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Novel p104 protein regulates cell proliferation through PI3K inhibition and $p27^{Kip1}$ expression

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The protein p104 was first isolated as a binding partner of the Src homology domain of phospholipase Cγ1, and has been shown to associate with p85α, Grb2. The ectopic expression of p104 reduced cellular growth rate, which was also achieved with the overexpression of only the proline-rich region of p104. The proline-rich region of p104 has been found to inhibit the colony formation of platelet-derived growth factor BB-stimulated NIH3T3 cells and MCF7 cancer cells on soft agar. Mutagenesis analysis showed that the second and third proline-rich regions are essential for growth control, as well as for interaction with p85α. Overexpression of p104 increased the level of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, and inhibited the activity of phosphoinositide 3-kinase (PI3K). In summary, p104 interacts with p85α and is involved in the regulation of $p27^{Kip1}$ expression for the reduction of cellular proliferation. [BMB reports 2010; 43(3): 199-204]

INTRODUCTION

Thirteen mammalian phospholipase C (PLC) isozymes have been cloned and classified into six types: β, γ, δ, ε, ζ, and η (1). Two regions of high sequence homology, designated X and Y, constitute the PLC catalytic domain for all isozymes, and only the γ-type PLC is known to have two Src homology 2 (SH2) domains and one SH3 domain.

It is well known that SH domains are involved in protein-protein interactions during intracellular signaling. The SH2 and SH3 domains both play important roles in controlling various enzymatic activities, such as gene expression, cytoskeletal localization of specific growth factor receptors, and the regulation of many other intracellular signals (2). Various proteins that contain SH2 domain recognize and bind to specific phosphorylated Tyr-containing peptide motifs, whose specificities are determined by the surrounding amino acids. Such binding is important for the maintenance of intracellular signaling machinery. SH3 domains recognize proline-rich motifs within their interacting proteins, and play important roles in oncoproteins, although SH2 domains are more common. For example, SH3 domains occur in oncoproteins such as phosphoinositide 3-kinase (PI3K), SRC and Crk (3, 4), and the SH3 domain of Grb2 has essential antiproliferative and anticancer activities (5). An SH domain-specific mitogenic response was elicited by microinjection of catalytically inactive PLCγ mutants into NIH3T3 cells, suggesting that SH domains of PLCγ itself appear to play a critical role in mitogenic signaling, independent of its PLC activity (6).

The $p27^{Kip1}$ protein was initially identified as part of a complex with cyclin E/cyclin-dependent kinase 2 (CDK2) during cell-cycle arrest induced by contact inhibition, transforming growth factor β (TGFβ) or lovastatin treatment (7). The activation of CDK2 by association with cyclin E is a key event driving the cell cycle from G_1 into S phase. CDK2 is inhibited when bound to $p27^{Kip1}$, and the downregulation of $p27^{Kip1}$ expression correlates with the mitogen-induced reentry of quiescent cells into the cell cycle (8), indicating that $p27^{Kip1}$ is one of the cyclin-dependent-kinase inhibitor (CKIs).

In this report, we identified p104 as a novel protein that interacts with p85α, Grb2, and PLCγ1. Given that p104 contains the recently reported KASH (Klarsicht/ANC-1/Syne-1 homology) domain, it may be classified as a member of the Syne/ANC-1 family (9). p104 is implicated in the regulation of cell-proliferation based on its binding to p85α via its putative SH domain-binding motifs and its regulation of $p27^{Kip1}$ expression.

RESULTS

Isolation of p104 as a novel binding partner of PLCγ1 and PI3K

To identify the proteins that physically interact with the SH domains of PLCγ1, we generated polyclonal rabbit antibodies directed against proteins that could interact with the SH2-SH2-SH3 domains of PLCγ1. Using these antisera, we expressed and screened a mouse brain cDNA library and isolated several positive clones (10). Three clones among these contained partial fragments of a novel gene, and a clone containing the entire open reading frame was isolated by further screening. This novel gene was predicted to encode a 104 kDa protein of 899

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amino acids long and was designated p104 (Supplemental data, Fig. 1; GenBank accession no. FJ848978).

A database search showed that the p104 protein shares significant homology with a human protein encoded by cDNA KIAA1011, which was recently classified as a member of the Syne/ANC-1 family. The p104 protein contains five spectrin repeats and several putative SH binding sites: one binding site for the amino-terminal SH2 domain of PLCγ1, one binding site for the SH2 domain of SEM-5/Grb2, three general SH3-binding motifs, and two binding sites for the SH2 domain of PI3K

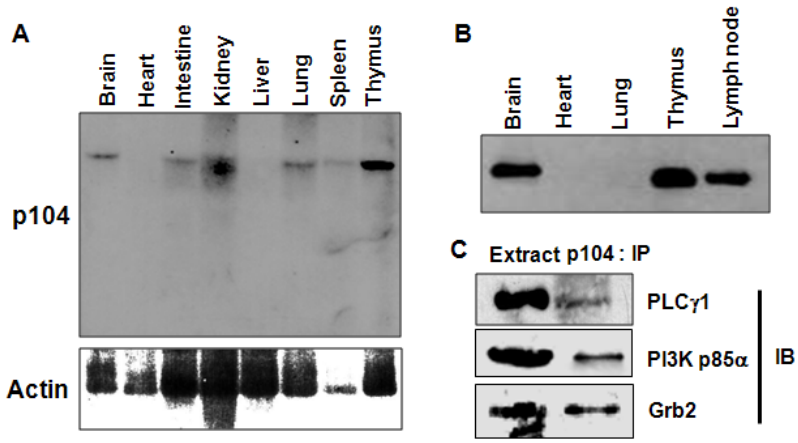

Fig. 1. Expression patterns of p104. (A) Northern blot analysis. Total RNA was isolated from several mouse tissues. RNA (50 μg) was resolved by electrophoresis, transferred to a nitrocellulose membrane, and then probed with an α-^{32}P-labeled DNA fragment of *p104*. (B) Western blot analysis. Cell extracts from different mouse tissues (100 μg/lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). p104 was detected by immunoblotting with anti-p104 antiserum. (C) The interactions of p104 with PLCγ1, p85α and Grb2. Cell extracts were immunoprecipitated with anti-p104 antiserum and the immunocomplexes were separated by 10% SDS-PAGE. PLCγ1, p85α and Grb2 were detected by immunoblotting with the respective antibodies.

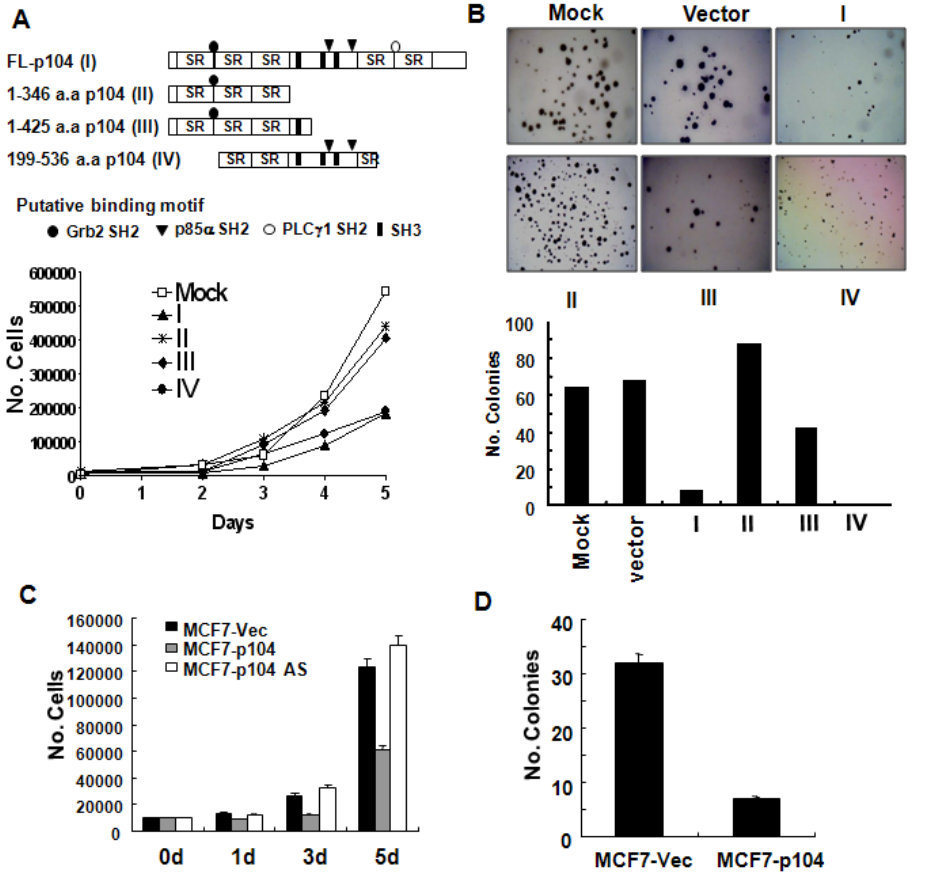

Fig. 2. Effect of p104 on cellular proliferation. (A) Reduction in cell growth by overexpression of p104. Full-length *p104* gene was cloned into pcDNA3.0 (I). Full-length p104 cDNA was digested and subcloned to generate constructs expressing the N-terminal half of the protein without PXXP motifs (II), a fragment with only the first PXXP motif (III), and a fragment with three PXXP motifs (IV). These constructs were introduced into NIH3T3 cells. After clonal selection, 4×10^3 cells were plated onto 30 mm plates. Cell numbers were counted at the indicated times. (B) Effects of several truncated p104 protein fragments on the anchorage-independent growth of NIH3T3 cells. Cells were transfected with *p104* or its derivatives as in (A), and cells were grown in soft agar in the presence of PDGF BB (200 ng/ml) for two weeks. The colonies were visualized with NBT staining and photographed (200× magnification). Colonies with a diameter of more than 100 μm were counted. (C) Growth inhibition of MCF7 human breast cancer cells by p104 overexpression. After mock, wild-type or antisense *p104* constructs were transfected into MCF7 cells, the numbers of cells were counted for five days. (D) Anchorage-dependant growth of MCF7 cells by p104. Vector- or *p104*-transfected MCF7 cells were cultured in soft agar medium for 10 days and the number of colonies was counted as in (B).

p85α (Supplemental data, Fig. 1) (11). The p104 transcript, approximately 4.5 kb in length, is expressed in various murine tissues, including the brain, kidney, lung, and thymus, but not in the heart or liver (Fig. 1A). Consistent with the Northern blot results, abundant levels of p104 protein (~104 kDa) were detected in the brain and thymus (Fig. 1B). However, p104 protein was not detected in the lungs despite the transcript being expressed. The interaction of p104 with PLCγ1 was confirmed by coimmunoprecipitation, as shown in Fig. 1C. p104 also co-precipitated with the p85α subunit of PI3K as well as Grb2 from the lysate of NIH3T3 cells.

Overexpression of p104 inhibits cell growth

The ectopic expression of p104 was investigated in NIH3T3 cells since p104 was shown to interact with p85α, a protein involved in the cell proliferation pathway. The overexpression of full-length p104 reduced the growth rate by approximately 60% compared with that of control cells (Fig. 2A). We next examined whether the putative SH domain-binding motifs of p104 contribute to the observed reduction in cell proliferation. Transfection of the three amino-terminal spectrin repeats of p104 (amino acids 1-346), which contain a putative Grb2 SH2 domain-binding motif, or the amino terminus with the first putative SH3-binding PXXP motif (amino acids 11-425) reduced cell proliferation by only 20%, whereas transfection of a construct encoding all three PXXP motifs and two p85α SH2 domain-binding sites (amino acids 199-536) reduced cellular proliferation similar to full-length p104 (Fig. 2A). PDGF BB has been reported to increase the transformation of NIH3T3 cells through the actions of PI3K and PLCγ1 (3). Therefore, the capacity of NIH3T3 cells to form colonies was investigated in semisolid agar containing 200 ng/ml PDGF BB after transfection with the modified forms of p104. Full-length p104, as well as a fragment containing amino acids 199-536, reduced colony formation in soft agar as cell proliferation was reduced (Fig. 2B). To test the effect of p104 on cancer cell proliferation, the sense or antisense *p104* gene was stably transfected into MCF7 breast cancer cells. Overexpression of p104 led to an approximately 50% reduction in the growth rate of MCF7 cells, whereas antisense *p104* gene had no effect (Fig. 2C). MCF7-p104 cells cultured in soft agar were checked for their capacity for anchorage-independent growth. It was found that colony formation of MCF7-p104 cells decreased by approximately 80% compared with that of vector-transfected MCF7 cells (Fig. 2D). These results suggest that p104 is involved in the regulation of cell proliferation through its SH domain-binding motifs, and that overexpression of p104 led to a reduction in the cellular growth rate.

PXXP motifs of p104 are essential for regulation of cell proliferation through interaction with p85α and inhibition of PI3K activity

Because the three proline-rich motifs of p104 appear to contribute to the regulation of cell growth (Fig. 2), the functional importance of these motifs was tested by substituting both proline residues of each PXXP motif with alanine. As shown in Fig. 3A, the ability of p104 to reduce cell proliferation was diminished by mutation of the second (2mP) or third (3mP) motif, both of which are conserved in the human p104 homologue, KIAA1011. We further examined which PXXP motif(s) is

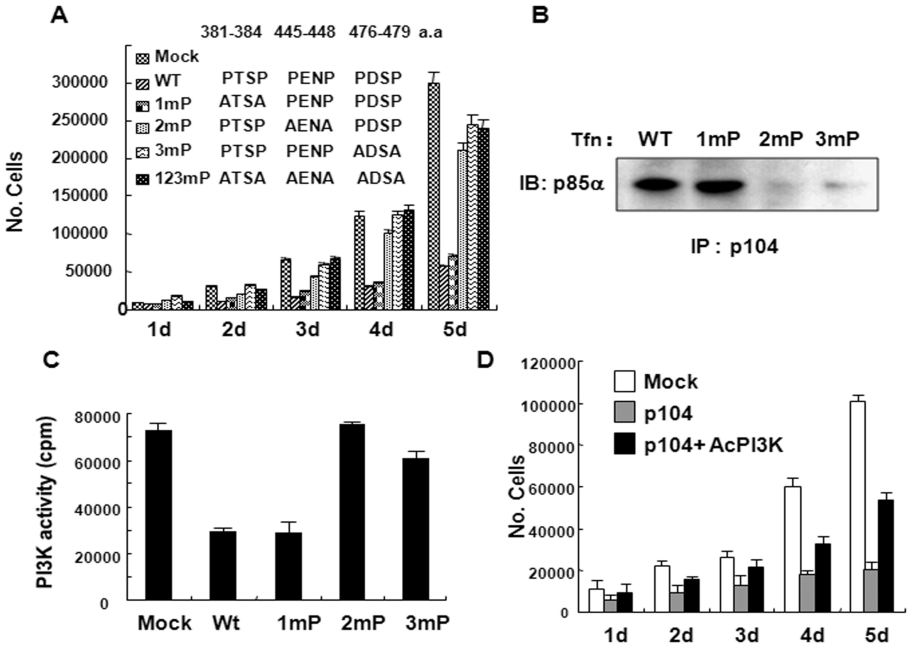

Fig. 3. The second and third PXXP motifs of p104 are essential for its function. (A) Roles of the PXXP motifs in cell proliferation. Several PXXP mutant forms of *p104* were generated by site-specific mutation of proline amino acids to alanine and transfected into NIH3T3 cells. Cells stably expressing the p104 mutant forms were established and their growth rates were checked for five days. (B) Interaction of the PXXP mutant forms of p104 with p85α. Extracts from NIH3T3 cells expressing the PXXP mutants were immunoprecipitated with anti-p104 antiserum, followed by Western blotting with anti-p85α antibody. (C) p104 overexpression reduces PI3K activity. PI3K was isolated from NIH3T3 cells transfected with various PXXP mutant forms using anti-p85α antibody. PI3K activity was measured with phosphoinositide as the substrate. (D) Effect of constitutively active PI3K in NIH3T3 cells expressing p104. A constitutively active form of PI3K was transfected into p104-overexpressing NIH 3T3 cells, and the cell numbers were counted every day for five days.

essential for the interaction of p104 with p85α. p85α co-immunoprecipitated with wild-type (WT) or the first motif-mutated p104 (1mP), but not with 2mP- or 3mP-mutated p104, indicating that the second and third PXXP motifs are essential for the interaction of p104 with p85α (Fig. 3B).

To assess how the activity of PI3K is affected upon interaction with p104, WT and mutated forms of p104 were transfected into NIH3T3 cells and PI3K activity was measured. As shown in Fig. 3C, the overexpression of the p104 WT or 1mP-mutant forms reduced the PI3K activity, whereas the 2mP and 3mP p104 mutants did not. This result suggests that p104 reduces cell proliferation by downregulating PI3K activity. To confirm this, we transfected an amino-terminal myristoylated form of PI3K p110, the constitutively active catalytic subunit of PI3K, into *p104*-transfected NIH3T3 cells (12). The co-transfection of constitutively active PI3K partially reversed the reduction in proliferation of *p104*-transfected NIH3T3 cells (Fig. 3D). These data also confirm the role of p104 in reducing the proliferation of NIH3T3 cells by modulating the activity of PI3K.

Induction of $p27^{Kip1}$ by p104

It has been suggested that PI3K and Akt/PKB activation may promote cell entry into S phase through the phosphorylation and inactivation of AFX, thereby reducing $p27^{Kip1}$ expression (13, 14). Since p104 interacts with p85α (Fig. 1C, 3B) and reduces the activity of PI3K upon overexpression (Fig. 3), the relationship between p104 and $p27^{Kip1}$ was investigated. The level of p104 mRNA was relatively low in growing NIH3T3 cells, but it was increased to a maximum level by serum deprivation within 12 h. When the serum was replenished, expression of p104 mRNA diminished to a basal level after 9 h (Fig. 4A). The protein level of p104 decreased after the addition of serum, as was the transcript levels. This reduction seemed to be in good correlation with the level of $p27^{Kip1}$, which decreased at similar time points (Fig. 4B). In addition, overexpression of p104 in NIH3T3 cells using the retrovirus-based vector, pLXSN, led to an increase in $p27^{Kip1}$ expression (Supplemental data, Fig. 2).

Since p104 is highly expressed in the thymus (Fig. 1B), we checked by Western blot analysis which type(s) of T cells expresses p104 in the thymus using D9, S49.1, and EL4 cell lines. As shown in supplemental data Fig. 3, p104 protein was specifically detected only in mature T-cell lymphoma EL4 cells (15), and not in D9 or S49.1 cells from an immature T-cell thymoma. This suggests that p104 is expressed only in mature, differentiated T cells. To address the relationship between p104 and $p27^{Kip1}$, EL4 cells were treated with phorbol myristate acetate (PMA) plus ionomycin, which mimics cell proliferation signaling via the T-cell receptor (TCR)/CD3 complex (16). A reduction in p104 levels was detected 2 h after treatment and it had completely disappeared after 3 h. Interestingly, the level of $p27^{Kip1}$ paralleled the pattern of p104 expression in EL4 cells (Fig. 4C). However, neither the level of

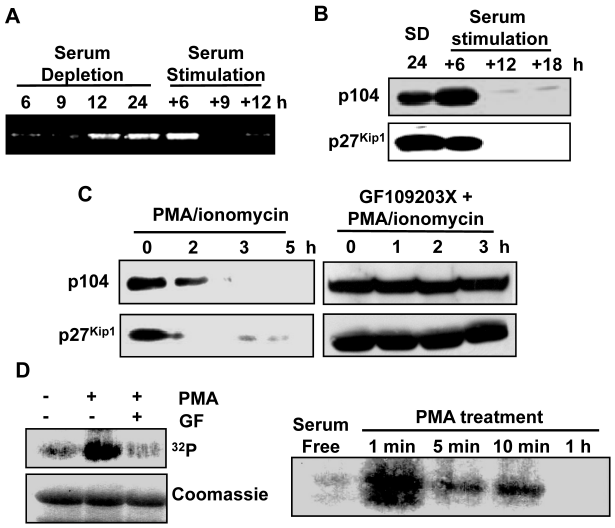

Fig. 4. Relationship between p104 and $p27^{Kip1}$. (A) Modulation of p104 transcripts following serum depletion and replenishment. Levels of p104 transcripts were checked by RT-PCR at the indicated time points under serum depletion and replenishment after 24 h of serum depletion. (B) Patterns of p104 and $p27^{Kip1}$ protein expression with or without serum. After serum depletion for 24 h, NIH3T3 cell extracts were prepared in the presence of serum for 6, 12 and 18 h. Total protein (50 μg) was used for Western blot analysis with the appropriate antibodies. (C) Reductions in p104 and $p27^{Kip1}$ induced by PMA/ionomycin in EL4 cells. After EL4 cells were pretreated with or without GF109203X (5 μM) for 15 min, the cells were treated with PMA/ionomycin (5 μM) for 1, 2 or 3 h. The levels of p104 and $p27^{Kip1}$ after treatment with PMA/ionomycin were determined by Western blotting. (D) Phosphorylation of p104 by treatment with PMA/ionomycin. GST-fused p104 was isolated and incubated with the NIH3T3 cell lysate along with [γ-^{32}P] ATP and the indicated chemicals. After affinity purification on a Glutathione Sepharose 4B column, the separated proteins were subjected to SDS-PAGE. The radiolabeled fusion proteins were detected by autoradiography, and the amount of loaded protein was confirmed by gel staining with Coomassie Blue (left panel). After NIH3T3 cells were cultured in isotope-labeled, orthophosphate- containing medium, the cells were treated with PMA/ionomycin for the indicated times, and p104 was immunoprecipitated with a specific antibody. The phosphorylation state was detected by SDS-PAGE and autoradiography (right panel).

p104 nor that of $p27^{Kip1}$ changed upon treatment with GF109203X, a well known protein kinase C (PKC) inhibitor, before the addition of PMA and ionomycin. Furthermore, we were able to observe the phosphorylation of p104 by PMA plus ionomycin treatment, which was diminished by co-treatment with GF109203X (Fig. 4D). Taken together, these data suggest that the downregulation of p104 is somehow related to the $p27^{Kip1}$-mediated downregulation of EL4 cell proliferation.

DISCUSSION

p104 was first isolated as a SH domain-binding protein of PLCγ1. Despite efforts, no biological significance could be

found between PLCγ1 and p104. We performed several experiments to unveil the role of p104 in cell signaling and did find that p104 interacts with p85α through its two SH3-binding motifs (Fig. 1C, 3B), as well as the SH domain of PLCγ1. We showed that the interaction of p104 with p85α plays an important role in modulating the activity of PI3K and regulating cell proliferation (Fig. 3C, D). The proline-rich domain of p104 is especially capable of inhibiting the anchorage-independent cell growth of PDGF-treated fibroblasts and cancer cells (Fig. 2). The second and third PXXP motifs of p104, which also participate in interaction with p85α, appear to be essential for the inhibition of cell proliferation (Fig. 3A, B). It appears that interactions between p104 and p85α are probably involved in the observed decrease in colony formation as well as cell-cycle regulation, although the exact mechanism is unknown. The levels of p104 transcript and protein expression were increased in NIH3T3 cells by serum depletion and then reduced again upon replenishment (Fig. 4A, B). The above data suggest that p104 potentially regulates the cell cycle, although the exact mechanism is still under investigation. One possible explanation is that p104 acts as a scaffolding protein that sequesters signal transduction molecules such as PLCγ1 and PI3K from their receptors and inhibits their activation. Upon extracellular signaling, several signal transduction molecules move to their phosphorylated receptors and are activated. These activated molecules activate secondary signaling pathways, such as the PKC pathway, which is involved in phosphorylation and modulation of p104. This hypothesis is supported by the phosphorylation and disappearance of p104 upon addition of the PKC activator, PMA/ionomycin, but not after pretreatment with the PKC inhibitor, GF109203X (Fig. 4C, D). It has been reported that many proteins such as $p27^{Kip}$ are subject to ubiquitination and proteosomal degradation after phosphorylation (17). It is possible that the phosphorylated form of p104 is degraded at the same time as $p27^{Kip}$ and by a similar mechanism.

The ectopic expression of p104 increased the expression of $p27^{kip1}$ (Supplemental data Fig. 2), which can induce cell-cycle arrest at the G_1-S transition via inhibition of the cyclin D-CDK4 and cyclin E-CDK2 complexes. It is well known that the duration of the G1-S transition is reduced in cancer cells, and that the overexpression of $p27^{kip1}$ can delay proliferation (18). In our study, the overexpression of p104 in MCF7 breast cancer cells led to a reduction in cell proliferation and soft agar colony formation (Fig. 2C, D). It is possible this was due to the ability of p104 to modulate downstream signaling pathways, such as those involving $p27^{Kip1}$. These results suggest the possibility that the *p104* gene could be used as a cancer hallmark or cancer cell cycle regulator. Since cellular proliferation is controlled by a complex network of signaling molecules, there are several possible p104-related signaling options that require further investigation.

MATERIALS AND METHODS

Construction of p104 mutants and expression plasmids

The 4-kb p104 cDNA was cloned into the pCDNA3.0 (Invitrogen Corp., Carlsbad, CA) or pLXSN (Clontech, Mountain View, CA) expression vector for use in various experiments. Fragments including nucleotides −46-1037 (*Eco*RI/*Sma*I), −46-1267 (*Eco*RI/*Bam*HI) and 594-1608 (*Xho*I/*Msc*I) of the p104 cDNA were cut with the indicated restriction enzymes and subcloned into pCDNA3.1 to express several truncated forms of p104. To generate several mutants p104 PXXP motifs with the proline to alanine mutation, the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used with the following mutagenic primers: 1mP: 5'-GAGAGCGAGGAG**G**C CACATCT**G**CCCAGTCACTGTGTC-3'; 2mP: 5'-GATGTAGAA ATC**G**CTGAAAAT**G**CTGAGGCTTATCTT-3'; and 3mP: 5'-CCG TGGCATGTT**G**CTGACAGC**G**CTTCCCATTCCAAG-3'. Two C→G substitutions (underlined) were introduced using each of these primers to change proline to alanine in the expressed peptide.

Measurement of anchorage-independent growth in semisolid agar

To evaluate the anchorage-independent growth of cells in semisolid medium, 1×10^4 cells were seeded into 0.3% soft agar (Noble agar; BD Biosciences, Franklin Lakes, NJ) containing 200 ng/ml platelet-derived growth factor (PDGF) BB. The resulting soft agar was layered onto 0.5% agar in 35 mm dishes. The cells were grown at 37°C for two weeks and colonies were counted after staining with 0.5 mg/ml nitroblue tetrazolium (NBT) in phosphate-buffered saline.

PI3K activity assay

NIH3T3 cells were washed twice with ice cold buffer A (137 mM NaCl, 20 mM Tris [pH 7.4], 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1 mM Na_3VO_4). After the addition of 1 ml of ice cold lysis buffer (buffer A containing 1% non-iodet P-40 and 1 mM phenylmethylsulfonyl fluoride) for 20 min, cells were scraped from the dish and centrifuged for 10 min at 10,000 × g. The supernatants were incubated with 5 μl of anti-p85α antibody (BD Biosciences) for 1 h at 4°C and the PI3K immunocomplex was isolated using Protein A Sepharose beads. The PI3K activity was assayed with the addition of 50 μl of reaction buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA), 20 μg of phosphatidylinositol, 10 μl of 100 mM $MgCl_2$ and [γ-^{32}P] ATP (6,000 Ci/mmol). The sample was incubated with agitation for 10 min at 37°C, and the reaction was terminated with the addition of 20 μl of 6 N HCl and 160 μl of $CHCl_3$: MeOH (1 : 1). The organic and aqueous phases of each sample were then separated by centrifugation for 10 min at 10,000 × g, and the radioactivity of the lower organic phase was measured by a liquid scintillation spectrometer.

See Supplemental materials and methods for additional methods.

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