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# The Inhibition of p85αPI3KSer83 Phosphorylation Prevents Cell Proliferation and Invasion in Prostate Cancer Cells

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## ABSTRACT

Phosphoinositide 3-kinase proteins are composed by a catalytic p110 subunit and a regulatory p85 subunit. There are three classes of PI3K, named class I–III, on the bases of the protein domain constituting and determining their specificity. The first one is the best characterized and includes a number of key elements for the integration of different cellular signals. Regulatory p85 subunit shares with the catalytic p110 subunit, a N-terminal SH3 domain showing homology with the protein domain Rho-GTP-ase. After cell stimulation, all class I PI3Ks are recruited to the inner face of the plasma membrane, where they generate phosphatidylinositol-3,4,5-trisphosphate by direct phosphorylation of phosphatidylinositol-4,5-bisphosphate. All pathways trigger the control of different phenomena such as cell growth, proliferation, apoptosis, adhesion and migration through various downstream effectors. We have previously provided direct evidences that a Serine in position 83, adjacent to the N-terminal SH3 domain of regulatory subunit of PI3K, is a substrate of PKA. The aim of this work is to confirm the role of p85αPI3KSer83 in regulating cell proliferation, migration and invasion in prostate cancer cells LNCaP. To this purpose cells were transfected with mutant forms of p85, where Serine was replaced by Alanine, where phosphorylation is prevented, or Aspartic Acid, to mimic the phosphorylated residue. The findings of this study suggest that identifying a peptide mimicking the sequence adjacent to Ser 83 may be used to produce antibodies against this residue that can be proposed as usefool tool for prognosis by correlating phosphorylation at Ser83 with tumor stage. J. Cell. Biochem. 114: 2114–2119, 2013. 

**KEY WORDS:** PROSTATE CANCER; REGULATORY SUBUNIT p85; PHOSPHORYLATION; LnCap

Prostate cancer is one of the most diffuse and deadly neoplasm among Western men. Its management is closely related to hormone dependence, or hormone resistance. More specifically, hormone dependence allows effective therapy by anti-androgens, while hormone resistance, generally joined to castration resistance, determines the necessity of new therapeutic strategies. Generally

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hormone resistance in CRPC (castration resistant prostate carcinoma) is not solely caused by loss of AR expression/mutation, but to AR loss of function. Additionally, activation of AR and AR downstream regulated genes could be induced by hormone-independent pathways. Even a contribution of tumor-associated fibroblasts is currently discussed. Yet, in spite of the partial knowledge of this phenomenon, physicians are still far from a suitable management of CRPC. PI3Ks are a group of kinases controlling different signal transduction pathways leading to proliferation, survival and migration of cells. They are divided into three major classes based on primary sequence, substrate preference, and regulation [Fruman et al., 1998; Cantley, 2002]. While class IA PI3Ks are heterodimeric enzymes composed by a catalytic subunit (p110α, p110β, or p110δ) complexed with one of five regulatory subunits (p85α, p55α, p50α, p85β, or p55γ), the class IB enzyme is a dimer made of p110γ catalytic subunit and p101 or p84 regulatory subunit [Cantley, 2002]. Upon growth factor stimulation, the nSH2 and cSH2 domains of p85 bind to phosphorylated tyrosine (YXXM motif) in activated receptors and adaptors that activate catalytic p110. Once activated, PI3Ks phosphorylate phosphoinositide 4,5-bisphosphate leading to the production of phosphoinositide 3,4,5-triphosphate, which serves as an important second messenger in the regulation of cell survival, growth, proliferation, and motility through a variety of downstream effectors [De Gregorio et al., 2007]. It was shown, in other cell lines, that p85A and p85D, respectively, inhibit or amplify cAMP biological effects on growth and survival [Cosentino et al., 2007]. In many steroid-dependent cancers, a close cross-talk exists between growth factor and steroid signaling which converge in the PI3K/AKT pathway; this signaling cascade is frequently deregulated in breast cancer [Altomare and Testa, 2005]. Particularly it was found that estrogen receptor signaling is profoundly altered when p85A is expressed [Cosentino et al., 2007] and that the substitution of Ser 83 with Ala inhibits cAMP effects on cell survival and G1 arrest in NIH3T3 fibroblasts. Conversely, the aspartic mutant of Ser 83 in p85/PI3K stimulated cell survival in the absence of cAMP and slowed down cell cycle progression, replicating the effects of cAMP. Ras binding to PI3K and AKT phosphorylation resulted inhibited by p85A and stimulated by p85D. Moreover, it has been shown that the expression of p85A abolished cyclic AMP/TSH-induced cell cycle progression and that it was lethal in thyroid cells (FRTL-5). Conversely, p85D inhibited apoptosis following TSH withdrawal. Moreover, the binding of the aspartic version of p85 PI3K to RIIb was independent of cAMP or TSH stimulation. Similarly, binding of PI3K to p21Ras and activation of AKT, a downstream PI3K target, were severely impaired in cells expressing the p85A mutant. It has also been described that the PI3K binding to estrogen receptor and PKA RIIβ subunit, is finely regulated by the PKA-induced phosphorylation of the p85αPI3K Ser83 [Cosentino et al., 2007; De Gregorio et al., 2007]. Since p85αPI3K binds several types of receptors and adaptors, the phosphorylation of Ser83 by PKA can induce a conformational change of the PI3K complex, which results in facilitated binding to these partners, thereby modulating PI3K activity [Cosentino et al., 2007; De Gregorio et al., 2007]. This site represents probably a nodal point, where information from several receptors, that regulate cell growth, survival, adhesion and motility, is channeled to PI3K. It has been well defined that the involvement of cytoskeleton with cell surface integrins and the extracellular matrix influences cell survival [Giancotti and Tarone, 2003; Spagnuolo et al., 2004; Meredith et al., 1993]. Detachment of cells can induce programmed cell death [Xu et al., 1998]. Conversely, increased cell adhesion and extracellular matrix interactions promote cell survival through a variety of signaling mechanisms in which activation of focal adhesion kinase (FAK) plays a central role [Xu et al., 1998; Parsons, 2003]. FAK is a non-receptor tyrosine kinase providing a scaffold for multiple signaling cascades, stimulating activation of tyrosine kinases, mitogen-activated protein kinases, and PI3K.

The aim of this work was to analyze the role of the SH3 domain of p85αPI3K in LNCap prostate cells expressing the mutant versions of p85α PI3K in LnCap cells, focusing on the role of p85α PI3K Ser83 in cell motility, growth and survival.

## MATERIALS AND METHODS

### CELL CULTURES AND PLASMID TRANSFECTION

LNCaP cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured as previously described [Horoszewicz et al., 1983]. LNCaP were grown and made quiescent as reported [Castoria et al., 1999], and then transfected by Superfect (Qiagen) with purified plasmids as report [Donini et al., 2012]. All experiments were performed in transfected cells grown for 48 h in phenol red-free RPMI (Lonza; Verviers, Belgium), 5% FBS (Biowest; Nuaillé, France), 1% piruvate Na and 1% non-essential aminoacids. Plasmids carrying bovine p85αPI3K wt tagged with FLAG (pSG5-FLAG-p85wt), or its Ser83 mutated forms, p85A (pSG5- FLAG-p85A) and p85D (pSG5-FLAG-p85D) were obtained as previously [Cosentino et al., 2007] described. Transfections were performed using Lipofectamine Plus, Gibco BRL, Life Technologies (Rockville, MD) following the manufacturer's instructions. In all transfections, pEGFPC3 plasmid was included to determine and normalize transfection efficiency. Experiments varying in the transfection efficiency >20% were discarded.

### CELL GROWTH ANALYSIS

Proliferation was assessed by MTT assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyltetrazolium bromide (MTT) (Sigma–Aldrich Co.) according the manifacturer's instructions.

### MIGRATION AND INVASION ASSAYS

Cell migration was measured using a Transwell migration chamber (diameter 5-mm, pore size 8 μm; Costar Corporation, Cambridge, MA). Cells grown to 70% confluence in Petri tissue culture dishes were detached using EGTA 1X (Sigma–Aldrich Co.) and resuspended in serum-free medium containing 0.2% BSA. LNCaP cells ($30 \times 10^5$ cells/100 μl) were seeded in the upper compartment of transwell and allowed to migrate through the polycarbonate filters, and invade the matrigel (1:1, v/v with complete medium) coated filters. The lower compartment was filled with phenol red-free DMEM with 10% charcoal-stripped serum. After 48 h of incubation at 37°C, relative numbers of cells transmigrated through the membrane were determined by staining cells on the undersurface of the Transwell membrane with Crystal Violet followed by cell lysis and measurement of absorbance values at 540 nm. The dected cells are those that

migrated from a chemotactic gradient thrusts, stained with crystal violet, and those do not migrate were removed after being quantified. The percentage of cells migrate were normalized with respect to a control represented by the empty vector.

### CONFOCAL MICROSCOPY

LNCaP cells transfected with pSG5-p85αWT, pSG5-p85αALA and p-SG5-p85αASP, grown on coverslips were fixed for 20 min with paraformaldehyde (3%, w/v in PBS), permeabilized for 20 min with Triton X-100 (0.2%, v/v in PBS) and incubated for 1 h with PBS containing FCS (1%, v/v). For cytoskeletal detection, coverslips were stained by incubation with anti-Vimentin and anti-RhoAantibodies diluted 1:1,000 in PBS for 3 h followed by three washings with PBS. Coverslips were then incubated with Alexa-Fluor 488 anti-rabbit 1:1,000 and Alexa-Fluor 633 anti-mouse 1:1,000 in PBS for 1 h. All coverslips were washed three times in PBS, incubated for 10 min with PBS containing Hoechst 33258 (Sigma) at a final concentration of 1 mg/ml and finally washed three times with PBS. The coverslips were mounted in Moviol (Calbiochem, CA) on glass slides. All images were captured with Zeiss confocal microscope 510. The microphotographs were analyzed with ImageJ.

### STATISTICAL ANALYSIS

All data are presented as the means ± SE of at least three experiments in triplicates ($n \geq 9$). Statistical significance between groups was determined using Student's *t*-test (matched pairs test or unmatched test as appropriate). All statistical analyses were performed using JMP Software (Statistical Discovery SAS Institute). $P < 0.05$, statistical significance; $P < 0.001$, high statistical significance.

## RESULTS

### ROLE OF SER83-P85A/PI3K PHOSPHORYLATION ON CELL PROLIFERATION

Since PI3K-AKT pathway promotes survival and cell growth, we assayed whether the Ser83p85α phosphorylation could affect cell proliferation. The MTT cell viability assay performed with LNCaP cells transiently transfected with p85wt, ala, asp or with the empty vector (at 0, 24, and 48 h), demonstrates that all cells that over-express the p85 mutants showed partly inhibition of cell growth suggesting that it acts as a negative regulator of the PI3K pathway at each time point examined. Moreover, the rate of growth observed in cells over-expressing the mutant Ala was significantly lower ($P < 0.05$) compared to that observed in cells over-expressing p85wt and asp (Fig. 1).

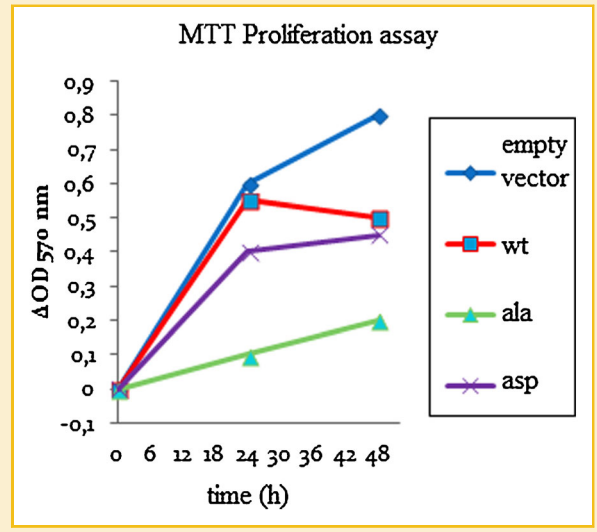


Fig. 1. Role of p85αPI3KSer83 in LNCaP proliferation. Cells transiently transfected with empty vector or with p85αPI3K mutants were assayed by MTS at 0, 24, and 48 h after transfection (* $P < 0.05$ compared to empty vector or p85αPI3K wt or p85D). The data are mean of three independent experiments performed in triplicate ($n = 9$).

### EFFECTS OF PHOSPHORYLATION OF P85-SER83 ON CELL MIGRATION

Cell migration plays an important role in a wide range of processes, both physiological and pathological. PI3K plays an important role in cell migration in different types and cellular systems, acting with other pathways in regulating cellular polarity. The role of phosphorylation of p85-PI3KSer83 in the LNCaP cells migration was investigated using a migration assay. Our results show that the expression of the mutant p85Ala in LNCaP cells results in a significant reduction ($P < 0.001$) of migration (15–20% vs. Vector empty), thus indicating that phosphorylation of p85-Ser83 is essential for the control of migratory behavior (Fig. 2).

### EFFECTS OF P85-SER83 PHOSPHORYLATION ON INVASIVE GROWTH

Malignant tumors, during the course of the disease, undergo a series of modifications. The most worrying turns out to be the formation of metastases. In this process the crucial step occurs when the neoplastic cells are detached from the primary tumor, degrade extracellular matrix proteins and eventually penetrate into the surrounding tissues and vessels. Prostate cancer cells in culture adhere to the substrate [Donini et al., 2012]. The percentage of invasive cells were normalized with respect to a control represented by empty vector. Even in the case of invasiveness, the LNCaP prostate cancer cells transfected with the ala mutant showed a reduced invasiveness compared to wt (Fig. 3).

### EFFECTS OF PHOSPHORYLATION ON CYTOSKELETON CHANGES

The evaluation of cytoskeletal modification was performed by double immunofluorescence for vimentin and RhoA. Vimentin belongs to the intermediate filament family proteins, while RhoA is a GTPase belonging to Rho family, whose role in cell migration and cancer metastases development has been recently described [Ridley, 2001]. In particular it has been hypothesized that RhoA is involved in lamellipodia dynamics and thus in migration and invasion [Vega and Ridley, 2008]. Confocal laser microscopy showed that LNCaP cells, upon transfection, lost their characteristic shape extensions and acquired a round shape with a visibly larger nucleus and reduction of the lamellopodia. We suppose that the ala-mutation could determine the cytoskeletal rearrangements, possibly by interacting with proteins such as 14-3-3, a particular kind of proteins that has the ability to bind a series of functionally diverse signaling proteins, including

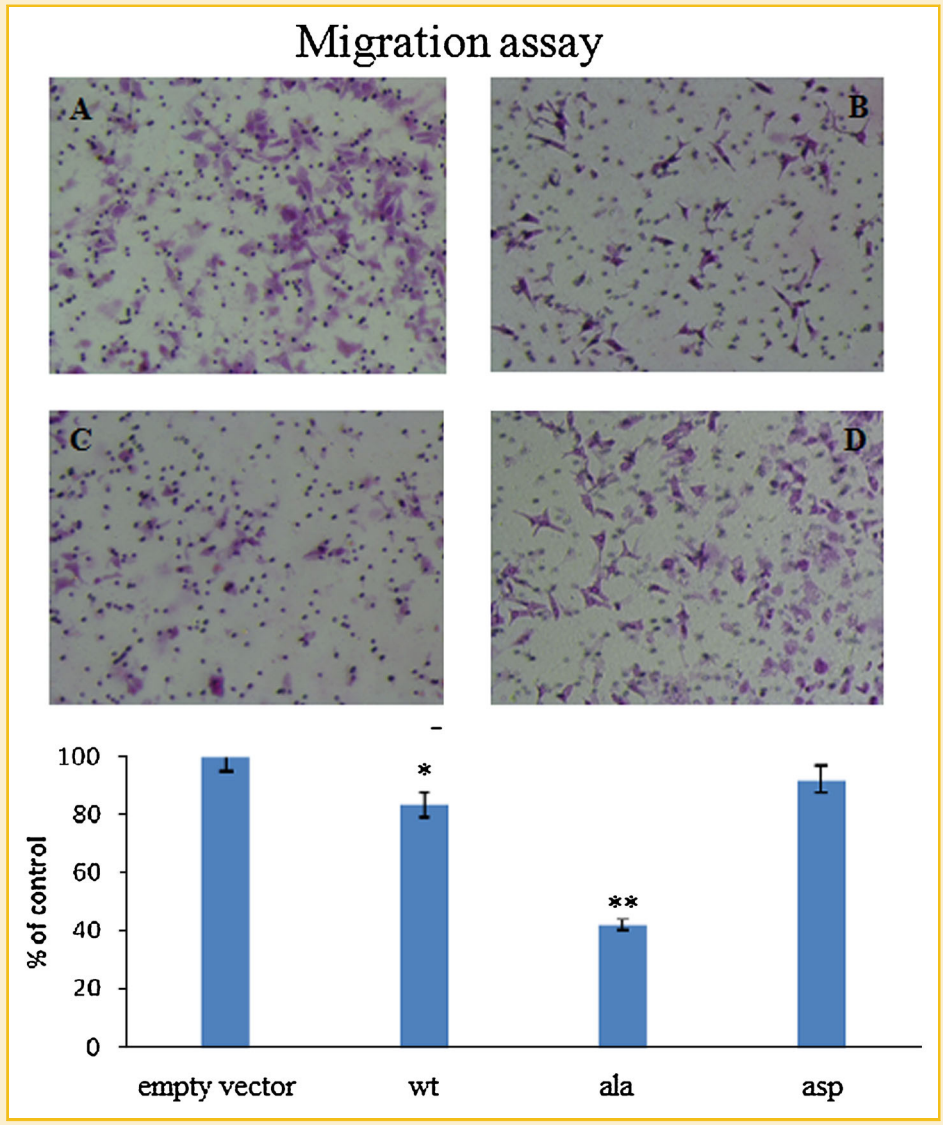


**Fig. 2.** Migratory behavior of LNCaP transfected cells. Migration assay on LNCaP cells overexpressing empty vector (A), p85αPI3K wt (B), ala (C) and asp (D). Histogram represents quantification of cell motility (% of migration; $^{**}P < 0.001$ comparing p85A vs. empty vector or p85αPI3K wt or p85D; $^{*}P < 0.05$ comparing p85A vs. pwt). Images are representative of three separate experiments performed in triplicate (n = 9).

kinases, phosphatases and transmembrane receptors. They play an important role in a number of vital regulatory processes, such as signal transduction, apoptosis, and cell cycle control [Kumagai et al., 1993; Hungerford et al., 1996; Teng et al., 1997; Xu et al., 1998; Parsons, 2003]. On the other hand the immunolocalization of RhoA shows that in wt cells the protein is localized to the nucleus and that, upon ALA transfection it is completely relocalized to the cytoplasm accompanied by a complete absence of lamellipodia (Fig. 4).

## DISCUSSION

Prostate cancer arises from cells that accumulate different mutations within their genome. Molecular events responsible for the development of the disease have not yet been clarified, but androgens play surely a key role.

Steroid hormones are indispensable for the maintenance of the normal development, growth and differentiation of prostate cells. Additionally, androgens play a central role in both the induction and progression of prostate tumor formation. The early stages of prostate cancer is hormone-responsive and sensitive. The most commonly used therapy for prostate cancer is based on androgenic deprivation: the lack of testosterone stimulus induce cancer cells to undergo apoptosis. In more advanced stages the tumor, cells can acquire additional mutations that make them hormone-independent. The knowledge on (AIPC) androgen independent castration resistant prostate cancer underlines that increasing the understanding of PI3Ks signaling pathway may highlight its importance for prognostic and therapeutic intervention in hormone-refractory prostate cancer.

In many steroid dependent a close interplay exists between growth [Vanhaesebroeck and Waterfield, 1999] factors and steroid signaling

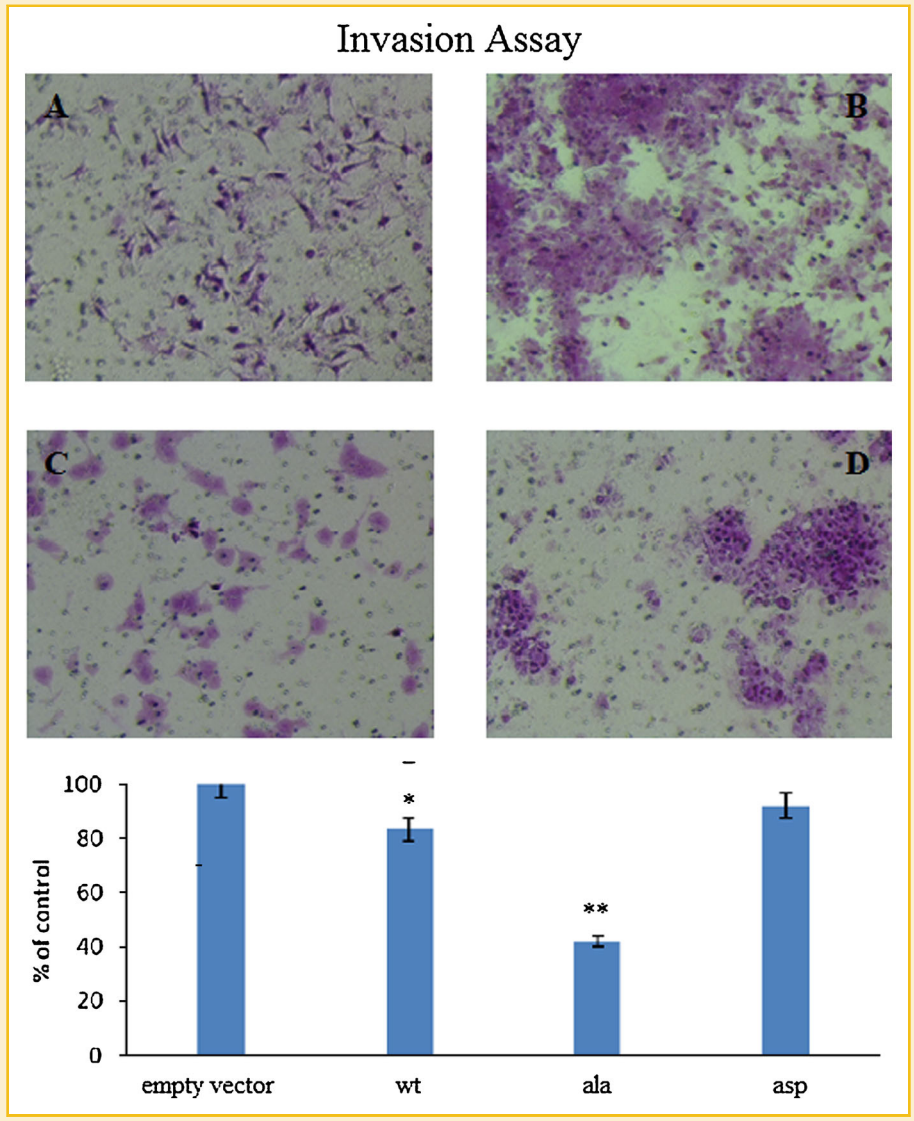


Fig. 3. Invasiveness behaviour of LNCaP transfected cells. Cell invasion assay in LNCaP cells transfected with empty vector (A), p85wt (B), ala (C), and asp (D). Histogram represents quantification of cell motility (% of migration; $^{**}P < 0.001$ comparing p85A vs. empty vector or p85αPI3K wt or p85D; $^{*}P < 0.05$ comparing p85A vs. bwt). Images are representative of three separate experiments performed in triplicate (n = 9).

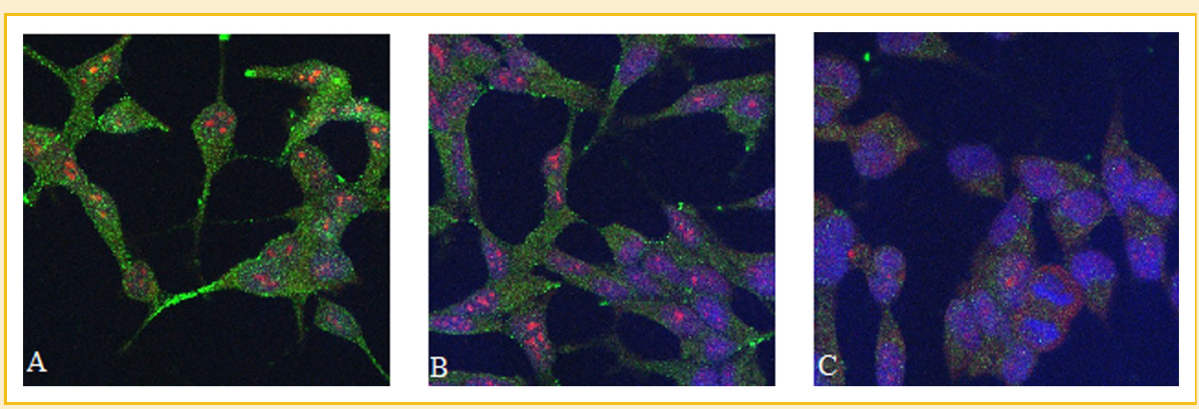


Fig. 4. Morphological and cytoskeletal changes afterwards p85α transfection. Double immunofluorescence for Vimentin (green)/RhoA (red) in LNCaP cells transfected with empty vector (A), pSG5-p85αwt (B), and pSG5-p85αala (C). Nuclei were counterstained with Hoechst 33258. The coverslips mounted in Moviol (Calbiochem, CA). All images were captured with Zeiss confocal microscope 510. The microphotographs were analyzed with ImageJ.

which thus converge in thePIK3/AKT pathway, and this cascade is deregulated in breast and prostate cancer [Altomare and Testa, 2005]. The control of migratory behavior is a focal node in these tumors.

In this respect, our data give direct evidences that phosphorylation of p85alfaPI3KSer83 and the N-terminal SH3 domain represent a crucial step for the modulation of different signals. Notably, several studies have showed that the members of the superfamily of nuclear receptors, including the estrogen receptor or the thyroid receptor interact with the regulatory subunit p85of PI3K, and activate the PI3K/AKT pathway. Consistent with this knowledge [Cosentino et al., 2007], it has been shown that the binding of Ser83 to the protein 14-3-3, facilitates the interaction of PI3K with other molecular adapters, by strengthening its activities and allowing the membrane localization of p85. The receptors binding p85 can cooperate with cAMP-PKA signal via Ser83 phosphorylation of p85, explaining the pleiotropic nature of the effects exerted by cAMP-PKA on seemingly unrelated signaling cascades. The residue Ser83 plays an important role in regulating the binding between ligands and their receptors induced by cAMP-PKA. Recent studies underlined the importance of the Ser83 residue in the control of cell growth in different cellular models, as FRTL-5, NIH3T3, and SMCs, but it is worthnoting that the same phosphorylation leads to phenotypic effects, depending on the cell type. The Ser83 phosphorylation inhibited cell proliferation in fibroblasts and resulted crucial for cell cycle progression in thyroid cells, while had no effect on proliferation of endothelial cells. Our results show that the Ser83 phosphorylation is essential for the proliferation and survival of prostate cancer LNCaP cells, as evidenced by the low growth rate observed in cells over-expressing the mutant p85Ala, where phosphorylation is inhibited. The isoforms of PI3K play an important role in cell migration in different types and cellular systems but its contribution depends on the state of the cell and by the combination of stimuli to which the cell is exposed. In breast cancer, the direct activation of PI3K is sufficient to induce cell motility and invasion [Cosentino et al., 2007]. So far, the role of Ser83 in cell invasion and migration, in prostate, had never been investigated. This study demonstrates for the first time that the overexpression of p85A mutant induces a significant suppression of motility and invasiveness of LNCaP cells, indicating that the phosphorylation of Ser83 is a critical regulator of this process. To date, inhibitors targeting PI3K or other nodes of this pathway, have reached clinical trials. In this study, we focused on the understanding of phosphorylation of PI3K-p85α regulatory subunit in parameters crucial for cancer metastasis, suggesting that this event is crucial for tumor survival and metastatic process in hormone refractory prostate cancer. A further goal will be to synthesize a peptide that mimics the surrounding adjacent to the Ser83 sequence in order to produce a new tool for the diagnosis of hormone refractory prostate tumors and for therapy with the intent to interfere with the control of proliferation/invasiveness of this tumor.

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