SPANDIDOS PUBLICATIONS

INTERNATIONAL JOURNAL OF ONCOLOGY 40: 1627-1635, 2012

The p85α regulatory subunit of PI3K mediates cAMP-PKA and retinoic acid biological effects on MCF7 cell growth and migration

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Received October 13, 2011; Accepted November 29, 2011

DOI: 10.3892/ijo.2012.1383

Abstract. Phosphoinositide-3-OH kinase (PI3K) signalling regulates various cellular processes, including cell survival, growth, proliferation and motility, and is among the most frequently mutated pathways in cancer. Although the involvement of p85α^{PI3K} SH2 domain in signal transduction has been extensively studied, the function of the SH3 domain at the N-terminus remains elusive. A serine (at codon 83) adjacent to the N-terminal SH3 domain in the PI3K regulatory subunit p85α^{PI3K} that is phosphorylated by protein kinase A (PKA) *in vivo* and *in vitro* has been identified. Virtually all receptors binding p85α^{PI3K} can cooperate with cAMP-PKA signals via phosphorylation of p85α^{PI3K}Ser83. To analyse the role of p85α^{PI3K}Ser83 in retinoic acid (RA) and cAMP signalling, in MCF7 cells, we used p85α^{PI3K} mutated forms, in which Ser83 has been substituted with alanine (p85A) to prevent phosphorylation or with aspartic acid (p85D) to mimic the phosphorylated residue. We demonstrated that p85α^{PI3K}Ser83 is crucial for the synergistic enhancement of RARα/p85α^{PI3K} binding induced by cAMP/RA co-treatment in MCF7 cells. Growth curves, colorimetric MTT assay and cell cycle analysis demonstrated that phosphorylation of p85α^{PI3K}Ser83 plays an important role in the control of MCF7 cell proliferation and in RA-induced inhibition of proliferation. Wound healing and transwell experiments demonstrated that p85α^{PI3K}Ser83 was also essential both for the control of migratory behaviour and for the reduction of motility induced by RA. This study points to p85α^{PI3K}Ser83 as the physical link between different pathways (cAMP-PKA, RA and FAK), and as an important regulator of MCF7 cell proliferation and migration.

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Key words: p85α, PI3K, retinoic acid, RARα, cAMP, cell growth, cellular motility

Introduction

The phosphoinositide-3-OH kinase (PI3K) family of lipid kinases regulates cell survival and growth, as well as migration (1). This signalling pathway is deregulated in most human cancers by differential gene expression, amplification or mutation (2-5). Most of p85α^{PI3K} mutations cluster in the inter-SH2 (iSH2) domain of the molecule (5,6), which interacts with the catalytic subunit p110α^{PI3K} (7,8). A serine (at codon 83) was identified adjacent to N-terminus SH3 domain in the PI3K regulatory subunit p85α^{PI3K}, that is critical for cell cycle progression and cell survival in normal epithelium (9). p85α^{PI3K}Ser83 is phosphorylated by protein kinase A (PKA) *in vivo* and *in vitro*, influencing the ability of SH3 domain to interact with different partners (10,11). Nearly all receptors binding p85α^{PI3K} can cooperate with cAMP-PKA signals via phosphorylation of p85α^{PI3K}Ser83, thus explaining the pleiotropic nature of the effects exerted by cAMP-PKA on several, apparently unrelated, signalling cascades.

In many steroid-dependent cancers, a close cross-talk exists between growth factor and steroid signalling which thus converge in the PI3K/AKT pathway; this signalling cascade is frequently deregulated in breast cancer (12). All-trans retinoic acid (RA) is a vitamin A derivative involved in the growth inhibition of breast cancer cell lines (13-19). It has been demonstrated that administration of RA resulted in activation of PI3K signalling through a mechanism in which a stable complex including retinoic acid receptor α (RARα) and p85α^{PI3K} occurs also in the absence of RA (20,21). 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 p85α^{PI3K} Ser83 (9,10). Since p85α^{PI3K} 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 (9,10). This site represents probably a nodal point, where information from

several receptors, that regulate cell growth, survival, adhesion and motility, is channelled to PI3K.

The aim of this work was to analyse the role of the SH3 domain of p85α^{PI3K} in RA and cAMP-PKA signalling, using as model the breast cancer cell line MCF7, which expresses functional RARα receptor. For this purpose, we tested whether p85α^{PI3K}Ser83 modulates the binding of RARα to PI3K after treatment of MCF7 cells with RA and cAMP. Furthermore, we analysed the biological consequences of the expression of mutant versions of p85α^{PI3K} in MCF7 cells, focusing on the role of p85α^{PI3K}Ser83 in cell motility, growth and survival.

Materials and methods

Cell cultures and plasmid transfection. MCF7 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as previously described (9). All experiments were performed in quiescent cells growing for 48 h in phenol red-free DMEM (Lonza; Verviers, Belgium) and 5% charcoal-stripped serum (Biowest; Nuaillé, France). All-trans retinoic acid, 8-Br-cAMP and H89 were from Sigma-Aldrich Co. (St. Louis, MO, USA).

Plasmids carrying bovine p85α^{PI3K} 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 described (9). pSG5-RARα plasmid was kindly provided by Prof. Domingo Barettino, Instituto de Biomedicina de Valencia. Transfections were performed using Lipofectamine Plus, Gibco BRL, Life Technologies (Rockville, MD, USA) 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.

Western blotting and immunoprecipitation. Lysates from transfected MCF7 cells were separated by SDS-PAGE and immunoblotted as previously described (10). Antibodies against RARα and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against p85α^{PI3K} and phosphorylated FAK were from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Antibody against FlagM2 was from Sigma-Aldrich Co. For immunoprecipitation, cell lysates (1 mg) were incubated with 8 μg of antibodies against anti-FlagM2 or RARα, or with 5 μl of antibody against p85α^{PI3K}; immunocomplexes were precipitated using anti-rabbit IgG beads ExactaCruzF, from Santa Cruz Biotechnology, Inc., following the manufacturer's instructions, and processed for western blot analysis as described.

Clonogenic assays. MCF7 cells (3x10^2) expressing p85α^{PI3K} wt or its mutants were seeded into 6-well plates that were then incubated at 37˚C for ~10 days until cells have formed sufficiently large clones (at least 50 cells). Clones were counted after 30 min fixing with a mixture of 6% glutaraldehyde and 0.5% crystal violet.

Cell growth analysis. Proliferation was assessed by cell counting and by MTT assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyltetrazolium bromide (MTT) (Sigma-Aldrich Co.) as previously described (10).

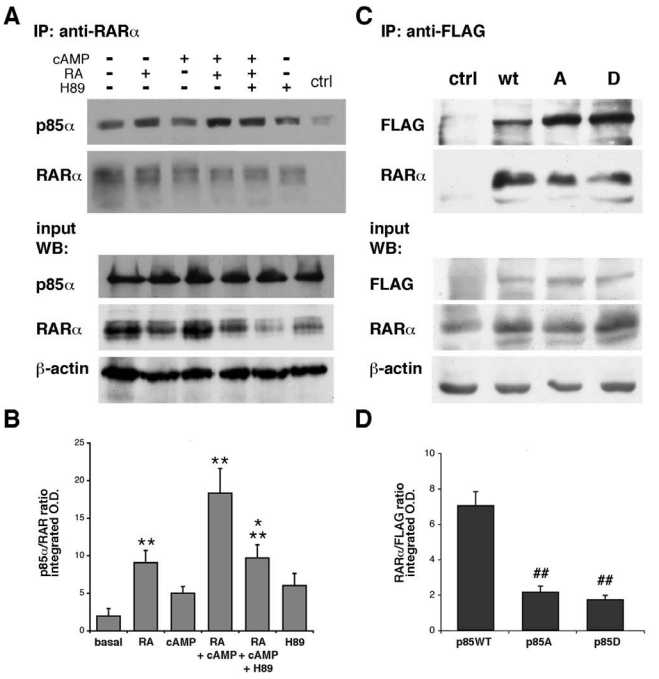

Figure 1. Interactions between RARα and p85α^{PI3K}. (A) Total lysates of MCF7 quiescent cells treated with RA 10 μM and/or cAMP 200 μM, and/or H89 10 μM or their vehicles for 30 min, were immunoprecipitated with RARα antibody. After western blotting the filter was sequentially developed with anti-p85α^{PI3K} and anti-RARα antibodies, using horseradish peroxidase-conjugated Rabbit ExactaCruzF as secondary antibody. (C) Total lysates of MCF7 quiescent cells transfected with p85α^{PI3K} wt, p85A or p85D were normalized for the transfection efficiency and immunoprecipitated with anti-Flag antibody. Sample aliquots were immunoblotted with anti-RARα and anti-Flag antibodies. The histograms represent the ratio of p85α^{PI3K}/RARα densitometric bands in the RARα immunoprecipitates (B) or the ratio of RARα/FLAG densitometric bands in the FLAG-p85α^{PI3K} immunoprecipitates (D), derived from three independent experiments performed in triplicate (n=9). Differences between treatments were tested for statistical significance using Student's matched pairs t-test: **P<0.001 vs. basal; *P<0.05 vs. RA+cAMP treated cells; $^{\#\#}$P<0.001 vs. p85α^{PI3K} wt.

Cell cycle analysis. Cell cycle analysis was performed by Fluorescence-activated cell sorting (FACS) as described (10). Fluorescence was determined by using the FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Wound healing assays. Confluent MCF7 cells were scratched with a p200 pipette tip, and cellular debris was removed by washing gently. Following wounding, culture medium was replaced with fresh medium and cells were exposed to RA 30 μM for 16 h. Images were acquired on a phase contrast microscope Axio Observer (Carl Zeiss, Inc., Oberkochen, Germany) and wound diameter was measured at 0 and 16 h using the computing software Axio Vision (Carl Zeiss, Inc.).

Migration 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. RA (30 μM) treated and untreated cells (30x10^5 cells/100 μl) were seeded in the upper compartment of transwell and allowed to migrate trough the polycarbonate filters coated with Fibronectin (40 μg/ml in serum-free medium

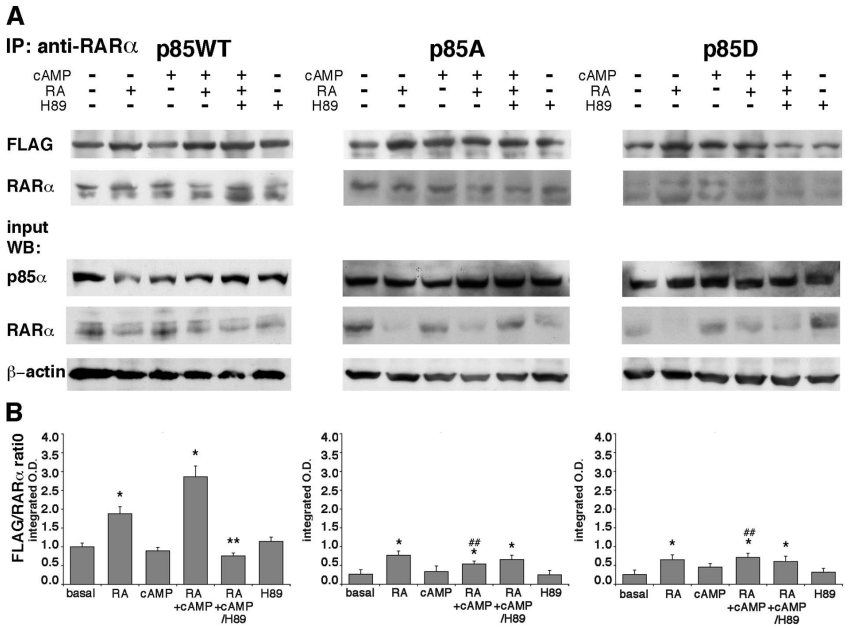

Figure 2. p85α^{PI3K} Ser83 in cAMP-PKA-modulation of the RARα/p85α^{PI3K} complex. (A) Quiescent MCF7 cells, transiently transfected with p85α^{PI3K} wt, p85A or p85D, were treated with RA 10 μM and/or cAMP 200 μM, and/or H89 10 μM or their vehicles for 30 min. Total lysates were normalized for transfection efficiency and then immunoprecipitated with RARα antibody, and after western blotting the filter was sequentially developed with anti-Flag and anti-RARα, using horseradish peroxidase-conjugated Rabbit ExactaCruzF as secondary antibody. (B) The histogram represents the ratio of Flag/RARα bands in the RARα immunoprecipitates, derived from three independent experiments performed in triplicate (n=9). Differences between treatments were tested for statistical significance using Student's matched pairs t-test: *P<0.05 vs. basal; **P<0.001 vs. RA+cAMP treated cells; $^{\#\#}$P<0.001 p85α^{PI3K} mutants co-treated vs. p85α^{PI3K} wt co-treated.

containing 0.2% BSA). The lower compartment was filled with phenol red-free DMEM with 10% charcoal-stripped serum. After 1, 2 or 4 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.

Statistical analyses. All data are presented as the means ± SE of at least three experiments in triplicates (n≥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

RARα and p85α^{PI3K} interactions. In order to study whether p85α interacted with RARα, immunoprecipitation experiments were performed in MCF7 cells made quiescent by charcoal-treated serum and medium lacking phenol-red for 48 h, and treated with RA 10 μM and/or cAMP 200 μM, and/or the PKA inhibitor, H89 10 μM, or their vehicles for 30 min. Immunoprecipitation of total cell lysates with anti-RARα antibody, showed a physical association between p85α^{PI3K} and RARα in all conditions examined (Fig. 1A, upper panel). In particular, RA treatment increased 4-fold the formation of p85α^{PI3K}/RARα complex, while cAMP stimulation showed 2.5-fold increase, with respect to untreated cells. Of note, the co-treatment with RA and cAMP induced a significant and synergistic enhancement of the binding (9-fold, P<0.001). Treatment of MCF7 cells with H89, did not modify the basal interaction, but abolished synergy with cAMP. Western blot on total lysates showed that RA treatment induced downregulation of RARα protein, as already reported by Bastien *et al* (22). p85α^{PI3K} and β-actin expressions in inputs were shown (Fig. 1A, lower panel). These findings collectively established that endogenous RARα and p85α^{PI3K} interact in MCF7 cells also in absence of RA, and that the co-treatment with RA and cAMP-PKA is required for the synergistic enhancement of this binding.

It has been previously identified that Ser83 in the p85α^{PI3K} molecule is phosphorylated *in vivo* and *in vitro* by PKA (23). To determine the relevance of this phosphorylation in the formation of the complex RARα/p85α^{PI3K}, it has been substituted Ser83 with alanine (p85A) to prevent phosphorylation or with aspartic acid (p85D) to mimic the phosphorylated residue (9,10). Immunoprecipitation experiments were performed in quiescent MCF7 cells transiently transfected with a construct containing p85α^{PI3K} wt or its Ser83 mutated forms. In order to minimize the negative regulation of PI3K signalling, caused by p85α^{PI3K} overexpression (24), we used only lysates from cells with comparable transfection efficiency (>70%). Immunoprecipitation experiments with anti-FLAG antibody demonstrated that overexpression of the mutated forms of p85α^{PI3K}, significantly (P<0.001) impairs the interaction between RARα and p85α^{PI3K} (Fig. 1C). Histograms, in Fig. 1B and D, represent the ratio of p85α^{PI3K}/RARα densitometric bands in the RARα immunoprecipitates, and the ratio of RARα/FLAG densitometric bands in the FLAG-p85α^{PI3K} immunoprecipitates, respectively, derived from three independent experiments performed in triplicate.

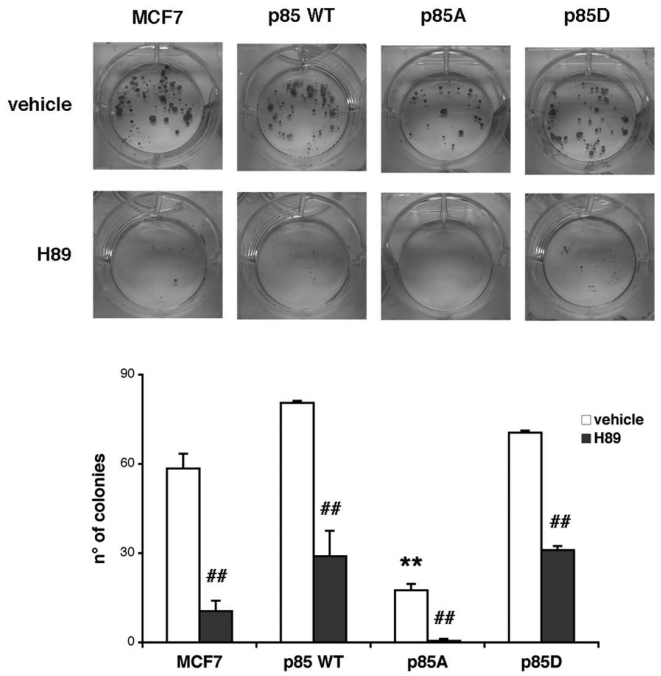

Figure 3. Role of p85α^{PI3K}Ser83 in MCF7 survival. Clonogenic assay of MCF7 cells expressing the p85α^{PI3K} wt or Ser83 mutants treated or not with H89 10 μM. The histogram represents the average number of colonies of four separate experiments performed in duplicate (n=8). (**P<0.001 p85A vs. empty vector or p85α^{PI3K} wt or p85D; $^{\#\#}$P<0.001 vs. basal).

Role of Ser83 in cAMP/RA modulation of RARα/p85α^{PI3K} binding. Quiescent MCF7 cells were transiently transfected with p85α^{PI3K} wt or mutant encoding vectors and treated for 30 min with RA 10 μM and/or cAMP 200 μM, and/or H89 10 μM or their vehicles. Immunoprecipitations with anti-RARα antibody, demonstrated that cells overexpressing p85α^{PI3K} wt formed the p85α^{PI3K}/RARα complex in all conditions examined and treatment with RA or with RA/cAMP, significantly (P<0.05) enhanced this binding (Fig. 2A and B). Cells overexpressing p85α^{PI3K} mutants, although maintained the ability to form the p85α^{PI3K}/RARα complex in all conditions examined, showed a significant (P<0.001) reduction of the synergistic effect of cAMP/RA treatment with respect to cells overexpressing p85α^{PI3K} wt. Moreover, the reduction of p85α^{PI3K}/RARα binding observed after H89 treatment in the cells overexpressing p85α^{PI3K} wt, was completely abolished in both p85α^{PI3K} mutant transfected cells. Taken together, these data demonstrated that p85α^{PI3K}Ser83 is crucial for the synergistic enhancement of RARα/p85α^{PI3K} binding induced by cAMP/RA.

Cell growth effects of p85α^{PI3K}Ser83 phosphorylation. PI3K-AKT is one of the most important pathways promoting survival and cell growth (25). In order to study whether the impairment of the RARα/p85α^{PI3K} binding modified the biological behaviour of MCF7 cells overexpressing the mutated forms of p85α^{PI3K}, we performed clonogenic and proliferation assays.

The overall survival of MCF7 cells transfected with the p85α^{PI3K} mutants, was evaluated by clonogenic assays in basal condition or after treatment with H89 10 μM. As shown in Fig. 3, overexpression of p85A induced a statistically significant (P<0.001 p85A vs. empty vector or p85α^{PI3K} wt or p85D) reduction of the ability to form colonies of at least 50 cells. Treatment with H89 significantly (P<0.001) inhibited the ability to form colonies in all conditions examined.

Growth curves, colorimetric MTT assay and cell cycle analysis by Fluorescence-activated cell sorting (FACS) were performed on MCF7 cells transiently transfected with p85α^{PI3K} wt, A or D or with the empty vector. Cells were counted at 0, 24, 48 and at 72 h. Our results demonstrated that all cells overexpressing p85α^{PI3K} showed an inhibition of cell proliferation at each time period examined. This effect was probably due to the overexpression of p85α^{PI3K} in the cells, that acts as a negative regulator of PI3K signalling and a potential tumour suppressor (23). Furthermore, the growth rate observed in cells overexpressing p85A was significant lower (P<0.05) with respect to that observed in cells overexpressing p85α^{PI3K} wt or D (Fig. 4A). Trypan blue test performed in all proliferation assays demonstrated that the growth inhibition was not due to reduction of cellular viability (data not shown). Similar results were obtained by colorimetric MTT assay (Fig. 4B). FACS analysis confirmed that only cells expressing p85A showed a lower proliferation rate, as demonstrated by a significant (P<0.001) reduction of the percentage of cells accumulated in G2 (Fig. 4C). Collectively these results demonstrated that phosphorylation of p85α^{PI3K}Ser83 plays an important role in the control of MCF7 cell proliferation.

The cell growth rate was also analysed in cells overexpressing p85α^{PI3K} mutants, untreated or treated with RA 30 μM, cAMP 200 μM or both of them for 12, 48 and 72 h. Our results demonstrated a consistent inhibition of cell replication in the cells that had undergone RA and/or cAMP treatment (Fig. 5A). In particular, a significant (P<0.001) reduction of proliferation was observed in MCF7 cells overexpressing p85α^{PI3K} wt or empty vector after 48 h of RA treatment, while cells overexpressing p85A or D, showed only a small inhibition of growth. Whereas cAMP treatment induced a significant (P<0.001) inhibition of proliferation in all conditions examined, co-treatment with RA and cAMP induced a significant (P<0.001) and synergistic inhibition of growth in cells overexpressing p85α^{PI3K} wt, D or empty vector, but not in those overexpressing p85A (Fig. 5B). These different behaviours could be a consequence of the decreased RARα/p85α^{PI3K} binding (Fig. 1B). Taken together these results indicated that Ser83 was involved in RA-induced inhibition of MCF7 cell proliferation, and that phosphorylation of this residue is required for the cAMP/RA synergistic inhibition of growth.

Role of phosphorylation of p85α^{PI3K}Ser83 in the control of migratory behaviour. PI3K has multiple roles in cell migration in many cell types and systems, acting redundantly with multiple other pathways to co-ordinate polarity (1). Moreover RA and other biologically active retinoids inhibit cellular migration in Airway SMCs (26) and in different breast cancer cell lines (27,28).

The role of phosphorylation of p85α^{PI3K}Ser83 in MCF7 cell migration, was investigated performing wound healing assays in presence or not of RA. Our results demonstrated that expression of p85A had an effect per se on MCF7 migration, displaying a statistically significant (P<0.001) reduction of migration (89% vs. empty vector, 84% vs. p85α^{PI3K} wt and

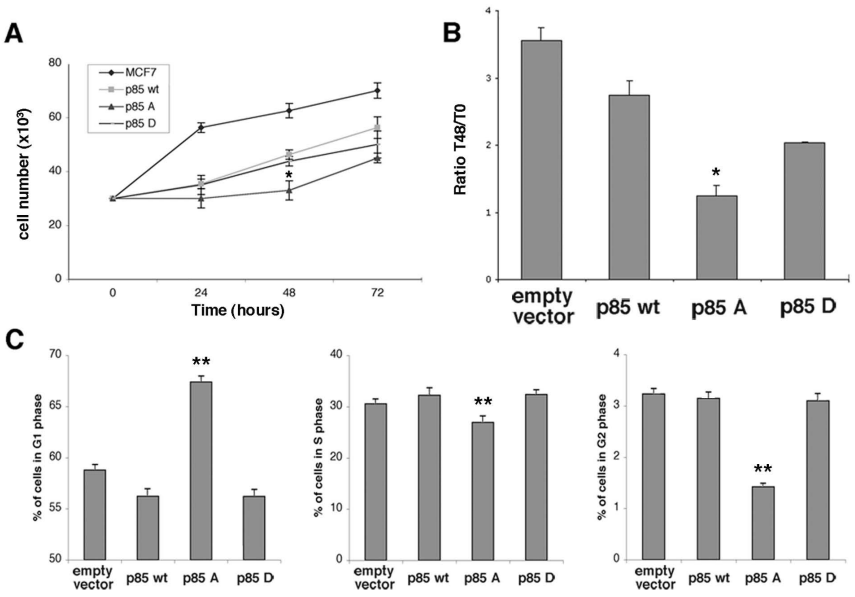

Figure 4. Role of p85α^{PI3K}Ser83 in MCF7 proliferation. (A) Cells transiently transfected with empty vector or with p85α^{PI3K} mutants were counted three times at 0, 24, 48 and at 72 h after transfection (*P<0.05 compared to empty vector or p85α^{PI3K} wt or p85D). (B) Using the colorimetric MTT assay, cell proliferation was evaluated 48 h after transfection (*P<0.05 compared to empty vector or p85α^{PI3K} wt or p85D). (C) Transfected MCF7 cells were serum starved for 12 h and then analyzed by FACS to determine the percentage of cells in G1, G2 and S-phase (**P<0.001 compared to empty vector or p85α^{PI3K} wt or p85D). The data are the mean of three independent experiments performed in triplicate (n=9).

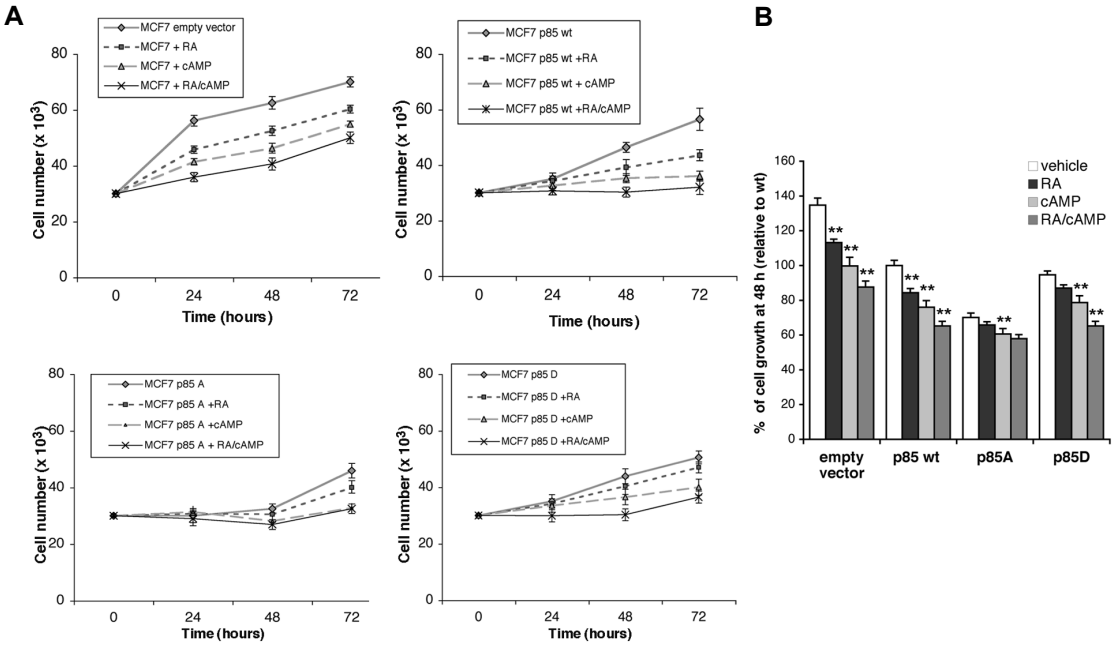

Figure 5. RA and cAMP effects on growth of MCF7 overexpressing p85α^{PI3K} mutants. (A) Cells overexpressing p85α^{PI3K} wt, A or D, or the empty vector, treated or not with RA 30 μM, cAMP 200 μM or RA/cAMP were counted three times by two independent investigators. Inter-observed variation was <5%. Values represent the mean of triplicate determination ± SD of three experiments performed in triplicate (n=9). (B) Histogram represents the percentage of growth inhibition after 48 h of RA and/or cAMP treatment in MCF7 cells overexpressing empty vector, p85α^{PI3K} wt or mutants (**P<0.001 vs. basal).

88% vs. p85D). RA treatment induced a significant (P<0.05) reduction of migration in cells overexpressing p85α^{PI3K} wt or empty vector, while it did not inhibit the motility in cells overexpressing p85D. The responsiveness to RA administration was not detectable in cells overexpressing p85A, because of their inability to migrate (Fig. 6). Similar results were obtained by Transwell experiments performed onto fibronectin-coated surfaces in presence or not of RA (Fig. 7A and B). Our data demonstrated that p85α^{PI3K}Ser83 was essential both for the control of migratory behaviour and for the reduction of motility induced by RA.

It has been demonstrated that p85α^{PI3K} interacts with focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase involved in integrin-mediated signal transduction pathways (29). FAK influences both focal adhesion assembly and disassembly, and is required for the dynamic regulation of integrin focal adhesions

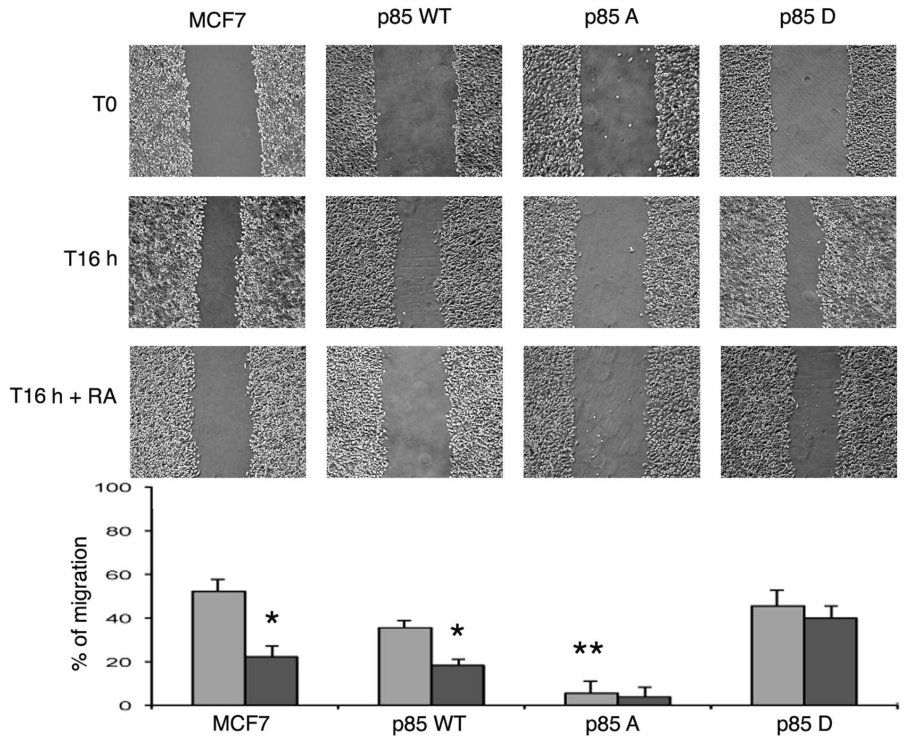

Figure 6. Migratory behaviour of MCF7 transfected cells. Wound-healing assay on MCF7 cells overexpressing p85α^{PI3K} mutants untreated or treated with RA 30 μM for 16 h. Histogram represents quantification of the RA treatment effect on cell motility (% of migration); (**P<0.001 comparing p85A vs. empty vector or p85α^{PI3K} wt or p85D; *P<0.05 comparing RA treated vs. basal). Images are representative of three separate experiments performed in triplicate (n=9).

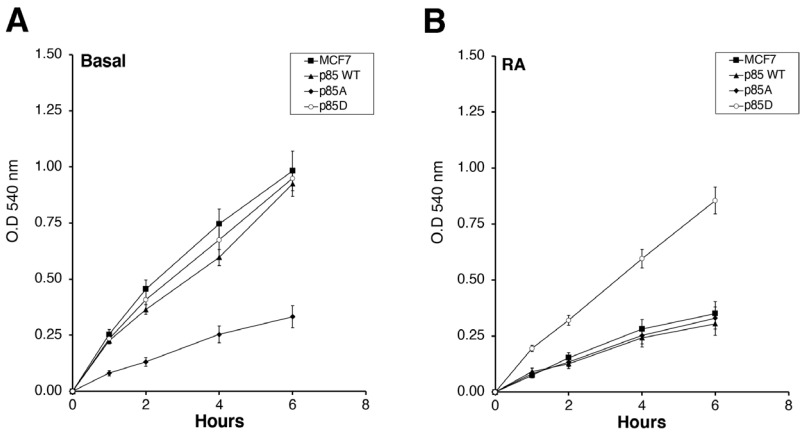

Figure 7. Cell migration toward fibronectin of MCF7 overexpressing p85α^{PI3K} mutants. Time course of cell migration of MCF7 transfected cells onto fibronectin-coated surfaces in the Transwell assay. The Transwell membranes were coated with fibronectin. After blocking with BSA, 3x10^5 transfected cells were plated in serum-free medium (in absence or in presence of RA 30 μM) into the upper well and incubated at 37˚C in a CO_2 incubator for 1, 2 or 4 h. 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. Dye levels are directly proportional to numbers of cells. Data are presented as means ± SD.

during cell migration (30). In order to clarify whether FAK was involved in the control of migratory behaviour mediated by p85α^{PI3K}, lysates from MCF7 cells transiently transfected with p85α^{PI3K} wt or p85A or p85D and treated with 30 μM RA for 10 min, were immunoprecipitated with anti-FLAG antibody and then sequentially analysed by western blotting with anti-P-FAK (Y397), anti-RARα and anti-p85α^{PI3K} antibodies. Our results demonstrated for the first time that p85α^{PI3K}, P-FAK and RARα co-immunoprecipitated; in particular, the complex formation was strongly enhanced in cells expressing p85A and reduced in those expressing p85D (Fig. 8). RA treatment strongly increased the formation of RARα/PI3K/P-FAK complex in all conditions examined. These results indicate that phosphorylation of p85α^{PI3K}Ser83 plays an important role in the formation of the PI3K/P-FAK complex, and in the modulation of this binding mediated by RA.

Discussion

The data presented herein indicated that p85α^{PI3K}Ser83 and the near SH3 domain represent a nodal point modulating information from several signalling cascades converging

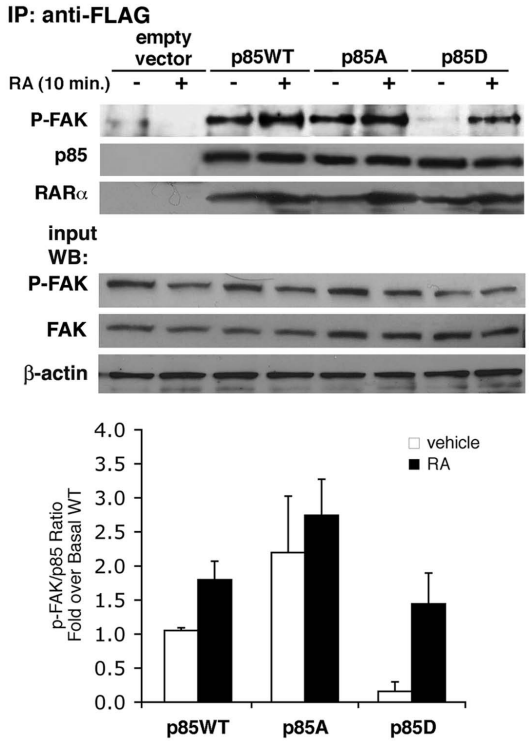

Figure 8. Effect of the substitution of p85α^{PI3K}Ser83 on the formation of P-FAK/PI3K complex. MCF7 cells, transiently transfected with p85α^{PI3K} wt, p85A or p85D, were treated with 30 μM RA for 10 min. Total lysates were normalized for transfection efficiency and then immunoprecipitated with FLAG antibody. After western blotting the filter was sequentially developed with anti-p85α^{PI3K}, anti-P-FAK (Y397) and anti-RARα antibodies, using horseradish peroxidase-conjugated Rabbit ExactaCruzF as secondary antibody. The histogram represents the ratio of P-FAK/p85α^{PI3K} densitometric bands in the p85α^{PI3K} immunoprecipitates, derived from three independent experiments.

on PI3K, such as RA and cAMP pathways. Several studies demonstrated that members of the superfamily of nuclear receptors, such as estrogen receptors (ER) (31-33) and thyroid receptors (34), interact with p85α^{PI3K} and activate the PI3K/AKT pathway. A stable physical interaction between RARα and p85α^{PI3K} has been demonstrated in different cell lines (20,35). p85α^{PI3K}Ser83 represents the physical link between Ras, ER and cAMP-PKA, since its phosphorylation stimulates both the activity of PI3K and its binding to ER and PKA RIIβ subunit (9,10). Recent studies demonstrated that Ser83 binding to 14-3-3ζ protein, facilitates the interaction of PI3K with other adapter molecules, enhancing its activity and p85α^{PI3K} membrane localization (11).

Nearly, all receptors binding p85α^{PI3K} can cooperate with cAMP-PKA signals via phosphorylation of p85α^{PI3K}Ser83, thus explaining the pleiotropic nature of the effects exerted by cAMP-PKA on several, apparently unrelated, signalling cascades (9). This study demonstrates that RARα interaction with p85α^{PI3K} depends on the presence of p85α^{PI3K}Ser83, and this residue plays an important role in the fine regulation of the binding induced by cAMP-PKA. The ability of the cAMP/PKA signalling to synergize with RA has been shown in different cellular models (36-38). Our results demonstrate that the co-treatment of MCF7 cells with RA and cAMP amplifies synergistically the RARα/p85α^{PI3K} binding, and that the presence of p85α^{PI3K}Ser83 is crucial for the maintenance of this synergy.

Recent studies highlighted the importance of p85α^{PI3K}Ser83 in the control of cell growth in different cellular models, such as FRTL-5 (10), NIH3T3 cells (9) and vascular SMCs (39), but it is worth noting that the same phosphorylation leads to opposing phenotypes, depending on the cell type. In fact the phosphorylation of p85α^{PI3K}Ser83 inhibits cell proliferation in fibroblasts and VSMC, while it is essential for the correct cell cycle progression in thyroid cells, and does not affect cell proliferation of endothelial cells (9,10,39). Our results show that phosphorylation of p85α^{PI3K}Ser83 is essential for MCF7 cell survival and proliferation, as demonstrated by the low growth rate observed in cells overexpressing the p85A mutant. Ser83 seems to play a role in mediating the growth inhibitory effects of RA. Retinoic acids, including vitamin A and its analogues, regulate growth and differentiation; they suppress tumour formation in animals and have shown to be effective chemotherapeutic agents in many cancers (26). The antitumour effect of retinoids is most often attributed to the induction of differentiation, but these compounds were also shown to arrest the growth of tumour cells (40). Many groups have found that retinoids potently inhibit the growth of breast cancer cell lines (15,16). In our model, we demonstrate that the expression of p85α^{PI3K} mutated forms in MCF7 cells is responsible of a robust reduction of growth inhibition induced by RA treatment, due to a significant reduction of the RARα/p85α^{PI3K} complex formation.

Another important mediator of proliferation is cAMP, which regulates cell proliferation in different manner depending on cell type (41). cAMP induces proliferation in FRTL5 thyroid cell (42,43), while it inhibits proliferation in NIH 3T3 (44). A differential regulation of VSM and endothelial cell proliferation, by cAMP/PKA-activated p85α^{PI3K} has also been described (39). In our model cAMP treatment is able to reduce cell proliferation in all conditions examined. Therefore, we believe that Ser83 is not essential to mediate the inhibitory effects of cAMP, since it can also inhibit cell proliferation in a PKA-independent manner (45). Nevertheless, we demonstrate that phosphorylation of p85α^{PI3K}Ser83 is required for the cAMP/RA synergistic inhibition of growth.

PI3K isoforms have multiple roles in cell migration in many cell types and systems, but the relative contribution of PI3Ks to different steps of migration depends on the cell state and the combinations of stimuli to which it is exposed (1). Direct PI3K activation is sufficient to induce cell motility and invasion (46). To date, the role of p85α^{PI3K}Ser83 in the migratory behaviour of cells has not yet been reported. This study demonstrates that the overexpression of p85A induces a highly significant suppression of MCF7 cells motility, indicating that the phosphorylation of p85α^{PI3K}Ser83 is a critical regulator of this process. Moreover, our data demonstrate that RA treatment causes a significant reduction of motility only in cells expressing p85α^{PI3K} wt, underlying that p85α^{PI3K}Ser83 is essential also for RA-induced inhibition of migration. Numerous studies indicate FAK as an important player in signalling cascades associated with cancer progression and metastasis (47). Moreover, FAK phosphorylation has been demonstrated in different tumour cell types (48-50), and several studies reported that p85α^{PI3K} interacts with FAK (29,33). Our data clearly demonstrate the existence of a ternary complex p85α^{PI3K}/RARα/P-FAK in which P-FAK Y397

participation is strongly enhanced in cells overexpressing p85A and reduced in those overexpressing p85D. We believe that these differences in the ability to bind P-FAK displayed by the $p85\alpha^{PI3K}$ mutants, may be responsible for the different migratory behaviour observed in our experiments. Although increased FAK expression has been correlated with increased cell motility, invasiveness and proliferation (16,51-52), it has been widely demonstrated that the activation of FAK/PI3K/Rac1 signalling inhibits MCF7 cell motility, reflecting differential activation of FAK via phosphorylation of additional sites (53-55). However, phosphorylation of FAK Tyr397 has been found correlated to inhibited cell migration in a human breast cancer cell line (56), and in squamous cancer cell lines (57). We think that the inhibition of migration observed in MCF7 cells after RA treatment, could be due to the enhanced formation of $p85\alpha^{PI3K}$/RAR/P-FAK Y397 complexes induced by RA. Further investigations are required to understand the discrepancy observed in MCF7 cells overexpressing p85D mutant, where RA treatment induces the P-FAK/$p85\alpha^{PI3K}$ complex formation, but is not able to inhibit migration.

This study provides evidence of the existence of a complex RARα/PI3K/P-FAK in MCF7 cells, that is fine-regulated by phosphorylation of $p85\alpha^{PI3K}$Ser83 and RA treatment. Changes in binding affinity of this complex are responsible for cell migration impairment. The data presented above have broad implications since they point to $p85\alpha^{PI3K}$Ser83 as the physical link between different pathways (cAMP-PKA, RA and FAK), and as an important regulator of MCF7 cell proliferation and migration.

Further work will be necessary to determine the mechanisms by which Ser83 interacts with other pathways in order to evaluate the clinical implication of these findings.

Acknowledgements

This work was partly supported by AIRC (Associazione Italiana Ricerca sul Cancro), and by MIUR (Ministry of the Education, University and Research of Italy). We thank Professor Laura Corbo for her excellent advise and helpful discussions, and Dr Silvia Bartollino for preparation of plasmids. This work is dedicated to the memory of Dr Davide Lazzereschi.

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