mutant (pTRE/NEPQNI) in the NEP cytoplasmic domain was obtained by PCR-based site-directed mutagenesis using a set of primer including point mutations (underlined) as follows: 5′-CT GAT ATC AAC ACT CCA AAG CCA CAG AAC ATA CAG CGA TGG ACT CCA CTG GAG-3′ and 5′-CTC CAG TGG AGT CCA TCG CTG TAT GTT CTG TGG CTT TGG AGT GTT GAT ATC AG-3′. This mutant was used to generate inducible TSU-GK27-QNI cells (QNI-12) (Papandreou et al., 1998). Mutant NEP-QNI cDNA was also cloned in pCISChENK vector for transient expression. N terminus FLAG-tagged PTEN wild-type and C-terminal deletion mutants ending at codons 254, 351, 363, 379, 385, and 398 were previously described (Georgescu et al., 1999). The truncation mutant starting at amino acid 201 and conserving the C terminus of PTEN (C-term) and the PTEN phosphorylation mutant disrupting the major phosphorylation site by changing Ser380, Thr382, and Thr383 to alanines (PTEN-3A) were similarly cloned in pFLAG-CMV-2 vector.

Protein extraction, immunoprecipitation, and Western blot analysis

Cells were lysed in 1 ml of RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1.2% aprotinin, 5 μM leupeptin, 4 μM antipain, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na_3VO_4), and 300–500 μg proteins were incubated 1 hr to overnight with 1–4 μg primary antibody, and then for 1 hr with 40 μl protein G-sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C. Immunoprecipitates were washed with RIPA buffer, resuspended in 2× Laemmli sample buffer, resolved on 8% or 10% SDS-PAGE, and transferred to nitrocellulose. Western blot analyses were performed as described (Sumitomo et al., 2000). For Far Western analysis, membranes were incubated with 1 μg/ml recombinant GST-PTEN fusion protein (Upstate) for 2 hr and then Western blotted using the anti-GST mAb (Santa Cruz, 1:2000) and anti-mouse secondary antibody. All experiments were performed at least twice using different cell lysates with similar results. For experiments using PP2A, cell lysates were incubated with 10 ng PP2A for 30 min prior to immunoprecipitation.

Protein fractionation

Fifteen T150 flasks of NEP WT-5 cells cultured with or without tetracycline were harvested and lysed in TNN buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA [pH 8.0]) as described (Vazquez et al., 2001). Cell lysates were passed through 0.8, 0.45, and 0.22 min filters, applied to a Sephacryl S-300 column (Amersham), and eluted with phosphate-buffered saline at 4°C. Molecular weights of eluted fractions were calculated using standard proteins supplied by Amersham. Individual eluted fractions (0.5 ml) were precipitated with cold acetone, dried, resuspended in Laemmli sample buffer, resolved on a 7% SDS PAGE, and transferred to nitrocellulose. Western blotting was performed as described above using anti-NEP (Novocastra Laboratories) and anti-PTEN (Upstate) antibodies.

Immunofluorescent microscopy

Cells were fixed with 3.7% formaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and soaked in PBS containing 1% BSA (BSA-PBS) and 5% skim milk for 30 min. Cells were then incubated with anti-NEP mAb (NCL, 1:100) and anti-PTEN pAb (FL-403, 1:100), diluted with BSA-PBS for 1 hr, washed 3 times for 5 min with PBS, and incubated with secondary antibodies FITC-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG (Santa Cruz, 1:200) for 1 hr. Labeled cells were observed using a fluorescent microscope, Axiovert 35 (Carl Zeiss Inc., Thornwood, NY).

Pulse-chase assay

Equal number of cells were cultured in RPMI1640 lacking methionine for 30 min, in the same media containing 300 μCi/ml [^{35}S]methionine (NEN) for 1 hr, washed with PBS, and then incubated in RPMI supplemented with 10% FCS and 0.15 mg/ml nonradioactive methionine for various time periods. Cells were lysed in RIPA buffer. For immunoprecipitation, 300 μg proteins from lysates were incubated for 1 hr with 1 μg of anti-PTEN antibody, then for 1 hr with 40 μl protein G-sepharose beads (Amersham) at 4°C. Immunoprecipitates were collected by centrifugation at 6000 × g for 1 min, washed with RIPA buffer, and resuspended in 2× Laemmli sample buffer. Samples were resolved on an 8% SDS-PAGE and transferred to nitrocellulose. Autoradiography and immunoblotting were performed using the same membrane. The relative intensity of each band obtained by autoradiography was measured by ImageJ software.

PTEN phosphatase assay

PTEN phosphatase assays were performed as described (Georgescu et al., 1999). The release of phosphate from the water-soluble diC_8-PIP_3 substrate (Echelon) was measured in a colorimetric assay by using 100 μl Malachite Green solution (Upstate) in accordance with the instructions of the manufacturer. Data are representative of one experiment performed in triplicate on at least two separate occasions.

NEP siRNA assay

Mewo cells were plated at a concentration of 3×10^5 cells/ml in 6-well plates (2 ml/well) and were transfected the following day with 300 pmol/well of NEP siRNA (smartpool from Dharmacon Research, Inc., Lafayette, CO) using lipofectamine Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 72 hours after transfection, cells were harvested and lysed in cold 0.1% CHAPS buffer (10 mM HEPES [pH 7.4], 5 mM DTT, 2 mM EDTA), supplemented with protease inhibitors. Equal amounts of total protein (20 μg) were separated on 8% polyacrylamide gels and transferred to nitrocellulose membrane. Western blotting was performed using antibodies to NEP (NCL-CD10-270), PTEN (A2B1), and actin.

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