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Coordinator: Professor G. Vecchio

Tutor: Professor V.E. Avvedimento

cAMP links PI3K to multiple signaling pathways

Candidate
Dr. C. Cosentino

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Administrative Location

Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano"
Università degli Studi di Napoli Federico II

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Carlo M. Croce, MD

Université Paris Sud XI, Paris, Francia

Martin Schlumberger, MD

“cAMP links PI3K to multiple signaling pathways”

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

Claudia Cosentino, Marina Di Domenico, Antonio Porcellini, Concetta Cuozzo, Giorgia De Gregorio, M.Rosaria Santillo, Savina Agnese, Rosina Di Stasio, Antonio Feliciello, Antimo Migliaccio and Enrico V. Avvedimento. **p85 regulatory subunit of PI3K mediates cAMP-PKA and estrogens biological effects on growth and survival.** Oncogene. 2006 Oct 2; [Epub ahead of print]

Giorgia De Gregorio, Anna Coppa, **Claudia Cosentino**, Severine Ucci, Samantha Messina, Arianna Nicolussi, Sonia D'Inzeo, Alba Di Pardo, Enrico V. Avvedimento, Antonio Porcellini. **The p85 regulatory subunit of PI3K mediates TSH-cAMP-PKA growth and survival signals.** Oncogene. 2006 Oct 9; [Epub ahead of print]

List of Figures and Tables

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Abstract

cAMP and PKA stimulate or inhibit cell proliferation, depending on the cell type. For example, cAMP rise induces G1 arrest in NIH 3T3 fibroblast, but it is an essential mitogenic signal in TSH-dependent cells. In both cell types, cAMP stimulates survival (Affaitati et al., 2003).

PI3K has been found essential for cAMP induced cell growth and survival in many cell types (Rameh and Cantley, 1999), but the exact contribution of PI3K signals and the mechanism(s) of actions are still largely unknown.

To determine the molecular mechanism(s) linking PI3K to cAMP-PKA, we identified and mutagenized a specific serine (residue 83) in $p85\alpha^{PI3K}$, which was phosphorylated *in vivo* and *in vitro* by PKA.

Serine 83 was substituted with alanine (p85A) or aspartic acid (p85D), respectively, to abolish or mimic the phosphorylated site. The effects of both mutants on cell proliferation and survival were tested both in NIH 3T3 and TSH-dependent cells. cAMP protected cells from apoptosis induced by substrate detachment (anoikis) or hormone (TSH) starvation. The expression of p85A impaired cAMP mediated cytoprotection and was lethal in thyroid cells FRTL5. p85D, on the contrary, amplified and replicated cAMP effects on survival. Analysis of cell cycle progression showed that the phosphorylation of the serine 83 mediated cAMP induced G1-arrest in NIH 3T3 and S-G2/M progression in TSH-dependent cells.

These results indicate that phosphorylation of $p85\alpha^{PI3K}$ controls G1 length and it is essential for cell cycle progression. The molecular mechanism triggering these effects involves : 1. stabilization of the complex Ras-PI3K and 2. stimulation of PI3K activity.

The data also shows that the regulatory subunit of PKA, RIIβ, converts cAMP from a negative regulator of proliferation (in NIH3T3) to a positive one (in endocrine cells). Interestingly, endocrine cells, including thyroid cells,

express significant amount of RIIβ. In this context RIIβ binds and targets PKA to PI3K and the membrane, stimulating growth and proliferation.

Moreover, phosphorylation of serine 83 of $p85\alpha^{PI3K}$ is essential also for estrogen signaling. p85A expression in MCF-7 impairs the ERα binding to $p85\alpha^{PI3K}$ and, as a consequence, abolishes estrogen induced AKT activity.

Taken together, the data suggests a general mechanism of PI3K regulation by cAMP, operating in various cell types under different conditions**.**

Background

The control of cell growth and survival is a very complex mechanism subject to different signals. Each "*signaling molecule*" (hormones and growth factors) interacts with a specific receptor, and this is the first step of the signal transduction (Alberts, c2002).

1. The cellular receptor may be of two types:

    1) *Intracellular*: typically steroid hormones and small peptides are able to diffuse through the plasma membrane and interact with their own receptor inside the cells. Usually the complex ligand-receptor enters the nucleus and modulates the expression of specific gene through the binding to specific responsive elements on the DNA (Alberts, c2002);

    2) *Extracellular*: Most of growth factors, proteic hormones and neurotransmitter interact with receptors present on the plasma membrane, which can be "*tyrosine kinase*" or "*G protein coupled*" receptor (Alberts, c2002).

    Briefly, the *tyrosine kinase* receptors (RTKs) are constituted of two subunits, their extracellular domain binds the ligand, while the catalytic domain is in the intracellular region. Upon the binding of the ligand this receptors dimerize and the tyrosine residues in the catalytic domain undergo autophosphorylation. The *G protein coupled receptor* (GPCRs), instead, are characterized by an extracellular domain, seven transmembrane segments and an intracellular domain. When the ligand binds the receptor it undergoes a conformational change that enables the activation of a specific trimeric G protein. The G proteins are constituted of three subunits: α, β, γ; in their inactive state the α subunit binds GDP. After the binding of the ligand to the receptor the GDP is substituted by GTP, the α subunit detaches from the β/γ complex and this is the starting point of the transduction pathway in the cell. The

GTP is soon hydrolyzed to GDP, and the G protein returns in its inactive state (Lodish, c1999 ; Alberts, c2002).

The signal transduced by the receptor is then amplified through a kinase cascade inside the cells.

Another important aspect of the signal transduction is that the different molecules regulating the cell behavior are not fully independent from the others. The activated pathway are overlapping, redundant sometimes and linked at many points, they establish a complicate network known as *crosstalk*. An example of crosstalk between receptors is the one between GCPRs leading to cAMP increase and RTKs that activate Ras and PI3K.

1. cAMP and Protein Kinase A (PKA)

GCPRs may bind different kind of G proteins (Table 1), which can act through various mechanisms and second messengers (cAMP, Ca^{2+}, inositol triphosphate, diacylglycerol or cGMP) (Lodish, c1999).

Specific receptors associated to G_s protein, such as TSH receptor (TSHR), activate adenylyl cyclase. This enzyme converts AMP in cAMP that, as a main effect, leads to the activation of PKA. PKA is a holoenzyme composed by two regulatory (R) and two catalytic subunits (cPKA) (Feliciello *et al.*, 2001, 2005). cAMP binds to the regulatory subunits leading to the release of the catalytic ones, which in turn phosphorylate many nuclear and cytoplasmic substrates controlling multiple cell functions, including motility, metabolism, differentiation, synaptic transmission, ion channel activities, growth and gene transcription (Edelman *et al.*, 1987; Haynes *et al.*, 1992; Meinkoth *et al.*, 1993; Feliciello *et al.*, 2001). There are two different types of PKA:

1) *PKA I*: it binds the regulatory subunit RIα that has an high affinity for cAMP, this entails that it is activated by low level of cAMP;

Table 1. Properties of Mammalian G Proteins Linked to GCPRs

G_α Subclass*	Effect	Associated Effector Protein	2nd Messenger
G_s	↑	Adenylyl cyclase	cAMP
	↑	Ca^{2+} channel	Ca^{2+}
	↓	Na^+ channel	Change in membrane potential
G_i	↓	Adenylyl cyclase	cAMP
	↑	K^+ channel	Change in membrane potential
	↓	Ca^{2+} channel	Ca^{2+}
G_q	↑	Phospholipase C	IP_3, DAG
G_o	↑	Phospholipase C	IP_3, DAG
	↓	Ca^{2+} channel	Ca^{2+}
G_t	↑	cGMP phosphodiesterase	cGMP
$G_{b\gamma}$	↑	Phospholipase C	IP_3, DAG
	↓	Adenylyl cyclase	cAMP

A given G_a may be associated with more than one effector protein. To date, only one major G_{sa} has been identified, but multiple G_{qa} and G_{ia} proteins have been described. In some cases (not indicated in this table) effector proteins are regulated by coincident binding to G_a and G_{bg}. KEY: ↑= stimulation; ↓ = inhibition. IP_3 = inositol 1,4,5-trisphosphate; DAG = 1,2-diacylglycerol.

SOURCE: A. C. Dolphin, 1987, Trends Neurosci. **10:**53; L. Birnbaumer, 1992, Cell **71:**1069.

(*Lodish H., Berk A., Zipursky S.L., Matsudaira P., Baltimore D., Darnell J. Molecular Cell Biology New York: W. H. Freeman & Co.; 1999*)

2) *PKA II*: binds the regulatory subunit RII that has a low affinity for cAMP; this implies that it is activated by high level of cAMP, typically upon G_s activation. Class II of PKA can be further divided into two subtypes depending on the kind of RII present in the holoenzyme: RIIα or RIIβ. RIIα is ubiquitous (as PKA I), while RIIβ is expressed mainly in endocrine, brain, fat and reproductive tissues (Edelman *et al.*, 1987; Haynes *et al.*, 1992). Moreover, RIIβ has a binding affinity to cAMP lower than RIIα.

2. Ras

The family of Ras proteins consists of ten highly conserved members, among these there are H-, N. and K-Ras, Rap1A and Rap1B. The common characteristic of all the proteins belonging to the Ras family is that they are small GTP binding protein synthesized in the cytosol, which translocate to the plasma membrane once they are activated. They are activated mainly by RTKs. Once the receptor is phosphorylated it recruits an adaptor molecule, such as Grb2. The adaptor molecule can bind the P-Tyr of the receptor through its SH2 domain and, through its SH3 domain, binds guanine nucleotide exchange factor such as SOS, which activate Ras replacing the GDP with the GTP.

The activity of Ras is limited to the time it needs to hydrolyse the GTP to GDP, and its GTPase activity is increased, many fold, by another class of protein, known as GTPase activating proteins (Kufe, c2003).

2a. Pathways downstream of Ras

The main pathway of Ras is represented by the MAPK (Mitogen Activated Protein Kinase) cascade. GTP-Ras binds Raf, a serine-threonine kinase, and localize it at the plasma membrane. This step is essential for Raf activation, that is reached by several phosphorylations on Ser-Thr and Tyr residues. Successively Raf phosphorylates MAPKK, which phosphorylates in turn ERK (Extracellular Signal Regulated Kinase). ERK can phosphorylate many transcriptional factors, among this Elk1 that increase the transcription of *fos,* an immediate early gene of the cell cycle. The net result is the induction of proliferation or differentiation depends on the activating stimulus and cellular system (Fig.1) (Kufe, c2003).

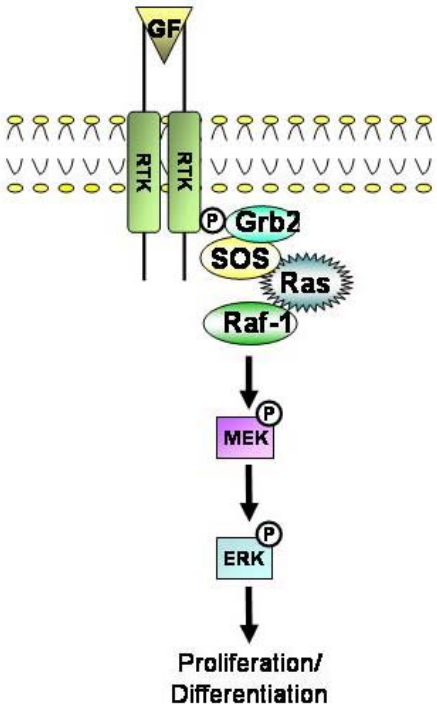

Figure 1: MAPK cascade. The binding of a growth factor (GF) to the tyorsine kinase receptor (RTK) induces the dimerization of the receptor and its autophosphorylation. An adaptor protein (Grb2) binds the activated receptor and recruits a nucleotide exchange factor (SOS), which binds and activates Ras. Ras activates Raf-1 that starts the phosphorylation cascade leading to Erk phosphorylation and activation.

Another pathway activated by Ras is the PI3K (Phosphatidyl-Inositol-3-Kinase) one, involved in the cell survival and growth control (Shaw and Cantley, 2006).

2b. Ras function and its role in cancer

Ras can induce proliferation or differentiation depending on the cell type and above all the stimulus by which it is activated. For example, in mouse fibroblast NIH3T3 the activation of Ras, due to the action of the PDGF, triggers to cell proliferation, while in rat pheochromocytoma cells PC12 induces terminal differentiation after exposure of the cells to NGF. Moreover, in PC12 Ras can even induce proliferation if activated by EGF, instead of NGF (Pollock *et al.*, 1990). Considering the role of Ras in the cell cycle control, it is

not surprising that different mutations of these genes are found in human cancers. The main hot-spots for activating Ras mutations are in the GTP binding domain, these alterations lead to the constitutive activation of Ras disabling it from hydrolyzing GTP. The main aminoacid for this function is the Gly in position 12, which is frequently substituted with Val or Asp. The nature of the mutation correlates even with the aggressivity of the cancer. In fact, Ras-Val12 is more frequently associated to advanced and metastatic colon-carcinoma, while Ras-Asp12 is more often present in benign human colorectal cancer. On a molecular point of view the difference between these two mutations is that Ras-Val12 activates the Erk pathway, on the other end Ras-Asp12 stimulates the PI3K and FAK pathway (Cespedes *et al.*, 2006).

3. PI3Ks

PI3Ks are some of the main players in the pathways regulating cell proliferation, survival and motility. They phosphorylate the inositol on the position 3 and can generate inositol 3 monophosphate, 1,3 diphosphate and 3,4,5 triphosphate. Depending on the structure and the substrate specificity PI3Ks are divided into 3 classes (Fig.2) (Walker *et al.*, 1999).

Class I PI3Ks preferentially phosphorylates phosphatidilinositol 4,5 diphosphate (PtdIns(4,5)P_2) *in vivo*, this class can be divided into two subclasses: IA and IB. The PI3Ks IA are p110α, β and δ, all of which bind an adaptor molecule of 85 KDa (p85) that is required for the binding to the tyrosine kinase receptor, by which the enzyme is activated. The class IB is activated by heterotrimeric G-proteins subunit and they require the binding to a p101 adaptor molecule for their full activation. All the enzyme belonging to class I are characterized by an N-terminal Ras Binding Domain (RBD), this implies that Ras can activate them.

Class II enzymes are large proteins (170-210 KDa) characterized by the PIK domain, 50% similar to the one of PI3Ks of class I, and a C2 C-term domain. Another characteristic domain is the PX, common to molecule such as

NADPH-oxidase, phox-40 and phox-47. *In vitro* class II enzymes preferentially phosphorylate PtdIns and PtdIns-4-P.

The prototype of class III enzymes, VPS34, was first identified in yeast in a screening for mutants defective in protein sorting. This protein is associated with a serine-threonine kinase, VPS15, which is essential for the intracellular trafficking. VPS15 recruits VPS34 under the cellular membrane and enhances VPS34 lipid kinase activity. The preferential substrate of VPS34 is PtdIns. The analog in mammalian cell has been identified; it is a heterodimeric protein and is associated with a phosphatidylinositol transfer protein, which stimulates its activity. This class of PI3Ks lacks the RBD (Fruman *et al.*, 1998; Vanhaesebroeck and Waterfield, 1999; Walker *et al.*, 1999).

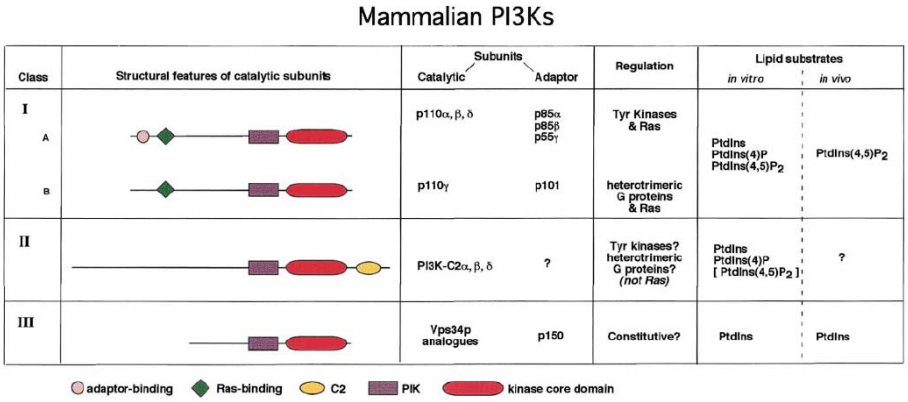

Figure 2: Classes of PI3Ks. The table illustrates the classes of PI3Ks and the defining characteristic of each one. (*Vanhaesebroeck B.,and Waterfield M. D., Experimental Cell Research,1999;* ***253,*** *239–254*)

3a. Structure and function of $p85^{PI3K}$

$p85^{PI3K}$ is the regulatory subunit of PI3K, there are three different isoforms, α, β and γ. They share a high homology and their characteristic domains are: a) an N-terminal SH3 binding domain; b) a Proline Rich Domain (PRD); c) a BCR domain, which is homolog to the GTPase of the Rho family; d) a second PRD; e) two SH2 domains separated by a region called iSH2 (inter-SH2 domain) (Fig.3).

Figure 3: Structure of p85α^{PI3K}. P1 and P2 are respectively the Proline Rich Domain 1 and 2.

It has been shown that the SH3 and the BCR domain are involved in the dimerization of p85^{PI3K}, that, in turn, may be involved in the stabilization of p110^{PI3K} (Harpur et al., 1999). The interaction between p85^{PI3K} and p110^{PI3K} is mediated by iSH2 domain, which binds the N-terminal of p110^{PI3K}. Previous studies have shown that p85^{PI3K} can both stabilize p110^{PI3K} increasing its activity and inhibit it. The actual model to explain the opposite effects of p85^{PI3K} on PI3K activity is that the binding of p110^{PI3K} to p85^{PI3K} is necessary to stabilize the protein. The binding to p85^{PI3K} itself is not sufficient to activate the lipid kinase activity of p110^{PI3K}, to reach the effect it is necessary a conformational change, which is induced by the phosphopeptide binding to the SH2 domain. Experiment with deletion mutant showed that the nSH2 is necessary and sufficient for the activation of p110^{PI3K}. In contrast, if the phosphopetides bind cSH2, to activate p110^{PI3K}, the first 322 aminoacids (SH3 domain, the first PRD and the BH domain) and the nSH2 are required (Carpenter *et al.*, 1993; Klippel *et al.*, 1993; Dhand *et al.*, 1994a; Holt *et al.*, 1994; Hu and Schlessinger, 1994; Yu *et al.*, 1998). Moreover, both the binding of phosphopeptide to the nSH2 and the one to the cSH2 induce a conformational change at the nSH2 (Fig. 4).

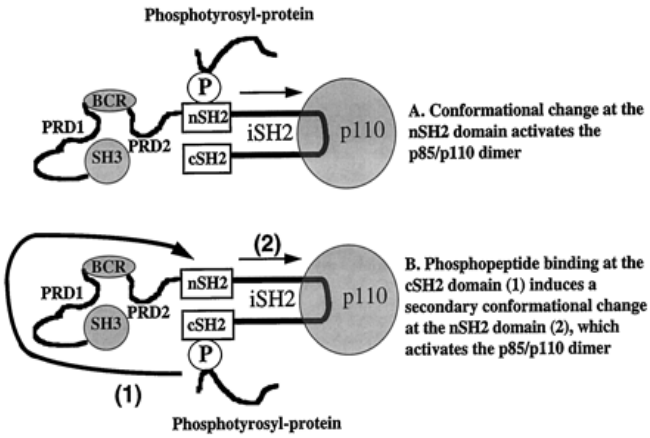

Figure 4: Activation of PI3K. The binding of phosphoprotein at the nSH2 induces a conformational change at the same domain activating PI3K (A), the binding at the cSH2 induces a conformational change at the nSH2 through the N-terminal of $p85\alpha^{PI3K}$ activating the enzyme (B). (*Yu J. et al, J. Biol. Chem., 1998; 273:30199-30203*)

3b. Pathways downstream of PI3K

The PIs generated by PI3K may act on different molecules involved in vesicle trafficking and budding, cell survival and proliferation and protein synthesis.

All PI3K's downstream effectors are characterized by a Pleckstrin Homology (PH) domain, which represents the binding site for the PI. The main effector of PI3K is the serine-threonine kinase AKT.

The binding of the PI to AKT induce a conformational change in the molecule that exposes the threonine 308 and the serine 473. These residues are substrate for two kinases: PI3K Dependent Kinase (PDK) 1 and 2. AKT, in turn, regulate many substrates involved in cell survival, proliferation and protein synthesis (Fig.5**)**:

1) *Cell survival*. AKT activate IKK, once activated this kinase phosphorylates IkB (the inhibitor of NFkB) inducing the realease of NFkB from the binding to IkB. AKT can even activate MDM2, resulting in the inhibition of p53, and can inhibit the pro-apoptotic protein bad. All these mechanisms contribute to induction of cell survival (Lodish, c1999).

2) *Cell proliferation*. PI3K activity is required at different steps of the cell cycle, first at the transition G0-G1, a second peak is at mid G1 and it is necessary for the entry in S-phase and induction of DNA synthesis and for the G2-M transition. AKT is involved at all the steps. Its main role in cell cycle control is the inhibition of Glycogen Synthase Kinase 3β (GSK3β) and the transcriptional factors FOXO. GSK is a negative regulator of cell cycle since it targets cyclin E, cyclin D and Myc for degradation. FOXO is the O subgroup of Forkhead transcriptional factors (TFs) family. These TFs induce the expression of molecule essential for quiescence maintenance, such as $p27^{KIP}$, p130 and cyclin G2 (Martinez-Gac *et al.*, 2004; Garcia *et al.*, 2006).

3) *Protein Synthesis*. AKT activates mTOR and p70S6K inducing protein synthesis, and, in turn, increase of cell mass (cell growth) that is essential for cell division (Garcia *et al.*, 2006).

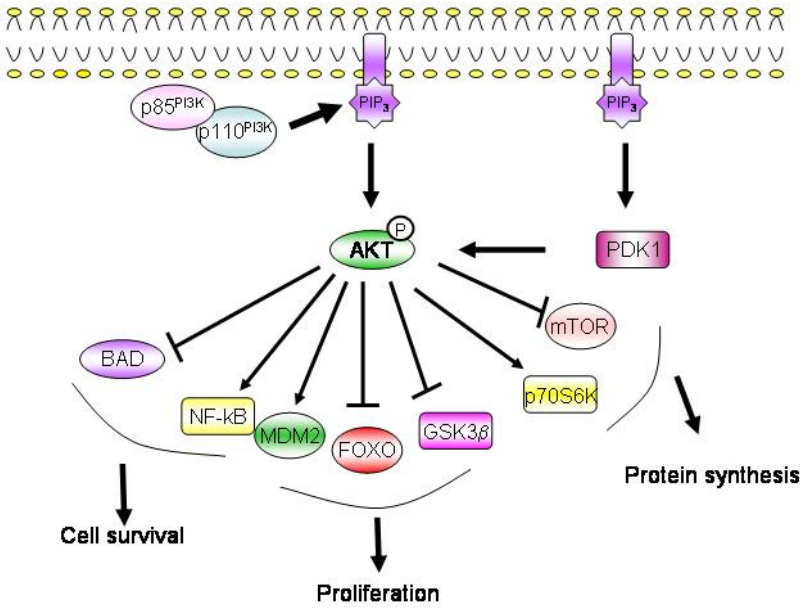

Figure 5: PI3K-AKT pathway. The activation of PI3K leads to the formation of PtdIns(3,4,5)P_3 (PIP_3) that activates directly or indirectly AKT. AKT phosphorylates different substrates controlling cell proliferation, survival and growth (protein synthesis).

3c. PI3K function and its role in cancer

Class IA PI3Ks play a main role in control cell replication, migration, survival and glucose homeostasis (Kufe, c2003). The oncogenic potential of PI3K and its target AKT was first revealed by two retroviruses (Bader *et al.*, 2005; Kang *et al.*, 2005):

1) the avian sarcoma virus ASV16, which encodes a constitutive active homolog of $p110\alpha^{PI3K}$, P3k, that is fused to the Gag sequence of the virus;

2) the murine lymphoma virus AKT8, that encodes for constitutively active AKT.

The oncogenicity of the two viral proteins is due to the constitutive membrane addresses, because of the myristoylation, and their constitutive kinase activity. In fact, if the wild type $p110\alpha^{PI3K}$ is overexpressed in normal chicken embryo fibroblast no alteration of cell growth is observed. In contrast, mutant $p110\alpha^{PI3K}$ can induce strong oncogenic transformation in the same cellular system.

In the few past years, different mutations of PIK3CA, the gene encoding $p110\alpha^{PI3K}$, have been identified, outlining the role of PI3K in human cancer. These mutations are somatic missense ones, they are tumor-specific and they map to a few hot-spots. The three most common mutations are E542K, E545K and H1014R. The H1014R mutation is in the substrate binding pocket and this suggest an increased binding affinity of mutant PI3K for $PtdIns(4,5)P_2$. The other two mutation, instead, are in the helical domain. It is not likely that they increase the catalytic activity of the enzyme since they are too far from the catalytic domain. Considering that both $p85^{PI3K}$ and Ras bind to the N-terminal of $p110^{PI3K}$ (even if not to the helical domain), it is possible that the E542K and the E545K substitution alter the binding to these proteins, or to other unknown regulatory proteins.

Other mutations that constitutively activate PI3K are the $p85^{PI3K}$ mutations. $p65\alpha^{PI3K}$ is the first oncogenic variant of $p85^{PI3K}$ that has been identified.

Jiemenez *et al*.in 1998 (Jimenez *et al.*, 1998), in fact, cloned p65α^{PI3K} from a murine lymphoma generated through X-Ray irradiation. This variant lacks part of the iSH2 and the cSH2, can still bind p110α^{PI3K} and can localize the PI3K complex at the plasmamembrane. This results in the constitutive activity of the enzyme and contributes to cellular transformation. Another oncogenic variant of p85α^{PI3K} is p76α^{PI3K}, this lacks the cSH2 and it has been found in a Hodgkin's lymphoma cell line (Jucker *et al.*, 2002). In human ovarian and colon cancer Philip *et al*. found a few mutation in splicing site leading to exon 13 skipping, so to the deletion of the region of the iSH2 proximal to serine^{608} (Philp *et al.*, 2001). The serine^{608} is an important auotregulatory site. Its phosphorylation by p110α^{PI3K} results in a three-sevenfold decrease of the lipid kinase activity (Carpenter *et al.*, 1993; Dhand *et al.*, 1994b). Even the oncogenic variants previously described impair the ser^{608} phosphorylation.

Other mutations frequently found in human cancer are:

1) *mutation of the upstream receptor*, such as PDGF, EGF, ErbB2. The increased expression and activation of the receptors result in increased activity of the downstream effectors (Osaki *et al.*, 2004);

2) *mutation of phosphatase PTEN* (phosphatase and tensin homologue deleted on chromosome 10). PTEN dephosphorylates PIP_3 to generate PIP_2 downregulating the PI3K pathway. It is a tumor suppressor gene frequently inactivated in primary cancers in thyroid, breast, prostate, uterus, central nervous system, soft tissue and above all colorectal cancer (Osaki *et al.*, 2004).

3) *mutation of AKT*, amplification of AKT2 are present in ≈15% of human ovarian cancer and ≈10% of human pancreatic cancer. Its amplification is not due to polysomy of the chomosome 19, where AKT gene is located (Cheng *et al.*, 1996). Moreover, AKT alteration in cancer correlates with a poor prognosis because of the increased cell motility,that results in an higher tumor invasiveness (Balsara *et al.*, 2004). In recent studies it has been shown a strong prognostic

significance for AKT constitutive activation in acute myeloid leukemia, it correlates, in fact, with a shorter overall survival (Min *et al.*, 2003).

4. Crosstalk between different signaling pathways: the paradox of cAMP

The different signaling pathways in the cell form complicate networks, where the many transduction systems communicate with each other in feed-forward and feed-back regulatory loops. Considering that it exists a wide range of specialized cell types, it is essential for the ubiqitously expressed signal transduction systems to be adapted to meet the specific requirements of the cell. It is paradigmatic, in this context, the role of cAMP. In fact, in cell such as rat thyroid cell FRTL-5 and Swiss 3T3 fibroblast cAMP induces proliferation (Lee *et al.*, 1998; Ariga *et al.*, 2000), while in most cell types (Magnaldo et al., 1989) it inhibits proliferation. How is it possible is not yet fully understood. It has been proposed that cAMP inhibits proliferation through the inhibition of ERK. This is due to mechanisms PKA-dependent and others cAMP, but not PKA, dependent. First, PKA can phosphorylate Raf-1 on serine 43, 259 and 621 blocking Raf-1 activation (Cook *et al.*, 1993; Mischak *et al.*, 1996; Dhillon *et al.*, 2002). Second, cAMP activates some cAMP dependent nucleotide exchange factor (EPAC) leading to the activation of Rap1. Rap1 is a small G-protein highly homologue to Ras and binds Raf-1 inhibiting it (Stork and Schmitt, 2002). This leads to the cAMP mediated inhibition of proliferation in cell as NIH3T3. In cell where proliferation is activated by cAMP, it has been proposed that ERK is activated by cAMP in a PKA- independent manner. In fact, it has been shown that Rap1 can stimulate the ERK pathway through B-Raf (Zwartkruis *et al.*, 1998). These observations suggest that the effect of cAMP on cell proliferation may depend on the differential expression of Raf isoforms in the distinct cell types (Fig.6).

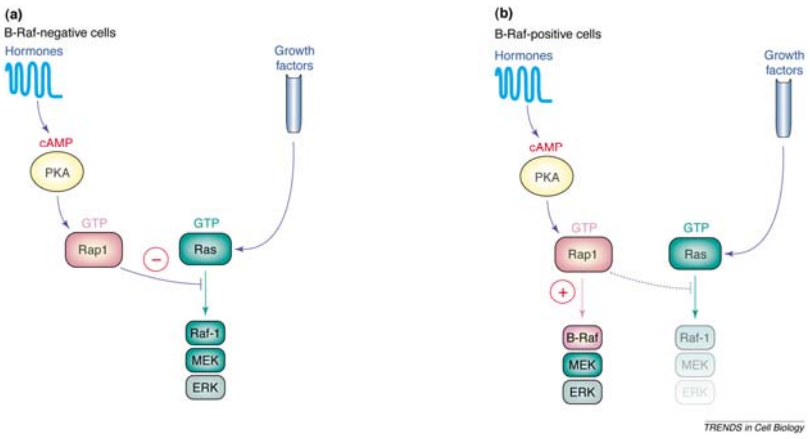

Figure 6: cAMP through Rap1 regulates ERK activity. cAMP activates Rap1, in B-Raf negative cells this leads to Raf-1 inhibition and suppresses ERK activity (**A**), instead, in B-Raf positive cells (**B**), Rap1 activates ERK.(*Stork and Schmitt, TRENDS in Cell Biology, 2002; 258-266*)

Another example of crosstalk between two pathways is the Raf-AKT one. RTKs activate both the PI3K and Ras pathways. In MCF-7 cells, a human breast cancer cell line, Insulin-like Growth Factor I (IGF-I) induces cell proliferation and survival through PI3K-AKT and growth arrest and differentiation through Ras-Raf. According to Moelling *et al* (2002), the effect of IGF-I on MCF-7 cells depends on the concentration of the growth factor. In particular, high doses of IGF-I activate AKT quickly and strongly enough to suppress Raf-1 activity. AKT, in fact, can phosphorylate Raf-1 on serine 259 inhibiting it. In this context proliferation is stimulated. Low doses of IGF, instead, are not enough to suppress Raf-1, so both pathways are active and the differentiation signals are favored over the mitogenic ones (Fig.7, (Moelling *et al.*, 2002).

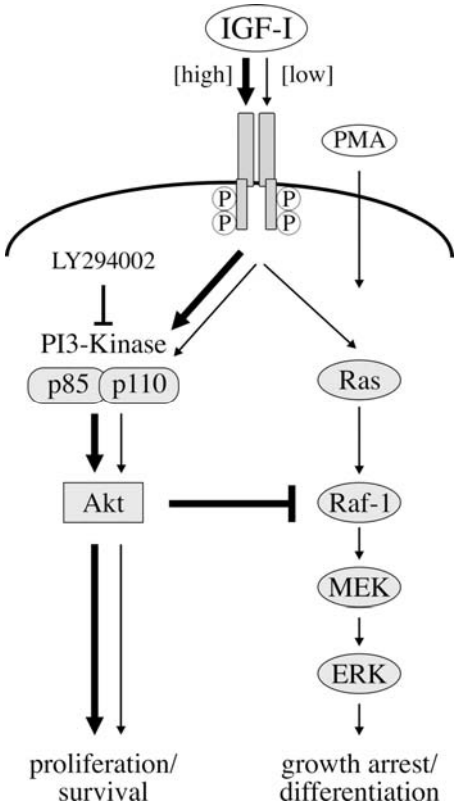

Figure 7: AKT-Raf crosstalk in MCF-7 cells. High concentration of IGF-I leads to the AKT dependent phosphorylation of Raf-1 and induction of the proliferation. This effect is reverted by LY294002, a specific PI3K inhibitor. Low dose of IGF-I activate both PI3K and Raf-1 (*Moelling et al, J. Biol. Chem, 2002; 31099-31106*).

Aims of the study

It has been previously shown in our laboratory that PI3K interacts with Ras upon cAMP stimulation, and this complex is essential for G1-S transition in these cells.

Moreover, the formation of the complex was H89 sensitive, indicating that PKA was involed in the regulation of the interaction. *In vitro* kinase assay revealed that $p85\alpha^{PI3K}$ was efficiently phosphorylated. These data suggested that cAMP-PKA selectively influences Ras effector pathway through $p85\alpha^{PI3K}$ phosphorylation.

1) The first part of the work presented here, focuses on the identification and validation of the PKA phosphorylation site on $p85\alpha^{PI3K}$.

2) The second part, instead, investigates the biological role of this phosphorylation in different cell types. It is worth noting that cAMP leads to opposite effects on proliferation depending on the cellular system (Pastan *et al.*, 1975). To investigate the role of phosphorylation of $p85\alpha^{PI3K}$ and its association with Ras in the regulation of cell proliferation, the experiments presented were performed both in cells where proliferation was inhibited (fibroblasts NIH 3T3) or stimulated (TSH-dependent cells) by cAMP.

Materials and Methods

1. Plasmid construction

The cDNA encoding for bovine p85α^{PI3K} wild type (acc. n.:163476) was cloned in the pSG5 vector (gift of Dr. J. Downward). The region of p85α^{PI3K} from the Xho I restriction site in position 1014 (acc. n.:163476) was amplified by PCR with a 3'end primer containing the Flag sequence (MDYKDDDDK) and a BamHI restriction site.

The PCR product was sub-cloned in the II-TOPO vector (Invitrogen), digested with Xho I and BamHI, extracted and ligated to a pSG5-p85α^{PI3K} vector previously digested with the same restriction enzymes. The vector encoding p85α^{PI3K}-flag was then used as template for site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene) to convert serine 83 in alanine or aspartic acid. All the constructs were verified by DNA sequence analysis.

2. Materials and Reagents

Unless otherwise specified, drugs and chemicals were obtained from Sigma Aldrich and cell culture supplies were purchased from standard suppliers, e.g. Falcon, Life Technologies inc., Hyclone.

The antibodies used were: anti-pan-Ras (clone 10, mouse monoclonal, UBI), anti-Raf1 (rabbit polyclonal, Santa Cruz), anti-p85^{PI3K} (rabbit polyclonal, UBI), anti-P-Serine (rabbit polyclonal, Zymed), anti-Erk 1/2 (rabbit polyclonal, Santa Cruz), anti-P-Erk 1/2 (mouse monoclonal, Santa Cruz), anti-P-Akt ser 473 (rabbit polyclonal, Cell Signaling), anti-Akt (rabbit polyclonal, Cell Signaling), anti P-Gsk ser 21/9(rabbit polyclonal, Cell signaling), ant GSKα/β (mouse monoclonal, UBI). The anti-flag antibody was the mouse monoclonal Sigma M2 antibody.

3. Cell culture and transfections

Cell lines used in the experiments were grown as follows:

1. murine fibroblasts NIH 3T3 in DMEM 10% bovine serum;
2. rat thyroid cells FRTL-5 in medium with 5% calf serum and six hormones (1mU/ml TSH, 1μg/ml Insulin, 3.6 μg/ml Hydrocortisone, 5 μg/ml Transferrin, 10 ng/ml Somatostatin, 20 μg/ml Glycil-histidil-lysine);
3. NTCRII cells in DMEM 10% foetal bovine serum. Considering that these cells were conditional stable clones of RIIβ and TSHR, the medium was supplemented with puromycin 2.5 μg/ml and geneticin (G418) 200 μg/ml to maintain the selection and tetracycline 1.0 μg/ml to keep the genes silenced. The selection and tetracycline were removed 48 hr before starting the experimental procedures described in the results (Porcellini *et al.*, 2003);
4. MCF-7 breast cancer cells in DMEM with 5% foetal bovine serum, supplemented with 6 ng/ml insulin and 3,75 ng/ml hydrocortisone;
5. HeLa cells in RPMI 10% foetal bovine serum.

All the media were supplemented with penicillin/streptomycin 100mU/mL and 2 mM glutamine.

Cells were transfected with lipofectamine according to the manufacturer's recommendations (Gibco Invitrogen). Briefly cells were transfected at 80% confluence with 4μg of DNA for each 100mm dish. The lipofectamine was used 1μL for each μg of DNA. The mix DNA-lipofectamine was incubated 45 minutes at room temperature to allow the formation of the precipitates. Before adding the mixture cells were washed with PBS and the growing medium was replaced with medium without serum and antibiotics. 5 hours after the adding of the mixture the normal concentration of serum was restored.

4. Cell lysis and immunoprecipitation

Cells were collected in ice-cold PBS and spun at 1500 rpm for 3 minutes. Pellets were re-suspended in lysis buffer (50 mM Tris-HCl pH 7.4, 1% Nonidet NP-40, 100 mM NaCl, 2 mM EDTA 50 mM NaF, 0.1 mM $NaVO_3$ 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate and a protease inhibitor cocktail). After 15 minutes incubation on ice the samples were spun at 13000 rpm for 10 minutes. Cell lysates were transferred to other tubes and quantified at the spectrophotometer using the Bradford assay (BioRad protein assay).

Protein lysates were diluted to 2 mg/ml and were incubated with 4 μg of antibody/0.5-1 mg of protein at 4°C in gentle rock agitation overnight. At the end of incubation, 20 μl of A/G plus were added to samples and the immunoprecipitates were washed three times with lysis buffer and then collected by centrifugation. The bound proteins were eluted with one volume of 2× Laemmli buffer or, when indicated, using 0.1 M glycine HCl pH 3.5. In the latter case, 1M Tris-HCl pH 8 was added to the eluted proteins to neutralize the pH.

5. GST pull-down

GST pull-down were performed as described by (Grieco *et al.*, 1996). Briefly, cells were lysed in 200mM NaCl, 50mM Tris-Hcl pH 7.5, 2mM $MgCl_2$, 10% glycerol, 1% NP 40, 10μg/ml Trypsin inhibitor, 1μg/ml aprotinin, 1μg/ml leupeptin, 10mM NaF, 10mM Na_3VO_4 (Pull Down buffer). 1 mg of protein extract was incubated with 1 microgram of GST-RIIβ fusion protein or the control protein (GST) for 4 hours at 4°C in gentle rock agitation. The pellets were washed 5 times in pull down buffer and re-suspended in one volume of 2× Laemmli buffer.

6. Western blot

Total cell extracts and immunoprecipitates were separated on 10% SDS-PAGE and transferred onto nitrocellulose filter. The filters were blocked in

TBS 0,1% TWEEN (TBS-T) 5% not-fat-dry-milk (NFM) for 1 hour at room temperature. The filters were washed three times with TBS-T and incubated with the indicated primary antibody. Primary antibodies were diluted according to the manufacturer's recommendations. The filters were successively washed three times with TBS-T and incubated with the peroxidase conjugated antibody diluted 1:3000 in TBS-T 3% NFM. The signal was detected with chemiluminescence system (Feliciello et al., 2000).

7. *In vitro* phosphorylation

HeLa cells were transiently transfected with $p85\alpha^{PI3K}$-flag and p85A. 48 hours after transfection, cell lysates were immunoprecipitated with non-immune IgG or anti-flag antibody 15 h at 4°C. Protein A/G bound immunoprecipitates were washed twice with lysis buffer and finally with Kinase buffer (Hepes 20 mM, $MgCl_2$ 10 mM, pH 7.4). The washed immunoprecipitates were treated with 0.4 μg of Protein Kinase A. Each aliquot was incubated in a final volume of 30 μl of Kinase buffer containing 10^{-5} M cAMP, 100 μM ATP and 10 μCi[γ^{32}P-ATP] for 30 minutes at 30°C. The reaction was stopped by adding one volume of 2× Laemmli buffer (Ciullo *et al.*, 2001).

8. *In vitro* protein synthesis

$p110\alpha^{PI3K}$ and $p85\alpha^{PI3K}$ wild type or p85A or p85D were co-transcribed and co-translated *in vitro* in [^{35}S]methionine-containing reticulocyte lysate according to the manufacturer's recommendations. The conditions of the reaction were optimized to reach the same efficiency of synthesis of the co-transcripted and co-translated proteins. The amount of template used was 1μg, and the optimal ratios of the two template were: $p85\alpha^{PI3K}$ wt / $p110\alpha^{PI3K}$ 1:2; p85A/ $p110\alpha^{PI3K}$ and p85D/ $p110\alpha^{PI3K}$ 2:1. The reactions were incubated for 1h 30' at 30°C and diluted 1:100 with PBS conatining 0.5% Triton X-100 and

protein inhibitors. The control reaction was performed using only $p110\alpha^{PI3K}$ as template.

The diluted lysates were immunoprecipitated with the anti-Flag antibody, as described previously. The immunocomplexes were washed three times with PBS-0.5% Triton X-100, solubilized in 2× Laemmli buffer and boiled. The samples were separated on SDS-PAGE. The gel was fixed 30 minutes in a solution containing 10% acetic acid and 20% methanol, washed three times in deionized water and treated with 100 mM salycilate to enhance the radioactive signal. The gel was then dried and expose on a autoradiography film. On the gel the input (1 µl of the reaction mix using 1 µg of plasmid as template) was loaded as control of the reaction.

9. Apoptosis assays

NIH 3T3 were co-transfected with GFP and the indicated vector. 48 hours later cells were plated in DMEM 0,1% bovine serum -/+ 200µM CPT-cAMP on plates covered with 2% agarose (*anoikis*) . After a 5 hours incubation cells were collected and washed three times with PBS before the 5 minutes incubation with propidium iodide. Successively cells were analyzed by Fluorescent Activated Cell Sorter (FACS) using CELLQuest software (Becton Dickinson). The percentage of death (PI positive cells, i.e. red population) was calculated on the population positive for GFP (green population). The experiments were performed in triplicate.

$5*10^5$ NTCRII cells were transfected as described. 24 hours later cells the normal medium was replaced by 0,5% serum medium with or without 10mU/mL TSH or 100µM cAMP. 18 hr after treatment, cells were fixed in 2% paraformaldehyde/1X PBS, 10 min, RT and washed one time in PBS + 50 mM glycine for 10 min at RT and 3 times for 5 min in PBS. Cells were permeabilized with 0.5% triton X-100/ 1X PBS for 10 min, washed 3 x 5 min in PBS and incubated with 100 µl of 1X TdT reaction mix. TUNEL reaction was carried out at 37°C for 60 min using 15 Units of TdT (ROCHE) and 2 µl

of 2mM BrdUTP. BrdUTP incorporation was revealed by anti-BrdU-FITC and the samples were then stained in Propidium Iodide. The data were acquired and analyzed by CELLQuest software for bivariate-analysis of DNA content versus BrdU. Experiments were performed in triplicate.

10. Cell growth analysis

$5*10^5$ cells were transfected with the indicated vectors. 48 hours later cells were plated in 60 mm dishes and growth in 0,5% serum containing medium. After 18 hr cells were induced into the cycle with 10 mU/ml TSH or EGF 100 ng/ml. Cells were collected and washed twice with PBS. Successively cells were fixed in 70% ethanol and stained for 30 min at room temperature in 0.1% triton-X100, 0.2 mg/ml DNase-free RnaseA, 20 µg/ml Propidium Iodide.

Cells were acquired using the FACScan Flow Cytometer (Becton Dickinson) and analyzed by Cell Fit Cell-Cycle Analysis Version 2 to define the percentage of cells in the different phases of cell cycle.

11. 5'-bromo-2'-deoxyuridine (BrdU) labelling

BrdU incorporation was assayed in a pulse-chase experiment. Cells were labelled for 30 min with BrdU to a final concentration of 20 µg/ml and harvested at 0, 90 and 270 min. After treatment, cells were fixed in ice-cold 70% ethanol for 4 hr at +4 °C and washed 3 times for in PBS. Cell pellet was re-suspended in 0.25 ml of 1N HCL and let stand 20 min at room temperature. After acidic denaturation of DNA, cells were washed 2 times in phosphate/citric buffer (0.2 M Na_2HPO_4; pH 7.4). BrdU incorporation was revealed by anti-BrdU-FITC and then stained for 30 min at room temperature in 0.1% triton-X100, 0.2 mg/ml DNase-free RnaseA, 20 µg/ml Propidium Iodide. Fluorescence was determined by using the FACScan Flow Cytometer. Experiments were performed in triplicate. The data were acquired and analyzed by CELLQuest software for bivariate-analysis of DNA content versus BrdU and by Cell Fit Cell-Cycle Analysis Version 2 for DNA content analysis.

12. Lipid kinase assay

Lipid kinase activity was determined as described by (Maier *et al.*, 1999). Briefly, the assays were carried out in a final volume of 50 µl containing 0.1% bovine serum albumin, 1 mM EGTA, 120 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM -glycerophosphate, 7mM $MgCl^2$ (buffer E). Lipid vesicles (30 µl containing 320 µM phosphatidylethanolamine, 300 µM phosphatidylserine, 140 µM phosphatidylcholine, 30 µM sphingomyelin, supplemented with 40 µM PI-4,5-P2 in buffer E) were sonicated 1 hour and incubated on ice 10 min. The immunoprecipitates were added to the lipid mixture and incubated for 10 min at 4°C in a final volume of 40 µl. The reaction was started by adding 40 µM ATP (1 µCi of [-32P]ATP in 10 µl of the assay buffer. The reaction was incubated 15 minutes at 30°C and then stopped with 150 µl of 1 N ice-cold HCl. The lipids were extracted by vortexing samples with 500 µl of chloroform/methanol (1:1). After centrifugation the organic phase was washed twice with 200 µl of 1 N HCl. Phosphorylated lipids were separated by TLC developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60:47:11.3:2), dried, and visualized by autoradiography and quantified with Phosphor-Imager.

RESULTS

1. PKA phosphorylates serine 83 of p85α^{PI3K}

It has been previously shown in our laboratory that PKA efficiently phosphorylates p85α^{PI3K} *in vitro* (Ciullo *et al.*, 2001). The sequence analysis revealed a PKA consensus in the sequence of bovine p85α^{PI3K} (KKIS). This consensus is highly conserved in evolution (Fig.8): KKIS in bovine/human and KRIS in mouse/rat. Moreover, no PKA consensus was found in p85β^{PI3K}.

Figure 8: Alignment of p85α^{PI3K} sequences from different species. p85α^{PI3K} presents an highly conserved PKA consensus at the residues 80-83.

To determine if this is a *bona fide* PKA phosphorylation site and its biologiacal role, the serine in the consensus (serine 83) was substituted with alanine to prevent the phosphorylation or aspartate to mimic it. To distinguish the exogenous from the endogenous protein, wild-type p85α^{PI3K} encoding cDNA was fused to a C-terminal Flag sequence (MDYKDDDDK) and subcloned in a pSG5 vector. The tagged wild-type cDNA was used as a template for the mutagenesis reaction (see Materials and Methods).

1a. In vitro phosphorylation

The wild-type and the alanine tagged proteins were transiently expressed in Hela cells. The cell lysates were immunoprecipitated with anti-Flag antibody or non-immune IgG (SNI) as described in Materials and Methods. The

immunoprecipitates were incubated *in vitro* in kinase buffer containing 10^{-5} M cAMP, 100 μM ATP and 10 μCi[γ^{32}P-ATP] for 30 minutes at 30°C, with or without recombinant PKA (cPKA). The immunoprecipitates were separated on SDS-PAGE and analysed by western blot with anti-p85 antibody (Fig. 9, upper panel) and by autoradiography (Fig.9, bottom panel).

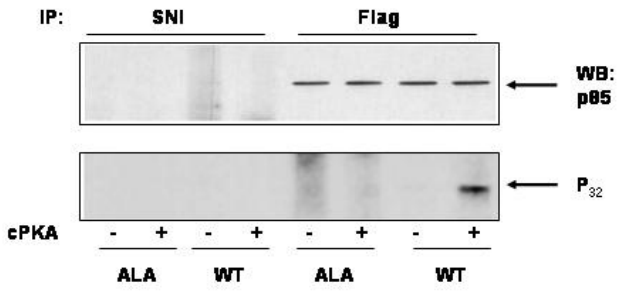

Figure 9: *In vitro* phosphorylation of serine 83 in p85α^{PI3K} by cAMP-PKA. HeLa cells were transiently transfected with p85α^{PI3K}-flag wild type or p85A; 48 hours after transfection, cell lysates were immunoprecipitated and *in vitro* phosphorylated with cPKA as described in Material and Methods. Sample aliquots were run on SDS-PAGE and subjected to immunoblot (upper panel) or autoradiography (bottom panel).

p85α^{PI3K} wild-type, but not p85A, was efficiently phosphorylated *in vitro* by PKA. This reaction was dependent on cPKA, because omission of cPKA from the mixture did not results in p85α^{PI3K} phosphorylation. The western blot in the upper panel shows that the immunoprecipitation efficiency was comparable in all the samples.

This result indicates that serine 83 is the PKA phosphorylation site on p85α^{PI3K}.

1b.In vivo phosphorylation

To verify that PKA phosphorylates p85α^{PI3K} not only *in vitro* but also *in vivo* quiescent breast carcinoma cells (MCF7), were transiently transfected with p85WT or p85A encoding vector. 24 hours after transfection cells were stimulated with 100μM CPT-cAMP or left untreated. Protein lysates were immunoprecipitated with anti-Flag antibody and the immunoprecipitates were

separated on SDS-PAGE. The proteins were transferred onto nitrocellulose filter and analyzed by western blot with anti-p85 and anti-phospho-serine (anti-P-ser) antibodies. Figure 10A shows that p85WT, but not p85A, was efficiently phosphorylated following cAMP treatment (bottom panel). The amount of protein in all samples was the same (upper panel).

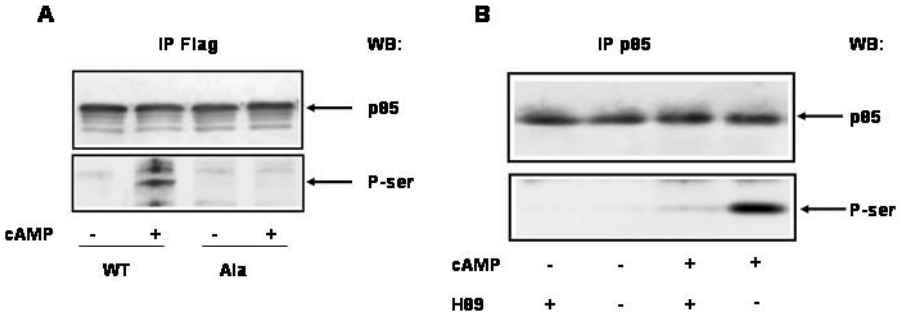

Figure 10: *In vivo* phosphorylation of serine 83 in p85α^{PI3K} by cAMP-PKA. (A). Quiescent MCF7cells were transfected with p85α^{PI3K} or p85A. 24 hours after the transfection cells were treated with 100 μM cAMP. The anti-Flag immunoprecipitate were analyzed by western blot with anti-p85^{PI3K} (upper panel) and anti-phosphoserine (lower panel) antibodies. (B) NIH 3T3 were serum starved in DMEM 0,1% calf serum, after 16 hours they were treated with or without 100 μM cAMP for 10' after a 30' minutes pretreatment with or without 10 μM H89. Cell lysates were immunoprecipatated with anti-p85 antibody and analysed by western blot with anti-p85 antibody (upper panel) and anti-phosphoserine (lower panel).

To monitor the phosphorylation of the endogenous protein, un-transfected murine fibroblasts NIH 3T3 were serum starved for 16 hours and treated with or without cAMP, after a pre-treatment with or without 10μM H89, a PKA inhibitor. Cell lysates were immunoprecipitated with anti-p85 antibody and analyzed with anti-p85 and anti-P-ser antibodies. cAMP induced p85α^{PI3K} phosphorylation (Fig 10B, bottom panel). This event was PKA dependent, since H89 abolished the phosphorylation. The amount of protein was the same in all the samples (Fig 10B, upper panel).

Taken together these data indicate that PKA phosphorylates p85α^{PI3K} on serine 83, and this site is the major, if not the only, PKA phosphorylation site on the protein, since p85A was not phosphorylated neither *in vitro* nor *in vivo*.

2. Biological effects following the phosphorylation of p85α^{PI3K} in NIH 3T3

Both cAMP and PI3K regulate cell survival and growth (see Background). In particular cAMP protects cells from serum deprivation induced apoptosis (Affaitati *et al.*, 2003) and regulates cell proliferation in different manner depending on cell type (Pastan *et al.*, 1975).In fact, cAMP induces proliferation in such cells as thyroid cell FRTL5 (Lee *et al.*, 1998; Ariga *et al.*, 2000), while it inhibits proliferation in most cell types, such as NIH 3T3 (Magnaldo *et al.*, 1989). The mechanisms underlying these effects are not yet defined.

On the other side, as discussed in the Background Section, PI3K-AKT is one of the most important pathway promoting survival and cell growth. These observations suggest that cAMP-PKA dependent phosphorylation of serine 83 may affect these important biological functions.

2a. cAMP induced-survival depends on phosphorylation of p85α^{PI3K}

To validate our hypothesis, inhibition of *anoikis* has been tested. *Anoikis* is an apoptotic pathway triggered by loss of cell adhesion to the extracellular matrix and strictly dependent on PI3K (Frisch and Francis, 1994; Khwaja *et al.*, 1997). In cell culture it can be easily assayed by culturing the cells on plates covered with a thin 2% agarose layer.

To test the effects of mutagenesis of serine 83 on the *anoikis*, NIH 3T3 cells were co-transfected with a GFP encoding vector and the wild type or mutant vesions of p85α^{PI3K}. 48h later, the cells were plated in 0,1% CS -/+ 200 μM CPT-cAMP medium on 2% agarose. After 5h cells were stained with propidium iodide and the percentage of apoptosis determined by FACS analysis. The histogram in figure 11B shows that cAMP protected cells from apoptosis, and the over-expression of p85WT amplified the effect of cAMP.

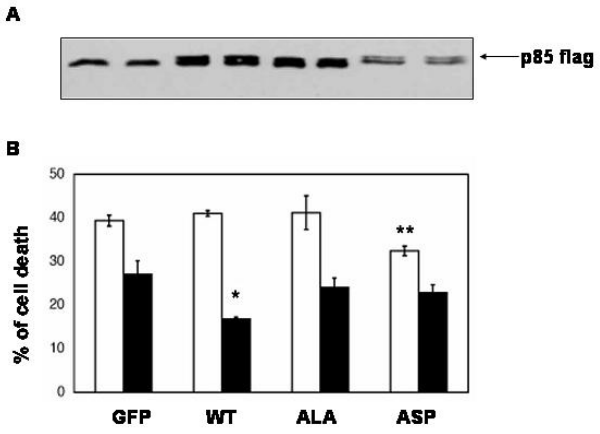

Figure 11: Analysis of cell survival. NIH 3T3 were co-transfected with a GFP encoding vector and the wild type and mutant of $p85\alpha^{PI3K}$. (A) The expression was analyzed by western blot. the exogenous molecule is the upper band of the doublet. (B) fraction of apoptotic cells after *anoikis* was determined by FACS analysis . The data are the mean of three independent experiments; * indicates $p \leq 0.01$ comparing cell death in the presence of cAMP in all samples.

The expression of p85A abolished the cAMP mediated protection (*). On the other side, the p85D expressing cells are more resistant to *anoikis* in absence of cAMP (**). This indicates that, at least in part, the S83D substitution can mimic cAMP effects on survival. It is worth noting that p85D expressing cells showed a reduced response to cAMP, probably caused by low expression of p85D (fig 11A).

These data indicate that $p85\alpha^{PI3K}$ mediates the cytoprotective effect of cAMP during *anoikis* and its phosphorylation is necessary for the cAMP induced survival.

2b. cAMP mediated G1-S arrest requires phosphorylation of $p85\alpha^{PI3K}$

To test if the phosphorylation of $p85\alpha^{PI3K}$ is involved in cAMP growth arrest, the wild type and the mutant $p85\alpha^{PI3K}$ were expressed in NIH 3T3. Cells were serum-starved 15 hours and induced into the cycle with 2 % serum in the presence or absence of cAMP 200 μM. 12 hours later cells were collected in ice cold PBS, fixed with 70% ice cold ethanol and stained with propidium iodide and then FACS analyzed. Fig.12A and 12B show that cAMP

accumulated cells in G1 and reduced the number of cells in S-phase. The expression of both mutants abolished the response to cAMP. p85A was not sensitive to cAMP arrest while the p85D expressing cells accumulated in G1 even in the absence of cAMP.

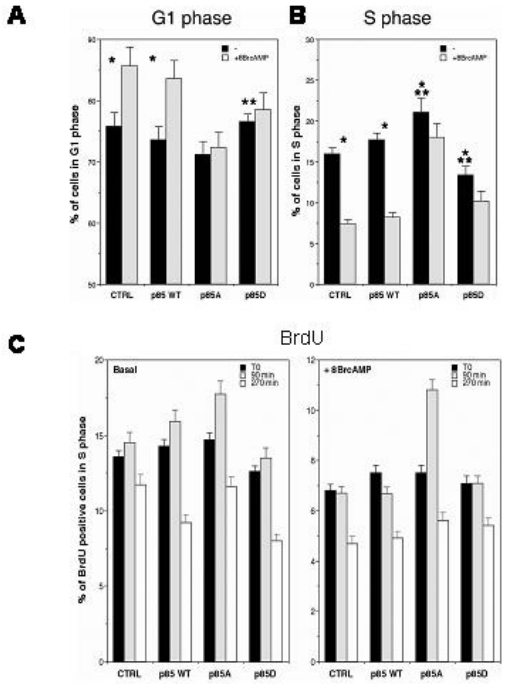

Figure 12: Analysis of cell proliferation. Transfected NIH 3T3 were serum starved in the presence or absence of 200μM cAMP for 12h and then analyzed by FACS to determine the percentage of cells in G1 (A) and S-phase (B) The data are the mean of three independent experiments. * indicates $p \leq 0.01$ basal versus cAMP; ** $p \leq 0.01$ basal p85A versus p85D; *** $p \leq 0.01$ basal p85α wild type, A or D-transfected cells versus control plasmid-expressing cells. (C) Pulse (30')-chase of BrdU incorporation. The columns represent the fraction of labeled cells stained with propidium iodide at 0 (black columns), 90 (gray columns) and 270 minutes (white columns) from the initial cell cycle induction (10% serum). The data are the mean of three independent experiments.

In order to test if these effect were caused by difference in the number of cells entering the S-phase, instead of the alteration of the S-phase progression, a BrdU pulse-chase experiment was performed (Fig.12C). The progression in S-phase was normal in all cell lines. At 90 minutes p85A expressing cells exhibited a higher percentage of BrdU positive cells. This was caused by an overall higher number of p85A expressing cells entering the S-phase compared to the controls.

These data indicate that PKA-dependent phosphorylation of p85α^{PI3K} mediates selectively G1 arrest induced by cAMP, since S phase progression was not influenced in cells expressing the mutant versions.

3. Molecular mechanisms regulated by phosphorylation of serine 83 of p85α^{PI3K}

It has been previously shown that cAMP-PKA stimulated the interaction between PI3K and Ras and this event was necessary for cell cycle progression in cells FRTL-5 (Ciullo *et al.*, 2001). Moreover, Ras and PI3K are among the major effectors in transduction pathway modulating cell cycle and survival. It is possible that cAMP promotes cell survival and induces G1 arrest in NIH 3T3 stabilizing PI3K/Ras complex through serine 83 phosphorylation and, in turn, activating PI3K-AKT pathway.

3a. The phosphorylation of p85α^{PI3K} increases the formation of the complex Ras-PI3K

In order to test our hypothesis, NIH 3T3 were transfected with p85WT or mutant encoding vectors. 36 hours later the cells were serum starved and, after 16 hours, were treated 10 minutes with or without 200μM cAMP. Cell lysates were immunoprecipitated with anti-Flag antibody. The immuno-precipitates were separated on SDS-PAGE and analyzed by western blot with anti-Flag and anti-Ras antibodies. Figure 13A, bottom panel, shows that Ras co-immunoprecipitated with p85WT after cAMP treatment, the substitution of serine 83 with aspartic acid amplified this effect. On the contrary, Ras was not present in p85A immunoprecipitates. Ras was barely detected in all the untreated samples, while the amount of p85α^{PI3K} was the same in all samples (upper panel).

The histogram in Figure 13B is the p85/Ras ratio determined as an average of 3 independent experiments.

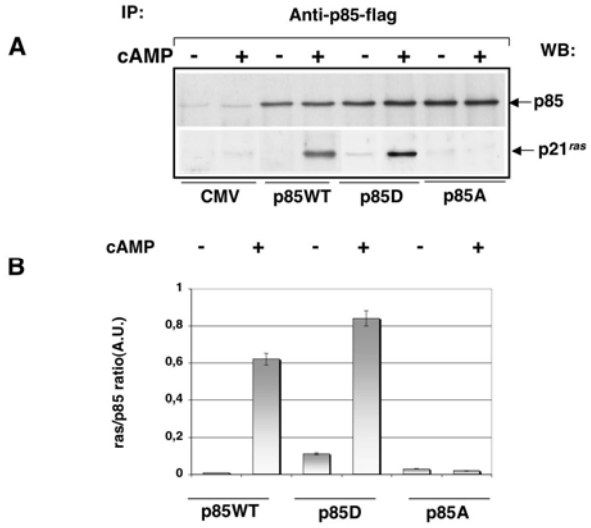

Figure 13: Effects of the substitution of serine 83 on the association Ras-PI3K. NIH 3T3 were transfected with p85 WT, A or D and straved for 16h. Later on , cells were treated 10 min with 200 μM CPT-cAMP. The total lysates were immunoprecipitated with anti-Flag antibody and analyzed by western blot with anti-$p85^{PI3K}$ and anti-Ras antibody (A). The histogram (B) represents the mean of the densitometric analysis of 3 independent experiments.

These data indicate that serine 83 phosphorylation was necessary to induce PI3K/Ras interaction, since p85A lost is ability to bind Ras, although it was not sufficient, because p85D/Ras association was still dependent on cAMP.

3b. The disruption of the phosphorylation site on $p85\alpha^{PI3K}$ did not abolish the binding $p85\alpha^{PI3K}$- $p110\alpha^{PI3K}$

Considering that $p110\alpha^{PI3K}$, and not $p85\alpha^{PI3K}$, mediates PI3K/Ras interaction (Rodriguez-Viciana *et al.*, 1996) it is possible that serine 83 substitution with alanine may alter the folding of $p85\alpha^{PI3K}$ disrupting the $p85\alpha^{PI3K}$- $p110\alpha^{PI3K}$ binding.

Since the commercial antibodies versus $p110\alpha^{PI3K}$ are not satisfactory in terms of specificity in immunoblot analysis, we decided to study the association between $p85\alpha^{PI3K}$ and $p110\alpha^{PI3K}$ using the recombinant proteins. To this end we transcribed and translated the vector encoding $p85\alpha^{PI3K}$ and

p110α^{PI3K} in reticulocyte lysates, adding ^{35}S-methionine to the reaction to label the products. Preliminary experiments indicated that the binding of the two proteins was not efficient when the *in vitro* translation was carried out separately.

To solve this problem, p85α^{PI3K} and p110α^{PI3K} proteins were co-translated. Successively, the samples were immunoprecipitated with the anti-Flag antibody, separated on SDS-PAGE and analized by autoradiography. Figure 14 shows that 1. The 2 proteins interacted very efficiently in vitro; 2. The complex p85α^{PI3K} -p110α^{PI3K} was specific since immunoprecipitation of the *in vitro* translated p110α^{PI3K} with the anti-Flag antibody was negative; 3. p85A interacted with p110α^{PI3K} as well as p85α^{PI3K} wild type, p85D instead appeared more efficient than the wild type in the formation of the complex with p110α^{PI3K}.

These results indicate that the S83A substitution did not alter the folding of p85α^{PI3K}, and suggest that the phosphorylation of serine 83 of p85α^{PI3K} stabilizes the complex PI3K/Ras. This in turn stimulates the association p85α^{PI3K}/ p110α^{PI3K}.

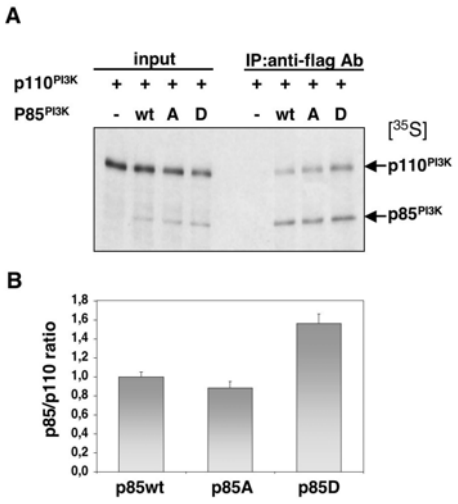

Figure 14: Effect of the substituion of serine 83 of p85α^{PI3K} on the association with p110α^{PI3K}. [^{35}S]methionine labeled p85α^{PI3K} p110α^{PI3K} where *in vitro* co-transcripted and co-translated. The samples where immunoprecipitated with the anti-Flag antibody and analyzed by autoradiography (A). The histogram (B) represents the mean of the quantitative analysis of 3 independent experiments.

3c. cAMP-PKA activates PI3K *in vitro*

To determine if the phosphorylation of serine 83 influenced PI3K lipid kinase activity, NIH 3T3 were transfected with the p85WT encoding vector. Cells were serum starved for 16 hours before a 10 minutes treatment with 200μM cAMP in the presence or absence of H89. Cell lysates were immunoprecipitated with anti-Flag antibody, and the immunoprecipitates divided into two aliquots. One aliquot was used to test the *in vitro* lipid kinase assay (see Material and Methods). Following a 15 minutes incubation the reaction was stopped with 1N HCl and separated by TLC. Considering that the lipid substrate added to the mixture was the PI 4,5 diphostate the only phosphorylated product detected was the PI 3,4,5 triphosphate (PIP_3). The other aliquots were used to normalize for total $p85\alpha^{PI3K}$ present in the immunoprecipitates, by western blot with anti-p85 antibody.

Figure 15A shows that cAMP activated PI3K in a PKA dependent manner, since this effect was reversed by H89 (upper panel and histogram). The amount of p85WT immunoprecipitated was comparable in all the samples (bottom panel).

To verify if cAMP activated PI3K through serine 83 phopshorylation, NIH 3T3 were transfected with the mutant versions of $p85\alpha^{PI3K}$. Figure 15B shows the result of the lipid kinase assay performed on the mutant enzyme. In p85A expressing cells cAMP did not activate PI3K, on the contrary in p85D expressing cells the basal activity was slightly higher compared to p85A. Moreover, the serine 83 substitution did not significantly affect PI3K activity induced by PDGF.

Taken together these data indicate that cAMP induces PI3K activity through serine 83 phosphorylation.

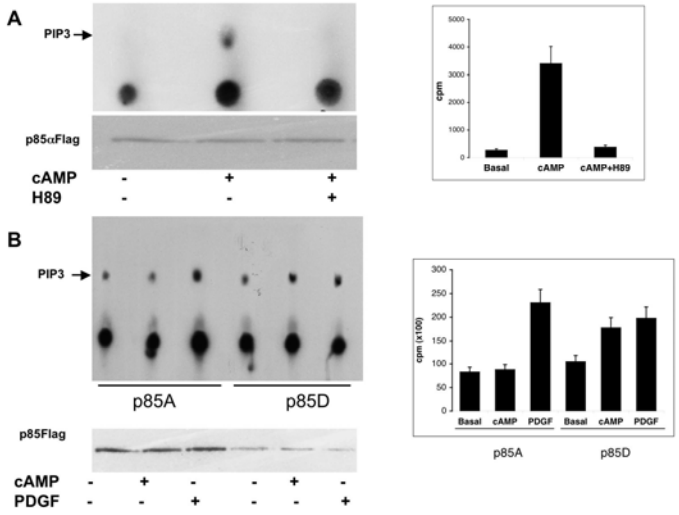

Figure 15: PI3K *in vitro* activity assay. NIH were transfected with p85α^{PI3K}-Flag encoding vectors. 24h after transfection cells were starved for 16h.(A) p85WT expressing cells were treated with or without 200μM CPT-cAMP for 10 min after a 30 min pretreatment with or without 10 μM H89. The cell lysates were immunoprecipitate with the anti-Flag antibody. The immunoprecipitates were divided into 2 aliquots. One was analyzed by western blot with anti-p85^{PI3K} antibody (bottom panel) and the other one was used for the activity assay (upper panel). The histogram shows the quantitative analysis from 3 independent experiments. (B) p85A or D expressing cells were treated 10 min with 200μM CPT-cAMP or 15 min with 100ng/mL PDGF. Cellular extracts were immunoprecipitated with anti-Flag antibody and divided into 2 aliquots. One of these was analyzed by western blot (bottom panel) and the other was used for the activity assay (upper panel). The histogram is the mean of the quantitative analysis from 3 independent experiments.

3d. Phosphorylation of serine 83 on p85α^{PI3K}alters cAMP induced PI3K signalling *in vivo*

To verify that cAMP activate *in vivo* PI3K, NIH 3T3 were serum starved for 16 hours and treated 10 minutes with 200μM cAMP in the presence or absence of H89. *In vivo* activity of PI3K can be measured determining the phosphorylation state of the downstream targets, such as AKT and GSK (Marte and Downward, 1997; Rameh and Cantley, 1999). Figure 16A shows the results of anti-P-AKT and anti-P-GSK antibodies. cAMP-induced phosphorylation of both proteins in a PKA dependent manner, since it was reversed by H89. The total amount of protein was comparable in all the samples, as shown by anti-AKT western blot (bottom panel).

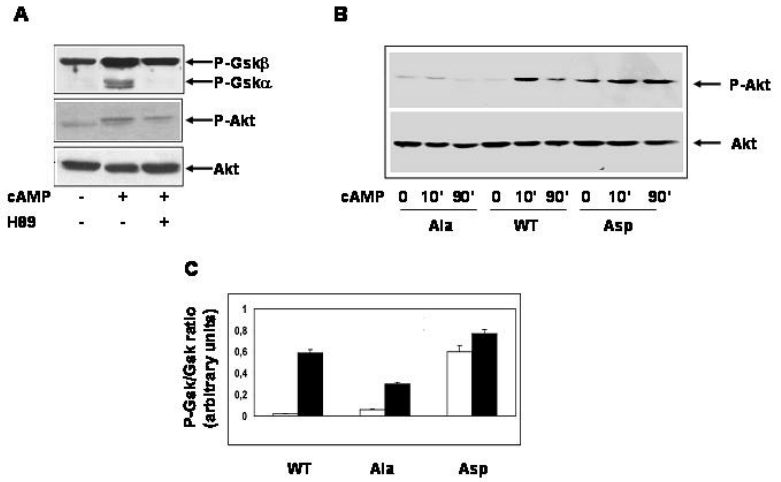

Figure 16 Activation of PI3K-AKT pathway in response to cAMP. (A)NIH 3T3 were serum straved 16h and successively treated 10 min with or without 200 μM CPT-cAMP following a 30 min treatment with or without 10 μM H89. Total lysates were analyzed by western blot with anti- PGsk, -Gsk and -AKT antibodies. (B) Serum starved p85WT or mutant expressing cells were treated 10 or 90 min with or without 200 μM CPT-cAMP. total lysates were analyzed by western blot with anti-PAKT and –AKT antibodies. (C) The white bars represent the basal P-Gsk/Gsk ratio in p85WT or mutant expressing cells, the black bars represent the same ratio in cells treated 10 min 200μM CPT-cAMP. These results are the average of 3 independent experiments.

To test that the effects of cAMP on PI3K *in vivo* activity were induced by serine 83 phosphorylation, NIH 3T3 were transfected with p85WT or mutant encoding vector. 36 hours later, the cells were serum-starved for 16 hours. Successively, protein lysates were separated on SDS-PAGE and analyzed by western blot with anti-P-AKT, -AKT,-P-GSK and –GSK antibodies.

Figure 16B shows the time course of activation of AKT in the transfected cells. In p85WT expressing cells cAMP activated AKT at 10 minutes, this effect was lost at 90 minutes. In p85A expressing cells the response to cAMP was absent. On the contrary, in p85D expressing cells the basal activity was higher compared to the p85WT expressing cells and the activation persisted up to 90 minutes. Similar results were obtained analyzing the P-GSK/GSK ratio (Fig. 16C).

Taken together the data presented indicate that cAMP-PKA phosphorylates

the serine 83 of p85α^{PI3K}, stimulating the formation of the Ras/PI3K complex. The association between these molecules triggers the activation of the PI3K-AKT pathway which results in inhibition of apoptosis and G1 arrest.

4. Biological effects of the phosphorylation of p85α^{PI3K} TSH-cAMP dependent cells: FRTL5 and NTCRII

Ciullo et al. in 2001 demonstrated that the association between PI3K and Ras was essential for the G1-S transition in thyroid cells FRTL5. Considering the data presented above concerning the role of serine 83 phosphorylation in the formation of Ras/PI3K complex and the regulation of cell survival and proliferation in NIH 3T3, we investigated the possibility that serine 83 was a key regulator of cAMP effects also in cAMP-dependent cells.

The following experiments were performed in thyroid cells FRTL5 and NTCRII cells. FRTL5 cells proliferate in response to TSH, an hormone who activates a Gs coupled receptor (see Background). NTCRII cells are modified NIH 3T3 fibroblasts that became TSH-cAMP dependent. Porcellini et al. in 2003 showed that NIH 3T3 expressing both TSH receptor and the PKA regulatory subunit RIIβ proliferated in dependence of TSH-cAMP, recapitulating the characteristics of thyroid cells (Fig. 17).

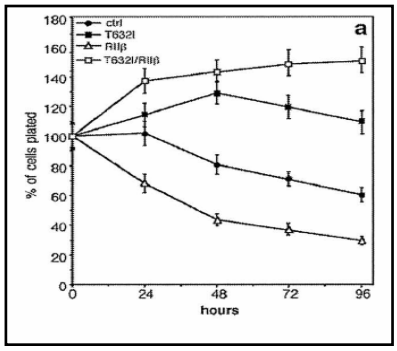

Figure 17: Effect of TSH on cell proliferation on engineered NIH 3T3. The expression of TSHR (T6321) or RIIβ induces cell death in response to TSH added to the culture media, while TSH induces proliferation in NIH 3T3 expressing both TSHR and RIIβ. (*Porcellini et al., JBC, 2003; 278, 40621-40630*)

4a. p85A is lethal in TSH-cAMP dependent cells

FRTL5 cells were co-transfected with a G-418 resistance encoding vector and p85WT or mutant encoding vector. 96 hours after the transfection 400 μg/ml of G-418 was added to the media but very few clones expressing p85A survived selection. To test if p85A expression in FRTL5 impaired cell survival, we tested the plating efficiency of the various transfected lines. $5x10^5$ cells were transfected with the G-418 resistance encoding vector and the indicated vector. 96 hours after the transfection 400 μg/ml of G-418 was added to the media. After 15 days, the G-418 resistant clones were counted. Figure 18 shows that the numbers of p85A expressing clones was significantly lower than the control and p85WT expressing clones. Moreover, the number of p85D expressing clones was slightly higher than the number of p85WT expressing clones.

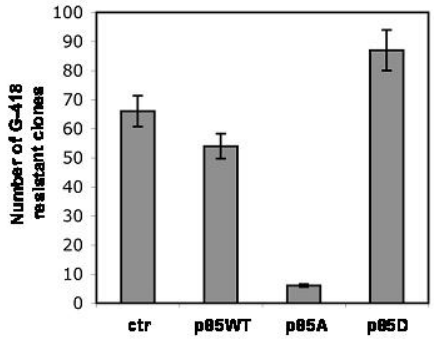

Figure 18: Plating efficiency of p85WT, A or D expressing clones. The ability to form G-418 resistant clones was determined by transfecting $5*10^5$ FRTL-5 cells with p85WT, A or D expressing vector and selecting the clones in the presence of 400 μg/ml of G-418 for 15 days. The histogram represents the number of clones obtained, and it is the mean of three experiments in triplicate.

The few surviving clones expressing p85A were expressed low levels of p85A protein (data not shown). These data indicate that p85A expression inhibited growth or/and survival in FRTL5, suggesting that phosphorylation of serine 83 of $p85\alpha^{PI3K}$ is a key step in the transmission of survival signals in FRTL5. To test this hypothesis, NTCRII cells were transiently transfected with p85WT, A or D or control vectors. Cells were grown for 18 hours in the

presence or absence of 10mU/mL TSH or 100μM cAMP. It is worth noting that the cells were grown in low serum medium (0,5%), because growth factors in the serum mask, at least in part, the effects of TSH deprivation (Porcellini *et al.*, 2003). The cells were collected and analyzed by TUNEL assay (see Materials and Methods). Figure 19 shows that TSH deprivation induced apoptosis in NTCRII cells that was reversed by TSH or cAMP. The expression of p85WT slightly reduced apoptosis induced by TSH deprivation. The expression of p85A, on the other hand, stimulated apoptosis under all conditions, while p85D expression protected cells from TSH-cAMP deprivation-induced apoptosis.

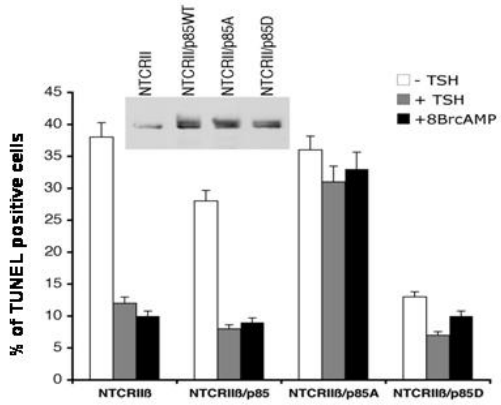

Figure 19: Effects of serine 83 phosphorylation on TSH-cAMP deprivation induced apoptosis in NTCRII cells. $5*10^5$ NTCRII cells were transfected with an empty vector or p85WT, A or D encoding vector. Cells were grown in 0,5% serum containing medium with or without TSH or cAMP. After 18 hours apoptosis was determined through TUNEL assay (see Materials and Methods). The western blot inset show the expression of p85 in all the samples, the upper band of the doublet is the exogenous protein.

Taken together these data indicate that phosphorylation of serine 83 of $p85\alpha^{PI3K}$ mediates cAMP induced survival both in FRTL5 and NTCRII cells.

4b. Phosphorylation of $p85\alpha^{PI3K}$ is necessary for S-G2/M transition

To test the role of the phosphorylation of serine 83 of $p85\alpha^{PI3K}$ on cell proliferation in TSH-cAMP dependent cells, NTCRII cells were transfected with an empty vector or the vector encoding p85WT or mutant versions. 24

hours after the transfection, the colture media was replaced with 0,5% serum containing medium. 18 hours later, 10mU/mL of TSH were added to the media to induce cell cycle entry. Cells were analyzed by BrdU labelling, as described in Materials and Methods. Figure 20 shows that only the expression of p85A affected cell cycle progression. In particular, p85A expressing cells accumulated in S-phase at 270 minutes (left panel). The S-phase arrest of p8A expressing cells correlates with the reduction of the number of cells in G2/M phase at 270 minutes (right panel).

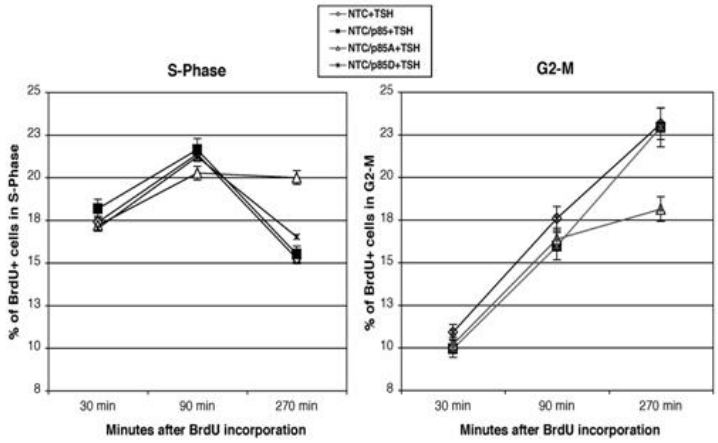

Figure 20: Effects of serine 83 phosphorylation on cell cycle progression. NTCRII cells were transiently transfected with the p85WT, p85A, p85D mutants or with the empty vector (control). 24 hours after transfection, the cells were starved in 0,5% serum containing medium. 18 hours later cells were induced with 10 mU/ml TSH. BrdU incorporation was assayed by a pulse-chase experiment. Cells were labeled for 30 min with BrdU and harvested at 0, 90 and 270 min. DNA content was determined by propidium iodide stainig. Cells were subjected to FACS analysis for bivariate-analysis of DNA content versus BrdU.

We also monitored EGF-induced proliferation, since NCTRII cells in the presence of serum respond to EGF. p85A affected selectively cAMP-TSH induced proliferation but not the EGF induced proliferation (data not shown, (De Gregorio *et al.*, 2006). These data indicate that phosphorylation of serine 83 is necessary for cell cycle progression.

5. Molecular mechanisms affected by phosphorylation of serine 83 of p85α^{PI3K} in FRTL-5 and NTCRII cells

As in NIH 3T3, cAMP induced the binding of Ras to PI3K, containing p85WT but not p85A (De Gregorio *et al.*, 2006). The formation of this complex was tested both in FRTL5 or NTCRII cells, respectively stably or transiently expressing p85WT or mutant versions. This indicates that the same molecular mechanism, i.e., stimulation of the complex PI3K/Ras, exerts opposing effects: cell proliferation in TSH-cAMP dependent cells and G1 arrest in NIH 3T3. One explanation of this paradox may involve the differential expression of RII isoforms in the cell lines analyzed. Both NTCRII and FRTL5, in fact, express RIIβ, while this protein is present at low level, if not absent, in NIH 3T3 fibroblasts (Porcellini *et al.*, 2003).

5a. Phosphorylated p85α^{PI3K} interacts with RIIβ

To verify if RIIβ influences the biological effects of serine 83 phosphorylation, the binding between RIIβ-PKA and PI3K has been tested. p85WT, A or D have been transiently expressed in NTCRII cells. 24 hours after the transfection, cells were serum starved for 6 hours before the treatment with 10mU/mL TSH (20 minutes). Cell lysates were incubated *in vitro* with GST or GST-RIIβ (Grieco *et al.*, 1996). Figure 21 shows the western blot with anti-Flag, -GST and -phosphoserine (P-Ser) antibodies on the bound proteins (pull down assay). p85WT formed a complex with RIIβ following incubation with TSH. The substitution S83A abolished the binding, while the substitution S83D determined constitutive (in the absence of cAMP) binding RIIβ/p85α^{PI3K}.

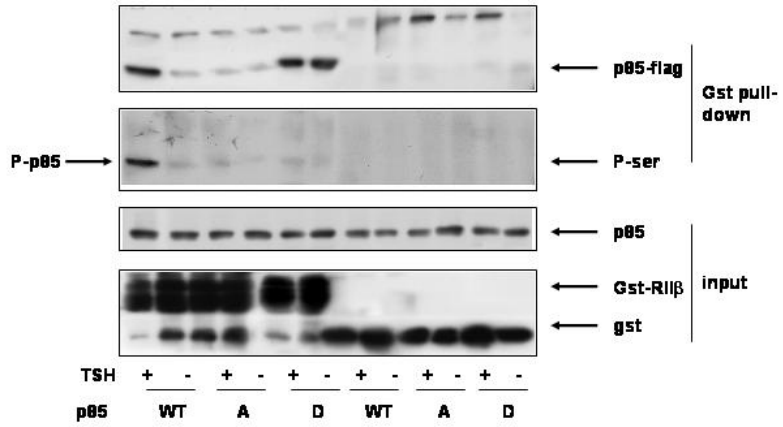

Figure 21: GST pull down in NTCRII cells. NTCRII cells were transiently transfected with the indicated vectors. Serum starved cells were treated 20 minutes with 10mU/mL TSH. Cell lysates were incubated with the indicated recombinant proteins. The samples were separated on SDS-PAGE and analyzed by western blot with the indicated antibodies.

Moreover, the anti-P-Ser western blot shows that p85WT bound to RIIβ was phosphorylated, and the phosphorylation was on serine 83 since p85D bound RIIβ without being phosphorylated.

In order to confirm that TSH induced RIIβ binding to $p85\alpha^{PI3K}$ *via* cAMP, the GST pull down assay was performed on NTCRII cells transfected with p85WT, A or D and treated with cAMP, instead of TSH. Figure 22 shows the western blot with anti-Flag and anti-GST on the bound proteins.

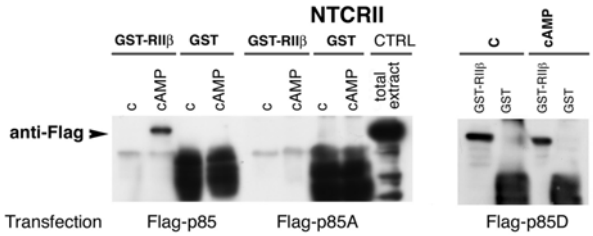

Figure 22: GST-RIIβ pull down in NTCRII expressing p85WT or mutant treated with cAMP. NTCRII cells were transiently transfected with p85WT (Flag-p85), p85A or p85D. 24 hours after transfection cells were serum starved in 0,5% serum. After 6 hours cells were treated with 100μM cAMP for 15 minutes. Cell lysates were incubated *in vitro* with GST-RIIβ or GST (see Materials and Methods). The pellets of the pull down were separated on SDS-PAGE and analyzed by western blot with the indicated antibodies.

Also cAMP stimulates the binding of p85WT to GST-RIIβ but not of p85A, the binding of p85D instead was constitutive.

To test if the binding between the two proteins was direct, p85WT or A was transiently expressed in NTCRII. Protein lysates from cAMP induced cells were immunoprecipitated with anti-Flag antibody and $p85\alpha^{PI3K}$ was eluted with 0,1M glycine pH 3,5. The eluted proteins were used for the GST-RIIβ pull down assay. Figure 23 shows that cAMP induced the binding to RIIβ of p85 WT, not of the mutated version, p85A.

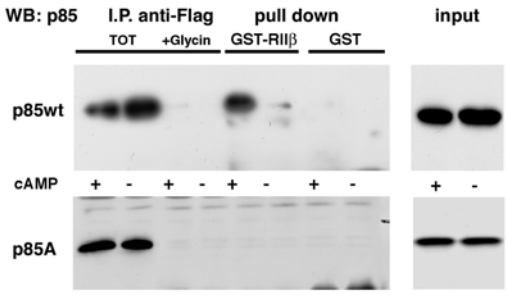

Figure 23: GST-RIIβ pull down on immunoprecipitated p85WT or A. Exogenous $p85^{PI3K}$ was immunoprecipitated with anti-FLAG antibody from NTCRII cells transfected with p85WT or p85A and exposed to 100 µM 8BrcAMP for 15 min. The immunoprecipitates were extensively washed in RIPA buffer containing 1% Triton X-100 -0.1% SDS and purified by elution with 0.1M glycine pH 3.5. **Input** indicates the eluted fraction; +glycine represents the pellet after elution. The purified $p85^{PI3K}$ was incubated *in vitro* with GST-RIIβ in a pull-down experiment as described in Materials and Methods.

Together these data indicate that phosphorylation of serine 83 in $p85\alpha^{PI3K}$ is important for the binding to RIIβ-PKA. This results, possibly, in the anchoring of PKA under the plasma membrane and the phosphorylation of a particular subset of substrates.

5b. Phosphorylation of serine 83 on $p85\alpha^{PI3K}$alters cAMP induced PI3K activity both *in vitro* and *in vivo*

To determine if phosphorylation of $p85\alpha^{PI3K}$ correlates with an increase of activity of PI3K, as in NIH 3T3, NTCRII cells were transiently transfected

with p85WT or A. Cells were then serum starved and induced 15 minutes with cAMP.

Exogenous p85α^{PI3K} was immunoprecipitated with the anti-Flag antibody and the immunoprecipitates divided into two aliquots: one aliquot was used to test *in vitro* lipid kinase activity and the other to control the immunoprecipitation efficiency.

Figure 24A show that cAMP activated p85WT- p110α^{PI3K} but not p85A-p110α^{PI3K}. Moreover, the phosphorylation of p85α^{PI3K} did not affect the EGF-induced PI3K activity (Fig. 24B).

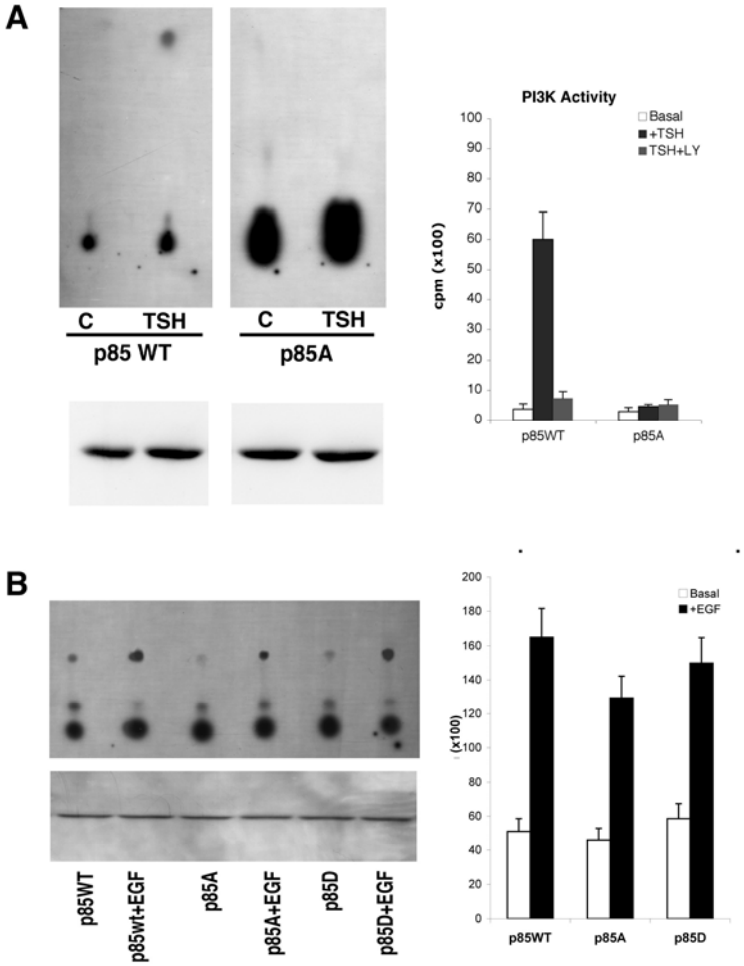

Figure 24: Effect of serine 83 substitution on *in vitro* PI3K activity. NTCRII cells were transiently transfected with p85WT or mutant. Cells were serum starved for 8 hours and treated 20 minutes with 10mU/mL of TSH (A) or 100nG/mL of EGF (B). The *in vitro* lipid kinase assay was performed as described in Materials and Methods. The histograms on the right represent the mean of the cpm counted on three independent experiments.

These data were confirmed *in vivo* by determining the level of phosphorylation of AKT in NTCRII cells transiently transfected with p85WT or mutant versions and treated with TSH.

Figure 25 shows that p85A expressing cells fail to activate AKT at all time points considered. On the other end, expression of p85D slightly amplified TSH signaling. P-AKT levels were, in fact, higher in p85D expressing cells than in the controls both at 0 and 5 minutes. These results confirm that phosphorlation of $p85\alpha^{PI3K}$ was necessary to activate PI3K through cAMP-PKA pathway.

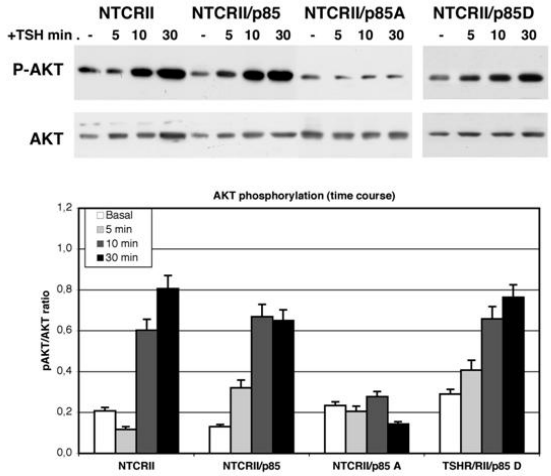

Figure 25: Effect of serine 83 phosphorylation on AKT activation in NTCRII cells. NTCRII cells were transfected with the indicated vector, 30 hours later the normal medium was replaced with a low serum medium (0,5% serum). After 18 hours cells were treated with 10mU/mL TSH for the indicated time. Protein lysate were analyzed by western blot with anti-P-AKT and –AKT antibodies. The histogram in B is the mean of three independent experiments.

6. cAMP-PKA amplifies estrogen binding and signaling to PI3K

Considering that PI3K is essential also in estrogen mediated AKT activation (Simoncini *et al.*, 2000; Castoria *et al.*, 2001) and that $p85\alpha^{PI3K}$ was efficiently phosphorylated in MCF-7 cells (Fig. 10A) it was possible that serine 83 phosphorylation was required for estrogen signaling. To verify this

hypothesis MCF-7 cells were made quiescent using charcoal-treated serum and medium lacking phenol-red for 3 days. Quiescent cells were transiently transfected with p85WT or mutant, after 24 hours, the cells were treated 3 minutes with 10 nM E_2. Cell lysates were separated on SDS-PAGE and analyzed by western blot with anti-P-AKT, -AKT and –p85 antibodies. Figure 26A shows that estrogen activated AKT both in cells transfected with the empty vector (nt) and in p85WT expressing cells. The expression of p85A abolished the induction of AKT phosphorylation. Conversely expression of p85D stimulated AKT phsphorylation.

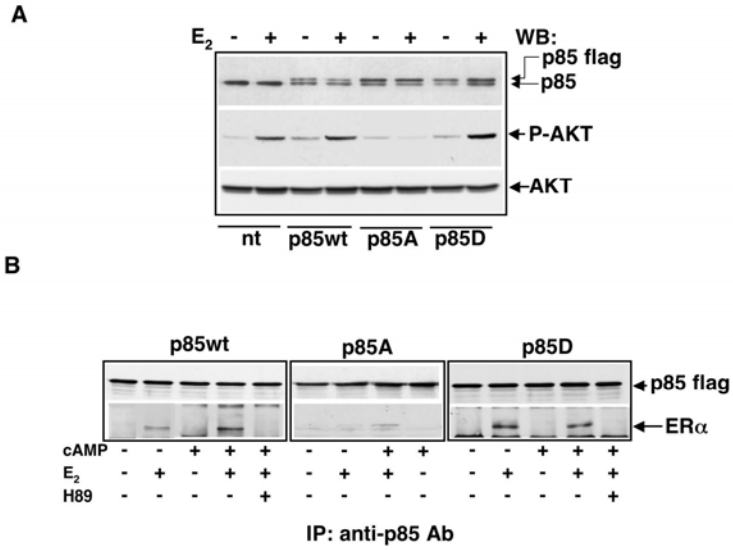

Figure 26: Estrogen signaling to AKT in MCF-7 cells transfected with p85WT or mutant. MCF-7 cells were made quiescent using charcoal-treated serum and medium without phenol-red for 3 days. Quiescent cells were transfected with the indicated vector. A) Transfected cells were treated without or with 10 nM E_2 for 3 minutes. Protein lysates were analyzed by western blot with the indicated antibodies. B) Transfected cells were treated with or without: 10 nM E_2 (3 minutes), 10μM H89 (30 minutes), 200μM CPT-cAMP (10 minutes). Protein lysates were immunoprecipitated with anti-Flag antibody and immunoprecipitates analyzed by western blot with anti-p85 and –ERα antibodies.

Since estrogens activate PI3K-AKT pathway through the direct binding of the receptor to $p85\alpha^{PI3K}$ (Castoria *et al.*, 2001) it was possible that phosphorylation of serine 83 was required for $p85\alpha^{PI3K}$ binding to ERα.

In order to test this hypothesis, quiescent MCF-7 cells were transiently transfected with p85WT or mutant. 24 hours later, cells were treated with E_2, H89 and/or cAMP. Protein lysates were immunoprecipitated with anti-Flag antibody and analyzed by western blot with anti-p85 and -ERα antibodies. Figure 26B shows that estrogen induced p85WT binding to the receptor, cAMP on its own did not induce the binding but amplified estrogen-stimulated binding. In fact, the amount of receptor bound to $p85\alpha^{PI3K}$ was significantly higher in cAMP and estrogen-treated cells than in cells exposed to estrogen alone. Moreover, the binding was PKA-dependent, since H89 inhibited the effects of estrogens.

cAMP-PKA modulates ERα- $p85\alpha^{PI3K}$ binding through the phosphorylation of $p85\alpha^{PI3K}$. In fact p85A failed to bind the receptor in all the conditions considered. Once more, it is worth noting that the phosphorylation of serine 83 of $p85\alpha^{PI3K}$ is absolutely necessary to mediate cAMP-PKA effects, but it is not sufficient. In fact, p85D binding to ERα in E_2 treated cells was inhibited by H89. These data indicate that phosphorylation of serine 83 is required for the binding of $p85\alpha^{PI3K}$ to ERα and the consequential activation of PI3K-AKT, but it still requires PKA, because these events are H89 sensitive. It is worth noting that ERα itself may be a PKA substrate (Cui *et al.*, 2004; Michalides *et al.*, 2004). These observations suggest the possibility that the receptor has to be phosphorylated by PKA to efficiently bind p85α.

Discussion

The data presented here indicate that phopshorylation of $p85\alpha^{PI3K}$ on serine 83 is a critical step in the cAMP-PKA signaling. Biological effects of cAMP and PKA on cell cycle and growth have been extensively studied (Pastan *et al.*, 1975; Lee *et al.*, 1998; Ariga *et al.*, 2000; Stork and Schmitt, 2002) but the molecular mechanism(s) underling their action are not yet defined. Our data may shed light on cAMP mediated phenotypes and may provide some mechanistic insights into transduction signalling in a variety of cell types.

1. cAMP-PKA selectively influences Ras signaling

Previous data indicate that cAMP inhibits Raf1 association with Ras and down-regulates the MAPK pathway through two different mechanisms.

The first is cAMP, but not PKA, dependent. In fact cAMP stimulates directly EPAC, a guanine nucleotide exchange factor for the Ras-like small GTPases (Rap1 and Rap2) (Bos, 2003). Rap1 binds Raf1 and inhibits its kinase function (Hu *et al.*, 1999). The importance of Rap1 mediated regulation of Raf-1 is confirmed by the complementary action of insulin. In fact, insulin, in the absence of cAMP-PKA, decreases Rap1 bound to Raf1 and increases the association Ras-Raf1, with a concomitant stimulation of Raf1-MEK-ERK cascade (Okada et al., 1998). The second mechanism is PKA dependent, PKA in fact phosphorylates serines 43 and 259 in Raf1. Phophorylation of serine 43 decreases the binding to Ras, while serine 259 appears to be the sole target for PKA inhibition of ERK1/2 (Dhillon et al., 2002).

The inhibition of Raf-1 signalling does not necessarily correspond to MAPK inhibition by cAMP. In fact cAMP may stimulate rather than inhibit MAPK (Stork and Schmitt, 2002; Norum *et al.*, 2003). Our data indicate that the major target of cAMP-PKA action is PI3K. Ciullo et. al in 2001 showed that Ras binding to PI3K increases when the cells were exposed to cAMP.

Moreover, the formation of this complex was dependent on PKA activity, since H89 reversed cAMP-induced association PI3K/Ras.

The results reported here indicate that PKA phosphorylates serine 83 of $p85\alpha^{PI3K}$ and this event is necessary for the binding to Ras, although not sufficient. This has been shown in NIH 3T3 and confirmed in TSH-dependent cells (De Gregorio *et al.*, 2006), suggesting that it is a general, not a tissue specific mechanism. Our data indicate that phosphorylation of serine 83 in $p85\alpha^{PI3K}$ stabilized the binding with $p110\alpha^{PI3K}$, resulting in stimulation of the enzymatic activity both *in vivo* and *in vitro* (Figs 14, 15 and 24). Stabilization of $p110\alpha^{PI3K}$-$p85\alpha^{PI3K}$ complex is important for two reasons: 1. It may facilitate translocation to the cellular membrane (see Background); 2. It increases local specific activity. We suggest that phosphorylated $p85\alpha^{PI3K}$ associates more efficiently to $p110\alpha^{PI3K}$, stabilizes the enzyme and localizes to the membrane. This results in an increased binding of PI3K to Ras and to the activation of the PI3K-AKT pathway.

2. cAMP-PKA regulates cell cycle progression through $p85\alpha^{PI3K}$ phosphorylation

cAMP inhibits proliferation in NIH 3T3. In fact the phosphorylation of $p85\alpha^{PI3K}$ leads to G1 arrest in fibroblasts, as shown by transfecting the mutant p85D. On the contrary, preventing the phosphorylation (p85A), NIH 3T3 cells become resistant to cAMP-induced G1 arrest (Fig. 12) . A different effect was observed in TSH-dependent cells. The phosphorylation of $p85\alpha^{PI3K}$ is in fact essential for a correct progression of the cells through S and G2/M phases (Fig. 20). How can the same phosphorylation lead to opposing phenotypes?

It is worth noting that NIH 3T3 fibroblasts display a very short cell cycle, approximately 24 hours, to complete 1 cycle. This implies a short G1. On the other hand, TSH-dependent cells display a 48 hours long cell cycle with an extended (ca. 36 h) G1. We suggest that cAMP-PKA regulates the length of G1. This effect is not detectable in TSH-dependent cells since they do not

proliferate in the absence of TSH or cAMP and in their presence the cells show a long G1. Moreover, cAMP activates PI3K-AKT pathway and one of the terminal targets of AKT is mTOR. mTOR induces protein synthesis that results in cell growth (Garcia *et al.*, 2006). On the other side, in *S. cerevisiae* iper-activation of cAMP-PKA can compensate the inactivation of PI3K-mTOR signalling (Rohde *et al.*, 2004; Zurita-Martinez and Cardenas, 2005). This indicates that cAMP-PKA induces cell growth in yeast, as well as mTOR stimulation. Cell growth depends on protein synthesis and cell size. Both these activities are robustly stimulated by cAMP-PKA (Zurita-Martinez and Cardenas, 2005). We believe that the increase of the length of G1 in NIH 3T3 by cAMP mimics the effects seen in thyroid cells, where a long G1 is accompanied by an increase in the cell size. TSH and cAMP are the driving force that accounts for PI3K stimulation and increase in cell size that ultimately represents the trigger of S phase (Baroni *et al.*, 1992). This is briefly outlined in Fig.27.

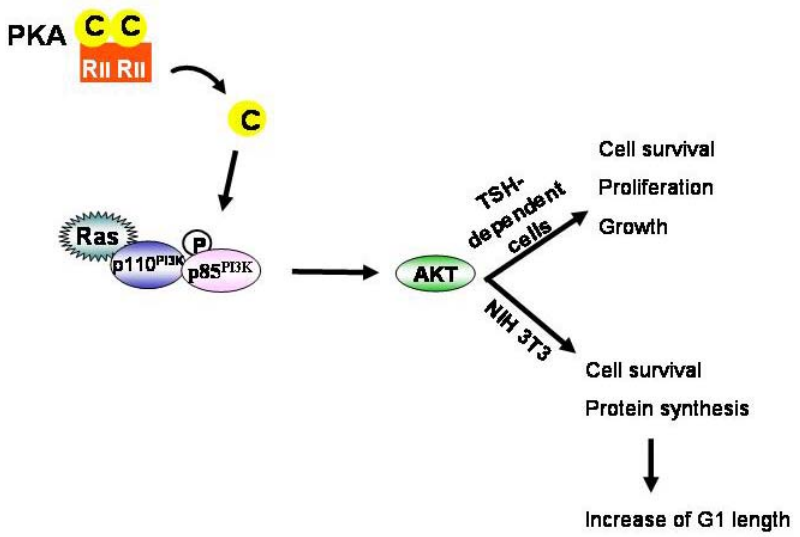

Figure 27: Model of the role of serine 83 phosphorylation. PKA phosphorylating serine 83 of p85α^{PI3K} stabilizes the ternary complex Ras-PI3K. This triggers to the activation of AKT pathway. It leads to: cell survival, growth (protein synthesis) and proliferation depending on the cell type.

3. cAMP cytoprotective action requires serine 83 phopshorylation

cAMP protects cells from apoptosis induced by different signals in different cell types (Affaitati *et al.*, 2003). In this study the resistance to *anoikis* has been evaluated. This apoptotic pathway is triggered by the loss of cell-matrix signals and it is highly dependent on PI3K. This system allowed us to define the role of serine 83 phopshorylation without the interferences of other survival pathway. The result was that phosphorylation of serine 83 was critical for cAMP mediated protection from *anoikis*. Moreover p85D expression replicated the effects of cAMP, at least in part (Fig. 11). This suggests that the phosphorylation of serine 83 of $p85\alpha^{PI3K}$ is the main player of cAMP cytoprotective signals.

The function of phosphorylated serine 83 was essential in TSH-dependent cells. In fact, the absence of TSH or cAMP induced apoptosis in p85WT but not p85D-expressing cells. Moreover thyroid and thyroid-*like* cells undergo apoptosis when expressed p85A, also in the presence of TSH (Figs 18 and 19) This further indicates that $p85\alpha^{PI3K}$ is a key player in TSH-cAMP induced survival.

4. PKA-RIIβ expression switches cells from cAMP-dependent proliferation to cAMP-dependent arrest

Different isoforms of PKA exist, depending on the characteristics of the regulatory subunit (Feliciello *et al.*, 2001). We can distinguish RI and RII subunits, the first has a higher sensitivity to cAMP. This indicates that RI-PKA responds to low level of cAMP while RII-PKA to high level of intracellular cAMP. Thyroid cells express high level of PKA-RII, and in particular PKA-RIIβ. Moreover, NIH 3T3 cells expressing TSH receptor but not RIIβ, do not proliferate in response to TSH. Only cells expressing in the meantime TSHR and RIIβ become TSH dependent cell for growth (Porcellini *et al.*, 2003). This indicates that RIIβ levels distinguish cAMP-dependent cells from independent cells.

Here we show that RIIβ binds phosphorylated p85α^{PI3K} and phosphorylation on serine 83 is the only requirement to this event, since p85D constitutively bind RIIβ (Figs 21 and 22). This association anchors PKA at the cellular membrane, determining a highly localized kinase activity (Feliciello *et al.*, 2001).

5. cAMP-PKA phosphorylates p85α^{PI3K} and amplifies estrogens signaling

The data presented in this study indicate that cAMP cooperates with estrogens in the stimulation of AKT. Moreover, this effect is dependent on p85α^{PI3K} phosphorylation. In fact, p85A was unable to bind the estrogen receptor and p85A expressing cells did not activate AKT in response to estrogens. On the other hand, the substitution S83D amplified the association ERα-p85α^{PI3K} induced by estrogens. Moreover, p85D expression enhanced estrogens mediated activation of AKT. These observations indicate that phosphorylation of serine 83 is essential for estrogens signaling. Our data also indicate that this step is necessary but not sufficient, because p85D expressing cells still respond to cAMP to amplify AKT signaling (Fig. 26). PKA can phosphorylate the receptor itself (Cui *et al.*, 2004; Michalides *et al.*, 2004), and this phosphorylation may be required for fully activation of the ERα-p85α^{PI3K} circuit. Considering that estrogens activate the adenylyl cyclase (Aronica et al., 1994) and H89 prevents the association ERα-p85α^{PI3K}, it is likely that PKA-dependent phosphorylation occurs even in cells treated only with estrogens, and that the bound p85α^{PI3K} is only the phosphorylated form. This may explain why p85A acts as a negative dominant.

Conclusions

The data presented above have broad implications since they point to $p85\alpha^{PI3K}$ as the physical link between different pathways: cAMP, Ras and virtually all the receptors that can bind PI3K (i.e. ERα), as schematically shown in Figure 28.

Moreover, the mechanisms described are relevant both in cAMP-dependent and –independent cells and contribute to explain the pleiotropic nature of the cAMP-PKA circuit in different cell types and under different conditions.

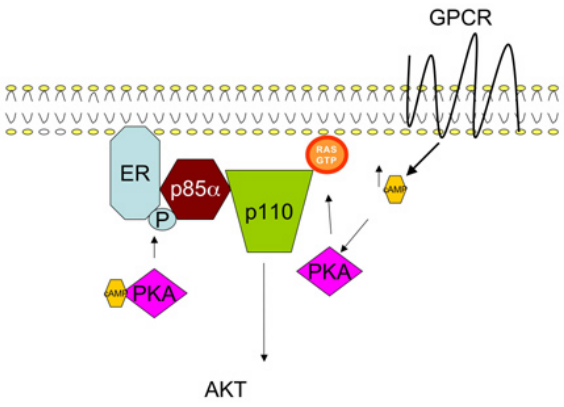

Figure 28: Amplification of estrogens and Gs protein-coupled receptors (GPCR) signaling to PI3K. A scheme outlining the effects of cAMP-PKA activation by Gs protein coupled receptors on PI3K signaling by estrogens. The pathway illustrated may link several receptor G protein coupled to estrogens and Ras-PI3**K** signals.

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ORIGINAL ARTICLE

p85 regulatory subunit of PI3K mediates cAMP–PKA and estrogens biological effects on growth and survival

C Cosentino[1], M Di Domenico[2], A Porcellini[3,4], C Cuozzo[1], G De Gregorio[4], MR Santillo[5], S Agnese[1], R Di Stasio[2], A Feliciello[1], A Migliaccio[2] and EV Avvedimento[1]

[1]*Dipartimento di Biologia e Patologia Molecolare e Cellulare, Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Università Federico II, Napoli, Italy;* [2]*Dipartimento di Patologia Generale, II Università di Napoli, Napoli, Italy;* [3]*Dipartimento di Medicina Sperimentale e Patologia, Università di Roma 'La Sapienza', Roma, Italy;* [4]*INM Neuromed, Pozzilli, Italy and* [5]*Dipartimento di Neuroscienze-Sezione di Fisiologia, Università Federico II, Napoli, Italy*

Cyclic adenosine 3′5′ monophosphate (cAMP) and protein kinase A (PKA) cooperate with phosphatidylinositol 3′ kinase (PI3K) signals in the control of growth and survival. To determine the molecular mechanism(s) involved, we identified and mutagenized a specific serine (residue 83) in p85α^{PI3K}, which is phosphorylated *in vivo* and *in vitro* by PKA. Expression of p85α^{PI3K} mutants (alanine or aspartic substitutions) significantly altered the biological responses of the cells to cAMP. cAMP protection from *anoikis* was reduced in cells expressing the alanine version p85α^{PI3K}. These cells did not arrest in G1 in the presence of cAMP, whereas cells expressing the aspartic mutant p85D accumulated in G1 even in the absence of cAMP. S phase was still efficiently inhibited by cAMP in cells expressing both mutants. The binding of PI3K to Ras p21 was greatly reduced in cells expressing p85A in the presence or absence of cAMP. Conversely, expression of the aspartic mutant stimulated robustly the binding of PI3K to p21 Ras in the presence of cAMP. Mutation in the Ser 83 inhibited cAMP, but not PDGF stimulation of PI3K. Conversely, the p85D aspartic mutant amplified cAMP stimulation of PI3K activity. Phosphorylation of Ser 83 by cAMP–PKA in p85α^{PI3K} was also necessary for estrogen signaling as expression of p85A or p85D mutants inhibited or amplified, respectively, the binding of estrogen receptor to p85α and AKT phosphorylation induced by estrogens. The data presented indicate that: (1) phosphorylation of Ser 83 in p85α^{PI3K} is critical for cAMP–PKA induced G1 arrest and survival in mouse 3T3 fibroblasts; (2) this site is necessary for amplification of estrogen signals by cAMP–PKA and related receptors. Finally, these data suggest a general mechanism of PI3K regulation by cAMP, operating in various cell types and under different conditions.
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Correspondence: Professor EV Avvedimento, Dipartimento di Biologia e Patologia Molecolare, Istituto di Endocrinologia, ed Oncologia Sperimentale del CNR, Via S. Pansini, 5, Napoli, Italy.
E-mail: avvedim@unina.it
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Introduction

Cyclic adenosine 3′5′ monophosphate (cAMP) regulates the growth of many cells types (Pastan *et al*., 1975). Although cAMP can promote the growth of some cells, such as Swiss 3T3 fibroblasts and thyrocytes (Lee *et al*., 1998; Ariga *et al*., 2000), it inhibits proliferation in most cells (Magnaldo *et al*., 1989). However, the precise mechanism by which cAMP inhibits cell cycle entry and progression remains undefined (Houslay and Kolch, 2000). cAMP prevents cells from entering S phase and arrests the cells in G1 (Kato *et al*., 1994). Furthermore, cAMP inhibits proliferation stimulated by either G protein-coupled receptors or receptor tyrosine kinases (Magnaldo *et al*., 1989), suggesting that it targets a signaling pathway that is central to cell cycle progression.

Stimulation of growth by cAMP–protein kinase A (PKA) in selected cell types, as thyroid cells, is tightly dependent on Ras and phosphatidylinositol 3′ kinase (PI3K) (Ciullo *et al*., 2001). Also, cAMP and PKA are powerful survival signals in several cell types (Affaitati *et al*., 2003). To date the mechanism(s) and the relevant players mediating cAMP effects on growth and survival are not completely known.

We have previously shown that cAMP and PKA regulate Ras signaling, by selectively stimulating Ras–PI3K complex. Also, we have found that p85α^{PI3K} was an efficient PKA substrate *in vitro* (Ciullo *et al*., 2001).

Here we report that Ser 83 in the p85α^{PI3K} is phosphorylated *in vivo* by PKA and that the corresponding mutants (p85A or p85D) inhibit or amplify cAMP biological effects on growth and survival. This site is also relevant for the interaction of p85α^{PI3K} with estrogen receptor α, as the mutants inhibit or amplify estrogen signaling to PI3K. Finally, we demonstrate that cAMP in the absence of serum stimulates PI3K and that p85α^{PI3K} mutants selectively interfere with cAMP stimulation of PI3K activity.

These data provide a molecular framework that explains the synergism between cAMP and different types of receptors, and add another level of complexity to the regulation of PI3K and Ras p21 by cAMP–PKA.

Results

PKA phosphorylates Ser 83 in p85α^{PI3K}

p85α subunit of PI3K can be efficiently phosphorylated by PKA *in vitro* (Ciullo *et al*., 2001). A possible PKA target sequence is Ser 83, which is highly conserved in evolution and is preceded by basic residues in bovine/human (KKIS) and mouse/rat (KRIS) sequences.

To determine the relevance of this site *in vivo*, we have substituted Ser 83 with alanine (p85A) to prevent phosphorylation or with aspartic acid (p85D) to mimic the phosphorylated residue. The tagged recombinant proteins were transiently expressed in HeLa or NIH3T3 cells and immunoprecipitated with anti-flag antibody. Immunoprecipitates were incubated *in vitro* with purified catalytic PKA subunit in the presence of [$^{32}\gamma$-P]ATP. p85α^{PI3K} wild type was efficiently phosphorylated by PKA whereas p85A was not, indicating that Ser 83 was the site phosphorylated by PKA *in vitro* (Figure 1a). PKA-phosphorylated p85α^{PI3K} was also identified *in vivo* in breast cancer cells MCF7 extracts immunoblotted with an antiphosphoserine antibody. Figure 1b shows that p85α^{PI3K}, not p85A, was recognized by the phosphoserine antibody in extracts of MCF7 cells treated with cAMP. Figure 1c shows the same experiment performed in NIH3T3 cells expressing p85α^{PI3K} and p85A. The p85A protein was not recognized by the antiphosphoserine antibody. Phosphorylation of p85α^{PI3K} was prevented by incubating the cells with the PKA kinase inhibitor H89 (Figure 1c). The endogenous protein p85α^{PI3K} was also phosphorylated in NIH3T3 cells stimulated with cAMP. Phosphorylation was inhibited by pretreating the cells with H89 (Figure 1d).

Taken together these data indicate that PKA phosphorylates p85α^{PI3K} at Ser 83.

p85α^{PI3K} mutants alter cAMP cytoprotection and growth inhibition

To test the biological relevance of Ser 83 phosphorylation of p85α^{PI3K}, we determined in mouse fibroblasts the rate of *anoikis*, a specific apoptotic pathway triggered by loss of cell adhesion to the extracellular matrix (Frisch and Francis, 1994; Khwaja *et al*., 1997). We co-transfected NIH3T3 cells with a GFP encoding vector and the wild type or the mutant versions of p85α^{PI3K} (Figure 2a and b). At 48 h after transfection, the cells were plated on 2% agarose in a medium containing 0.1% calf serum in presence or absence of 200 μM cAMP. After 5 h, we determined the fraction of apoptotic cells by fluorescent activated cell sorter (FACS) analysis. Figure 2b shows that treatment with cAMP significantly reduced the number of apoptotic cells. cAMP cytoprotection was abolished by wortmannin, a PI3K inhibitor (data not shown and Khwaja *et al*., 1997). Expression of wild-type p85α^{PI3K} amplified cAMP response (see ∗ in Figure 2b), whereas expression of p85A completely eliminated cAMP amplification, displayed by wild-type p85α^{PI3K} (Figure 2b). Cells expressing p85D, on the other hand, were significantly more resistant to *anoikis* in the absence of cAMP (see ∗∗ in Figure 2b). The low response to cAMP of these cells was probably dependent on the low levels of p85D protein (Figure 2a). These data indicate that p85α^{PI3K}

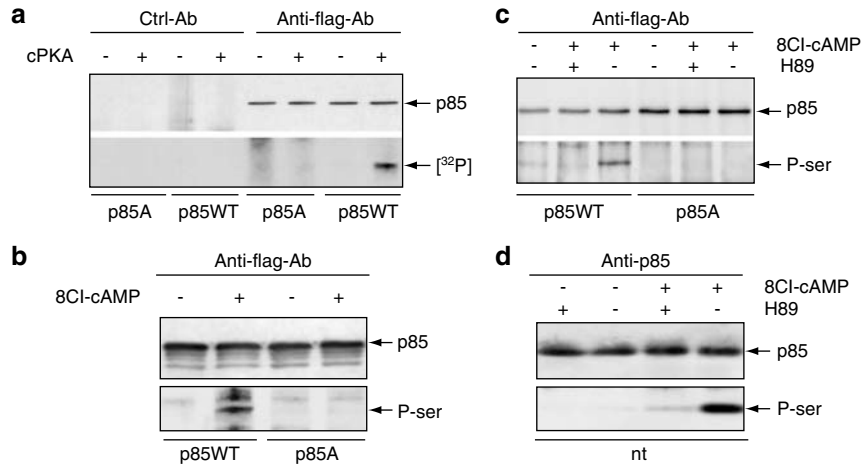

Figure 1 Phosphorylation *in vivo* and *in vitro* of Ser 83 in p85α^{PI3K} by cAMP–PKA. (**a**) *In vitro* phosphorylation of wild-type p85α^{PI3K} or p85A. HeLa cells were transiently transfected with p85α^{PI3K}-flag wild type or p85A; 48 h after transfection, cell lysates were immunoprecipitated with non-immune IgG or anti-flag antibody for 15 h. A/G bound immunoprecipitates were *in vitro* phosphorylated with cPKA as described in Material and methods. Sample aliquots were run on SDS–PAGE and subjected to immunoblot (upper panel) or autoradiography (bottom panel). (**b**) *In vivo* phosphorylation of wild-type p85α^{PI3K} or p85A. MCF7 cells were made quiescent using charcoal-treated serum and medium lacking phenol-red for 3 days. Quiescent cells were transfected with p85α^{PI3K} or p85A. At 24 h after the transfection cells were treated with 100 μM cAMP. Protein lysates were immunoprecipitated with anti-flag antibody for 15 h, separated on 10% SDS–PAGE and immunoblotted with anti-p85^{PI3K} (upper panel) or antiphosphoserine (lower panel) antibodies. The arrow indicates p85α^{PI3K} band. (**c**) NIH 3T3 were transfected with p85α^{PI3K} or p85A. At 24 h after transfection, the cells were treated with 100 μM cAMP in the presence or absence of the protein kinase A inhibitor, H89 (10 μM). In parallel cultures (**d**) un-transfected (nt) NIH3T3 cells, starved in DMEM 0.1% calf serum for 16 h, were treated with 100 μM cAMP for 10 min in the presence or absence of 10 μM H89. Cell lysates were immunoprecipatated with anti-p85α antibody and immunoblotted with anti-p85α (upper panel) or anti-phosphoserine (lower panel) antibodies.

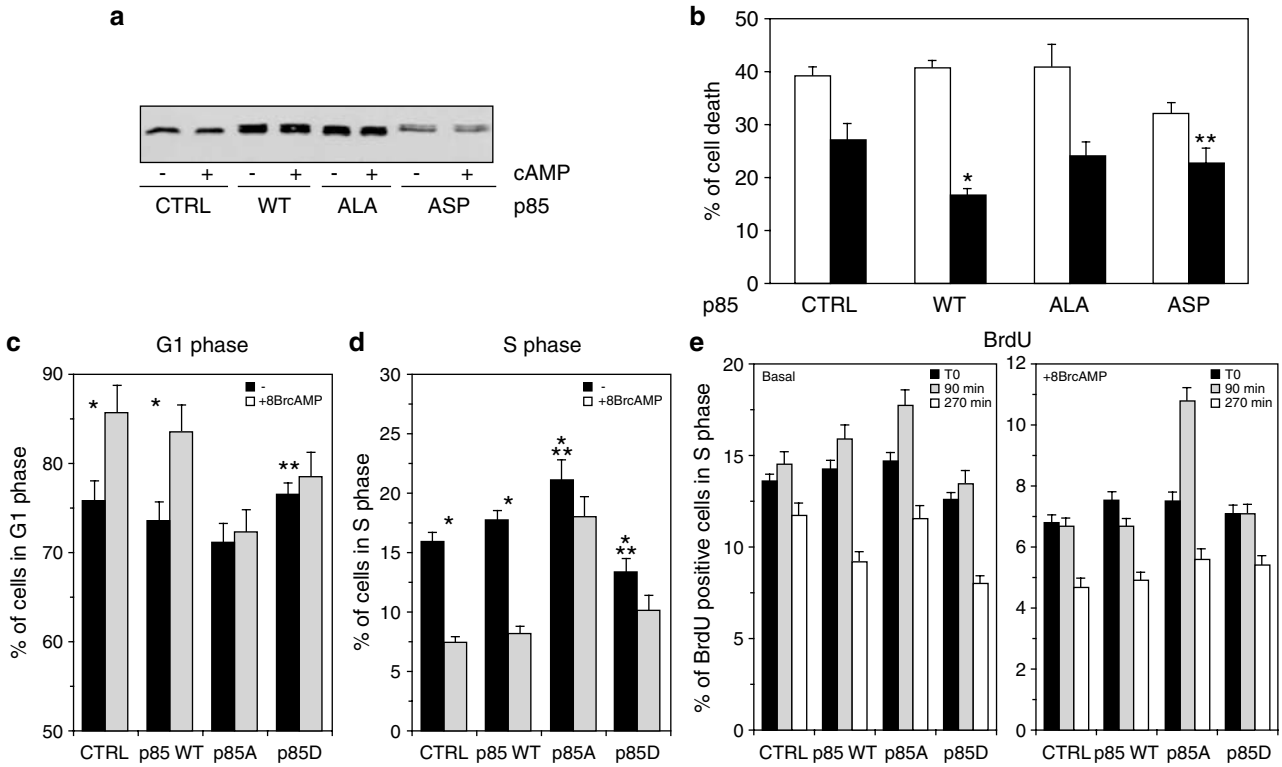

Figure 2 Phosphorylation of p85α^{PI3K} Ser 83 mediates cAMP biological effects on growth and survival. NIH3T3 cells were transiently transfected with p85α^{PI3K}-flag wild type or mutant versions. A GFP expression vector encoding green fluorescence protein was used as reporter. At 48 h after transfection, cells were plated on 2% agarose in DMEM 0.1% CS without or with cAMP 200 μM for 5 h and the fraction of apoptotic cells was determined by FACS analysis. (**a**) Extracts of transfected cells immunoblotted with anti-p85α^{PI3K} antibody. Exogenous p85α^{PI3K} is represented by the upper band in the doublet. (**b**) Quantitative analysis of FACS data. White and black columns represent, respectively, untreated or cAMP-treated samples. The data are the mean of four independent experiments; * indicates $P \leqslant 0.01$ comparing cell death in the presence of cAMP in all samples. The p85A (ALA) sample is significantly different from p85α^{PI3K} (WT), but not from CTRL or p85D (ASP). To analyse the growth, NIH3T3 cells were transiently transfected with the constructs indicated above. At 24 h after the transfection, the cells were serum-starved 15 h and induced into the cycle with 2% serum in the presence or absence of cAMP (200 μM for 6 h). After 12 h the cells were subjected to FACS analysis. The quantitative analysis of FACS data relative to G1 phase (**c**) or S phase (**d**) was derived from three independent experiments and analysed by Student paired *T*-test. Black and gray columns represent respectively untreated or cAMP-treated samples. * indicates $P \leqslant 0.01$ basal versus cAMP; **$P \leqslant 0.01$ basal p85A versus p85D; ***$P \leqslant 0.01$ basal p85α wild type, A or D-transfected cells versus control plasmid-expressing cells. (**e**) Pulse (30′)-chase of BrdU incorporation in transfected cell lines in the presence or absence of cAMP. The columns represent the fraction of labeled cells stained with propidium iodide at 0 (black columns), 90 (gray columns) and 270 min (white columns) from the initial cell cycle induction (10% serum). The data are the mean of three independent experiments.

mediates cAMP inhibition of *anoikis* and that phosphorylation of Ser 83 is an important signal for cAMP-induced-survival.

To test if p85α^{PI3K} mediated also cAMP inhibition of growth in mouse NIH3T3 fibroblasts, we analysed the biological effects of p85α^{PI3K} mutants on growth arrest induced by cAMP. NIH3T3 fibroblasts were cultured in low serum in the presence or absence of cAMP. Under these conditions, cAMP induced a robust and reversible inhibition of cell growth by accumulating the cells in G1 phase and reducing the fraction of cells entering S phase (see * in Figure 2c and d). Cells expressing p85A did not efficiently arrest in G1 in the presence of cAMP (Figure 2c). Conversely, in cell lines expressing the aspartic mutant, cAMP response was lost and the fraction of cells accumulating in G1 was significantly higher than in cells expressing p85A (see ** in Figure 2c). The fraction of cells arrested in S phase in the presence of cAMP was robustly stimulated in the control and cells expressing the wild-type p85α^{PI3K} (see * in Figure 2d). Cells expressing the mutant versions of p85α^{PI3K} did not respond to cAMP and the fraction of cells transiting S increased (see Figure 2d, p85A***) or decreased (see Figure 2d, p85D***) relative to control or wild-type p85α^{PI3K} cells. These data indicate a higher (p85D) or lower rate of G1 arrest (p85A) of these cell lines, independently on cAMP presence in the medium. The high statistical significance of S phase values, relative to G1, is due to the lower value of S phase cell fraction. To discriminate more precisely the effects of cAMP in G1 or S phases in cells expressing p85α^{PI3K} mutants, we performed a kinetic analysis of the cell cycle by labeling the cells with BrdU. Under these conditions, the entry and the progression in S phase of transfected cells can be precisely determined. Figure 2e shows that p85A or p85D did not alter S phase progression,

although p85A-expressing cells transited S phase very rapidly, compared to control or p85D expressing lines in the presence of cAMP. Cells expressing p85A were unrestrained in G1 in the presence of cAMP and entered S phase more efficiently than control or p85D-expressing cells. Taken together, these data indicate that Ser 83 in the p85α^{PI3K} is critical for cAMP-induced G1 arrest on cell cycle progression. Alanine substitution of Ser 83 abolished G1 arrest by cAMP. p85D, on the other hand, increased the number of cells arrested in G1 in the absence of cAMP (see ** in Figure 2d).

p85α^{PI3K} (ser83) phosphorylation increases association of to p21 Ras

We have previously shown that cAMP PKA selectively increased the association between PI3K and p21 Ras (Ciullo *et al*., 2001). As this complex can mediate cAMP–PI3K effects on growth and survival, we set out to determine the formation of p21 Ras–PI3K complex in cells expressing wild-type p85α^{PI3K} or p85A or p85D. NIH3T3 were transfected with p85α^{PI3K} wild type or p85A or p85D and 36 h after transfection, the cells were serum starved for 16 h before 10 min treatment with cAMP. Cell lysates were immunoprecipitated with anti-flag antibody and then analysed by Western blot with anti-Ras antibody. In the presence of cAMP, p85α^{PI3K} wild type efficiently was found associated with p21 Ras. This association was significantly inhibited in cells expressing p85A and stimulated in p85D expressing cells. In the absence of cAMP, Ras–PI3K complex was barely detectable in all the samples. In p85D-expressing cells, however, cAMP stimulated robustly association of PI3K to p21 Ras, suggesting that Ser 83 in p85α^{PI3K} cooperates with another cAMP–PKA signal to regulate PI3K association with p21 Ras (Figure 3a and b). We also tested the effects of H89 on the formation of the Ras–PI3K complex. H89 treatment reduced the complex Ras–PI3K to the levels found in p85A-expressing cells (Figure 3c). These data indicate that under conditions of cAMP stimulation, the interaction Ras–PI3K is significantly favored.

p85α^{PI3K} (Ser 83) phosphorylation selectively amplifies PI3K signaling by cAMP

To directly test the hypothesis that cAMP stimulation amplified Ras–PI3K signalling, we stimulated the cells with cAMP or PDGF and determined AKT and GSK phosphorylation, downstream targets of PI3K. Figure 4a shows that cAMP and PKA stimulated the phosphorylation of AKT and GSKα and that H89 reversed cAMP effects. To test if p85α^{PI3K} mutants interfere also with other pathways that signal to PI3K, we determined PDGF stimulation of AKT and ERK1/2, the two major kinases downstream to PDGFR. Figure 4b shows that expression of p85A and p85D did not significantly influence PDGF stimulation of ERK1/2 or P-AKT accumulation. p85A-expressing cells, on the other hand, poorly phosphorylated AKT or GSKα in response to cAMP. Conversely, expression of p85D increased the basal P-AKT and P-GSKα and robustly amplified cAMP response (Figure 4c and d).

So far we have analysed the effects of p85α^{PI3K} variants on indirect effectors of PI3K signaling. To directly test the relevance of cAMP induced phosphorylation of p85α^{PI3K} on PI3K activity, we have measured the activity of the enzyme in cells expressing wild type or the mutant versions of p85α^{PI3K}. We have previously reported that cAMP in the presence of serum did not stimulate PI3K activity (Ciullo *et al*., 2001). As the presence of serum can mask some important biological effects of cAMP–PKA (Porcellini *et al*., 2003) and (De Gregorio *et al*., 2006, in press), we carried out the

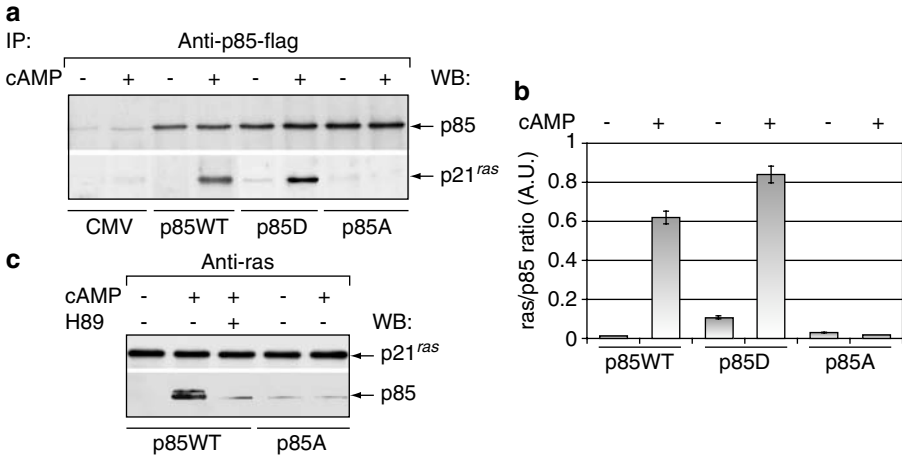

Figure 3 cAMP stimulates the formation of PI3K/Ras complex. NIH-3T3 cells were transiently transfected with wild type or mutant versions of p85α^{PI3K}-flag. At 36 h after transfection, the cells were starved for 16 h in DMEM, 0.1% calf serum and treated with cAMP 200 μM for 10 min. (**a**) Cells lysates were immunoprecipitated with anti-flag antibody and blotted with anti-Ras or anti-p85α^{PI3K} antibodies. (**b**) The histograms represent the ratio of Ras-p85α^{PI3K} bands in the p85α^{PI3K} immunoprecipitates, derived from three independent experiments. (**c**) NIH-3T3 were transiently transfected with wild-type p85α^{PI3K}-flag or p85A. At 36 h after transfection, the cells were starved for 16 h in DMEM, 0.1% calf serum and treated with cAMP 200 μM for 10 min in the presence or absence of H89. Cells lysates were immunoprecipitated with anti-Ras antibody and blotted with anti-Ras or anti-flag antibodies.

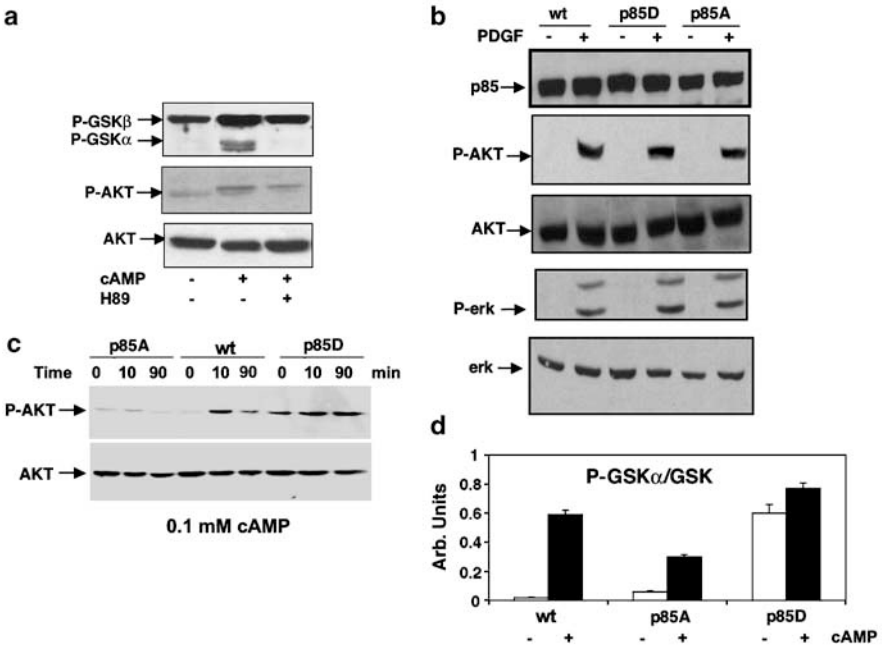

Figure 4 Serine phosphorylation of p85 selectively amplifies cAMP signaling to AKT and GSK. (**a**) NIH3T3 cells were stimulated with 8-Cl-cAMP (100 μM) in the presence or absence of H-89 (10 μM) for 10 min. Total extracts were prepared and immunoblotted with the specific antibodies indicated. (**b** and **c**) Immunoblots of extracts derived from cells transiently transfected with the p85α^{PI3K} expression vectors indicated. In (**b**) the cells were stimulated with PDGF (100 ng/ml) for 15 min, 48 h after the transfection; in (**c**) the cells were stimulated with 8-Cl-cAMP (100 μM) for 10 and 90 min, respectively. (**d**) The histograms of the pGSKα/GSK ratio, evaluated by densitometric analysis of Western blot with appropriate antibodies of extracts derived from cells transfected with the indicated p85α^{PI-K} plasmids and stimulated with (black) or without (white) cAMP (100 μM) for 10 min. Basal P-AKT in cells expressing p85D is lower in (**b**) than in (**c**), because the cells were starved 24 h (**b**), instead of 16 h (**c**).

stimulation of transfected cells with cAMP in low serum (0.2%). Figure 5 shows that cAMP stimulated PI3K activity. The activation of PI3K was inhibited by treatment of the cells with the PKA inhibitor, H89. Moreover, cells expressing p85A did not activate PI3K in response to cAMP, whereas efficiently activated PI3K, when treated with PDGF. Conversely, p85D amplified PI3K activity induced by cAMP and did not significantly influence PI3K stimulated by PDGF (Figure 5, lower inset).

To determine if p85A altered the structure of the protein and inhibited the formation of the PI3K complex, we tested the ability of *in vitro* synthesized p85α^{PI3K} to interact and form a stable complex with p110α^{PI3K}. Supplementary Figure 1S shows that co-translated p85α^{PI3K} and p110α^{PI3K} interact very efficiently. When tested under the same conditions, p85A ability to interact with p110α^{PI3K} was comparable to that of the wild-type p85α^{PI3K} (Supplementary Figure 1S). p85D, on the other hand, formed a complex with p110α^{PI3K} synthesized *in vitro*, with a higher affinity than p85α^{PI3K} wild type or p85A. These data indicate that p85A does not disrupt the folding of p85α^{PI3K} protein or the ability to interact with p110α^{PI3K}, whereas p85D induces a conformational change that improves the interaction with p110α^{PI3K}. As p85D stimulates cAMP-induced PI3K activity, we suggest that this conformational change activating the enzyme is mediated by phosphorylation of p85α^{PI3K} in Ser 83 by PKA.

cAMP–PKA amplify estrogen binding and signaling to PI3K

PI3K mediates also AKT activation by estrogens (Simoncini *et al*., 2000; Castoria *et al*., 2001). As p85α^{PI3K} was efficiently phosphorylated by PKA in MCF7, the breast carcinoma cell line, we asked whether p85α^{PI3K} phosphorylation on Ser 83 also affected PI3K interaction with upstream effectors such as estrogen receptor. Therefore, we analysed the phosphorylation of AKT stimulated by estrogen**.** Cells expressing p85α^{PI3K} wild type or p85A or p85D were treated with estrogens and stimulation of AKT was monitored with specific P-AKT antibodies. Figure 6a shows that p85D amplified AKT phosphorylation induced by estrogens and that p85A abolished this stimulation. Also, induction of GSK phosphorylation by estrogens was inhibited by p85A expression (data not shown).

As estrogen stimulation of AKT was inhibited in cells expressing p85A, we measured the fraction of the estrogen receptor α bound to wild type and mutant versions of p85α^{PI3K}. Figure 6b shows that wild-type p85α^{PI3K} immunoprecipitated efficiently the receptor. The binding of estrogen receptor to p85α^{PI3K} was stimulated by estrogens, as expected. cAMP did not stimulate the binding *per se* in the absence of estrogens, but amplified the action of estrogens on p85α^{PI3K} binding. H89, a PKA inhibitor, eliminated the binding of the receptor to p85α^{PI3K}. The p85A protein did not bind the estrogen receptor in the presence or in the absence of estrogens. Expression of p85D substituted

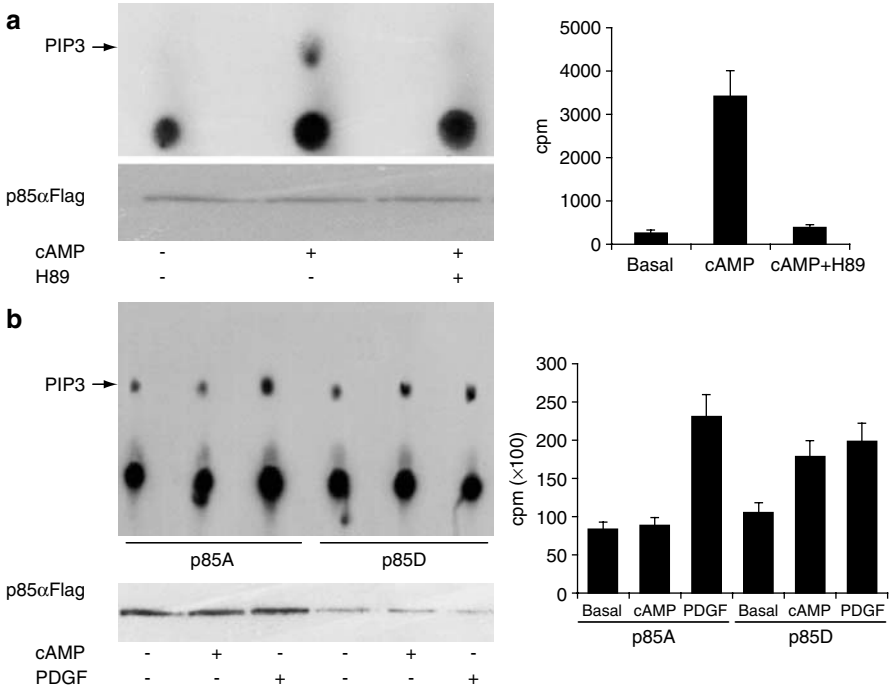

Figure 5 cAMP stimulates PI3K activity in the absence of serum. Effects of p85α^{PI3K} mutants on PI3K catalytic activity. NIH3T3 cells were transfected with the p85α^{PI3K} expression vectors indicated and stimulated with cAMP (100 μM) 20 min in the presence or absence of H89 (10 μM) or PDGF (100 ng/ml) 15 min, 48 h after transfection. Total extracts were prepared and subjected to immunoprecipitation with anti-flag antibody. Immunoprecipitates were quantified by immunoblot with anti-p85α^{PI3K} antibodies (p85α^{PI3K}-flag) and assayed for PI3K activity by using a mixture of lipids containing PI4-5 (see Materials and methods). PI-4-5 labeled at position 3 is indicated by the arrow. The upper inset (**a**) shows PI3K activity associated with p85α^{PI3K}-flag wild type and the immunoblot with anti-p85α^{PI3K} antibody of the p85α^{PI3K}-flag immunoprecipitate. The lower inset (**b**) shows PI3K activity associated with p85A and p85D, immunoprecipitated with the anti-flag antibody. The immunoblot with the p85α^{PI3K} antibody is shown below. The histograms on the right show the statistical analysis of (**a**) and (**b**) derived from three experiments.

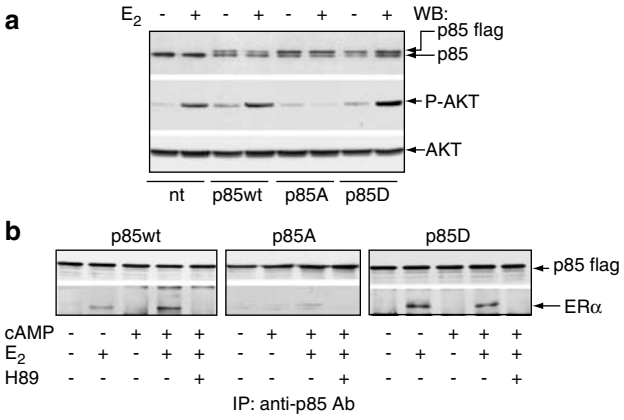

Figure 6 cAMP phosphorylation of p85α^{PI3K} amplifies estrogen binding and signaling to PI3K. MCF-7 cells were made quiescent by charcoal-treated serum and medium lacking phenol-red for 3 days. (**a**) Quiescent cells were transfected with p85α^{PI3K} wild type or p85A or p85D. After 24 h, the cells were treated with 10 nM E_2 for 3 min. Cell lysates were analysed by Western blot with anti-p85α^{PI3K} (upper panel), P-AKT (middle panel) or AKT (lower panel). (**b**) Quiescent cells were transfected with the wild type or the mutant versions of p85α^{PI3K}. After 24 h, the cells were treated with 10 nM E_2 for 3 min or for 10 min with 200 μM cAMP, or with 10 nM E_2 for 3 min following a 10-min stimulation with 200 μM cAMP. The cell lysates were immunoprecipitated with anti-flag antibody 15 h, as described above. Sample aliquots were immunoblotted with anti-ERα and anti-p85α^{PI3K} antibodies to detect the PI3K/ER complex. Lanes 5 and 14 show the complex estrogen receptor-p85α^{PI3K} in cells pretreated with 10 μM H89 for 30 min.

cAMP by amplifying estrogen stimulated binding of the receptor to p85α^{PI3K}. H89 inhibited the binding of p85D to estrogen receptor, indicating that another phosphorylation PKA-dependent was necessary for the formation of the complex estrogen-p85α^{PI3K}.

These data indicate that phosphorylation by cAMP–PKA of Ser 83 in p85α^{PI3K} is required for estrogen binding and signalling to PI3K.

Discussion

The data presented here indicate that the phosphorylation of p85α^{PI3K} in a critical serine residue mediates cAMP action on growth and survival. More specifically, phosphorylation of Ser 83 in p85α^{PI3K} shifts the signaling flow towards PI3K-driven pathways. Such a conclusion is supported by several observations. The substitution of Ser 83 with alanine inhibited cAMP effects on cell survival and G1 arrest in NIH 3T3 fibroblasts. Conversely, the aspartic mutant of Ser 83 in p85α^{PI3K} stimulated cell survival in the absence of cAMP and slowed down cell cycle cycle progression, replicating the effects of cAMP. Ras binding to PI3K and AKT phosphorylation were inhibited by expressing p85A and were stimulated by p85D.

cAMP effects on growth and cell cycle progression are very selective. cAMP–PKA act on at least three phases

of cell cycle. cAMP slows down G1, blocks S phase and G2–M transition (Kato *et al*., 1994; Kurokawa and Kato, 1998; Houslay and Kolch, 2000). Our data indicate that p85α^{PI3K} phosphorylation of Ser 83 controls selectively G1–S transition (Figure 2).

PI3K is an important regulator of cell growth as it links cell size, growth and cycle progression. For example, constitutive expression of p110α increases cell mass and size by stimulating the biosynthetic capacity of the cells. However, if not restrained, p110 activity impairs exit from the cell cycle (Alvarez *et al*., 2003). We suggest that Ras–PI3K signals regulate the length of G1 phase. cAMP by amplifying and stabilizing PI3K, lengthens G1 and slows down G1–S transition. The net result is an apparent G1–S block, which results in increased biosynthetic activity and cell mass prior to division. This is better shown in thyroid cells, FRTL5, which are exquisitely dependent on TSH and cAMP for growth and differentiation. In these cells, Ras and PI3K are selectively required for G1 phase progression (Ciullo *et al*., 2001). In thyroid cells, TSH induces PI3K activity and stabilizes the complex Ras–PI3K. Also, expression of p85 mutant (p85A) inhibits selectively the stimulation of growth and PI3K activity induced by TSH (De Gregorio *et al*., in press). In thyroid cells and in cells expressing high levels of the regulatory subunit of PKA, RIIβ, p85α^{PI3K} binds and anchors PKA. This binding is amplified in cells expressing p85D and it is H89 resistant (De Gregorio *et al*., in press). This suggests that anchoring PKA to the membrane is a prerequisite for cAMP–PKA biological effects.

Also, cAMP–PKA stimulate in the absence (Figure 5), not in the presence of serum (Ciullo *et al*., 2001), PI3K activity. This stimulation requires phosphorylation of Ser 83 in in p85α^{PI3K}, probably because phosphorylated p85α^{PI3K} induces a conformational change in the p110α^{PI3K} molecule (Supplementary Figure 1S) or increases its affinity to PKA (De Gregorio *et al*., in press). The stimulation of PI3K activity by cAMP appears to be very selective, as p85A or p85D mutants did not interfere with PDGF (Figure 4) or EGF (De Gregorio *et al*., in press) induced PI3K activity.

Although the cell lines we have used are not robustly dependent on cAMP signaling for growth or differentiation, our data indicate that PI3K-Ras signals are amplified by cAMP both in cAMP-independent (NIH3T3 fibroblasts or breast MCF7 cells) or dependent (thyroid) cells. In these latter cells, amplification of PI3K by cAMP is mainly triggered by TSH and PKA, which binds p85α^{PI3K}. The amplification of the pathway Ras–PI3K induced by cAMP is redundant in 3T3, but not in thyroid cells, because in these latter cells expression of p85A triggers apoptosis (De Gregorio *et al*., in press).

We suggest that Ser 83 in p85α^{PI3K} is an important point of convergence of two parallel pathways: cAMP–PKA and PI3K signals. In this framework, it is worth noting that in *Saccharomyces cerevisiae* inactivation of PI3K–TOR signalling is partly compensated by iper-activation of cAMP–PKA (Rohde *et al*., 2004; Zurita-Martinez and Cardenas, 2005).

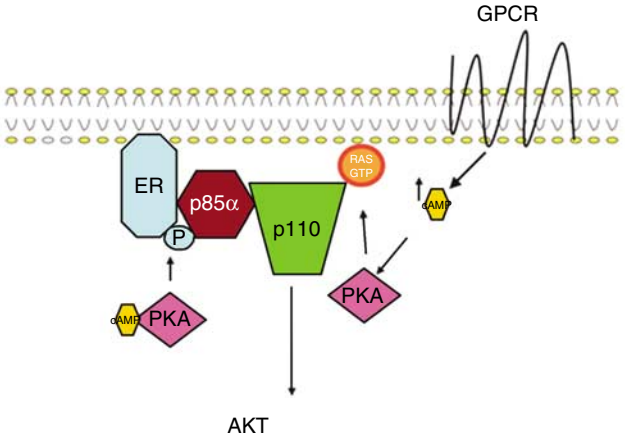

Figure 7 Amplification of estrogen and Gs protein-coupled receptors (GPCR) signaling to PI3K. A scheme outlining the effects of cAMP–PKA activation by Gs protein coupled receptors on PI3K signaling by estrogens. PKA and cAMP arrows on the right side indicate other cAMP–PKA targets that amplify Ras–PI3K interaction. p85α^{PI3K} links receptors coupled to G proteins to estrogens and Ras–PI3K signals.

Our data indicate that estrogen receptor signalling was profoundly altered when p85A was expressed. Accordingly, p85α^{PI3K} binding to estrogen receptor was severely impaired. Although PKA phosphorylates directly AKT and estrogen receptor (Cui *et al*., 2004), the effects we described were mainly dependent on p85α^{PI3K}, as they were inhibited or amplified by expressing the p85α^{PI3K} alanine or aspartic mutants, respectively. Moreover, we have data indicating that also induction of estrogen responsive genes is inhibited by H89 and it is significantly altered in p85A-expressing cells (data not shown). Estrogens stimulate adenylyl cyclase (Aronica *et al*., 1994) and cAMP–PKA stimulate differentiation of granulosa cells (Knecht *et al*., 1984). Our data indicate that phosphorylation of Ser 83 in p85α^{PI3K} by cAMP PKA stabilizes the binding of activated receptor to PI3K and stimulates PI3K activity.

General implications

The data presented above have broad implications because they point to p85α^{PI3K} as the physical link between Ras, estrogen receptor and cAMP–PKA (Figure 7). Also, we find in many and unrelated cell types that cAMP amplification of PI3K is essential for negative or positive cAMP effects on growth and survival. Apparently, all receptors binding p85α^{PI3K} can cooperate with cAMP–PKA signals via phosphorylation of Ser 83 in p85α^{PI3K}. This may explain the pleiotropic nature of the effects exerted by cAMP–PKA on several, apparently unrelated, signaling cascades and illustrates how cAMP–PKA can influence Ras p21 effectors at multiple levels.

Materials and methods

Plasmid construction

The cDNA encoding for p85α^{PI3K}-flag wild type was generated by fusing the flag sequence (MDYKDDDDK) to the C-

Terminus of bovine p85α^{PI3K} cDNA in the pSG5 vector (gift of Dr J Downward). The region of p85α^{PI3K} from the *Xho*I restriction site in position 1014 (Acc. No: 163476) was amplified by PCR with a 3′ end primer containing the flag sequence and a *Bam*HI restriction site. The PCR product was subcloned in the II-TOPO vector (Invitrogen), digested with *Xho*I and *Bam*HI, extracted and ligated to a pSG5-p85α^{PI3K} vector previously digested with the same restriction enzymes. The vector encoding p85α^{PI3K}-flag was then used as template for site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene) to convert Ser 83 in alanine or aspartic acid. All the plasmid constructs were verified by DNA sequence analysis.

Cell culture and transfections
HeLa and NIH3T3 cells were grown in RPMI with 10% fetal calf serum or Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum, respectively. MCF-7 cells were grown in DMEM with 5% fetal bovine serum, supplemented with: penicillin/streptomycin 100mU/ml, 2 mM glutamine, 6 ng/ml insulin and 3.75 ng/ml hydrocortisone. Cells were transfected with lipofectamine (Gibco-Invitrogen, Carlsbad, CA, USA). In all transfections, RSV-LacZ was included to determine and normalize transfection efficiency. Experiments varying in the transfection efficiency above 20% were discarded.

Antibodies, immunoprecipitation and immunoblot
The antibodies used were: anti-Ras (mouse monoclonal pan-Ras clone 10, UBI (an affiliate of Millipore, Billerica, MA, USA)), anti-p85PI3K (rabbit polyclonal, UBI), anti-P-Serine (rabbit polyclonal, Zymed, San Francisco, CA, USA), anti-Erk 1/2 (rabbit polyclonal, Santa Cruz, Santa Cruz, CA, USA), anti-P-Erk 1/2 (mouse monoclonal, Santa Cruz), anti-P-Akt Ser 473 (rabbit polyclonal, Cell Signaling, Beverly, MA, USA), anti-Akt (rabbit polyclonal, Cell Signaling), anti-P-Gsk Ser 21/9(rabbit polyclonal, Cell signaling), anti-GSKα/β (mouse monoclonal, UBI). The anti-flag antibody was the mouse monoclonal Sigma M2 antibody.

Immunoprecipitation
Total extracts were prepared by lysing the cells on ice 5 min with 50 mM Tris-HCl pH 7.4, 1% Nonidet NP-40, 100 mM NaCl, 2 mM EDTA 50 mM NaF, 0.1 mM $NaVO_3$ 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate and a protease inhibitor cocktail. Cell lysates (2 mg/ml) were incubated with 4 μg of antibody/0.5–1 mg of protein at 4°C in gentle rock agitation 15 h. At the end of incubation, 20 μl of A/G plus were added to samples and the immunoprecipitates were collected by centrifugation. SDS–PAGE and immunoblots were performed as previously described (Feliciello *et al*., 2000).

Anoikis
At 48 h after transfection, the cells were collected and maintained in suspension on dishes covered with a thin layer of 2% agarose. Cells were plated in medium containing 0.1% serum with or without 200 μM CPT-cAMP (Sigma) for 5 h. At the end of incubation, the cells were collected, washed twice with phosphate-buffered saline (PBS) and incubated 5 min with propidium iodide and analysed by FACS.

Analysis of DNA content and 5′-bromo-2′-deoxyuridine (BrdU) incorporation
2×10^6 transfected cells (48 h after transfection) were plated in 100 mm dishes and grown in low serum (0.5% fetal bovine serum) for 18 h in the presence or in absence of 50 μM 8-Cl cAMP. Cells were labelled for 30 min with BrdU to a final concentration of 20 μg/ml and harvested at 0, 90 and 270 min. After treatment, the cells were fixed in ice-cold 70% ethanol for 4 h at +4°C and washed three times in PBS. Cells were re-suspended in 0.25 ml of 1 N HCl and maintained 20 min at room temperature. After acidic denaturation of DNA, the cells were washed two times in phosphate/citric buffer (0.2 M Na_2HPO_4; pH 7.4). BrdU incorporation was revealed by anti-BrdU-FITC (Becton Dickinson, Franklin Lakes, NJ USA) and then stained for 30 min at room temperature in 0.1% Triton X-100, 0.2 mg/ml DNase-free RnaseA, 20 μg/ml propidium iodide. Fluorescence was determined by using the FACScan Flow Cytometer (Becton Dickinson). Experiments were performed in triplicate. The data were acquired and analysed by CELLQuest software (Becton Dickinson) and by Cell Fit Cell-Cycle Analysis Version 2 for bivariate analysis of DNA content versus BrdU incorporation.

In vitro *phosphorylation*
HeLa cells were transiently transfected with p85α^{PI3K}-flag and p85A. At 48 h after transfection, cell lysates were immunoprecipitated with non-immune IgG or anti-flag antibody 15 h at 4°C. Protein A/G bound immunoprecipitates were washed twice with lysis buffer and finally with kinase buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES) 20 mM, $MgCl_2$ 10 mM, pH 7.4). The washed immunoprecipitates were treated with 0.4 μg of partly purified catalytic subunit of protein kinase A. Each aliquot was incubated in a final volume of 30 μl of kinase buffer containing 10^{-5} M cAMP, 100 μM ATP and 10 μCi[γ^{32}P-ATP] for 30 min at 30°C. The reaction was terminated by adding 2 × Laemmli buffer.

Lipid kinase assay
Lipid kinase activity was determined as described by Maier *et al*. (1999). Briefly, the assays were carried out in a final volume of 50 μl containing 0.1% bovine serum albumin, 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid), 120 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM glycerophosphate, 7 mM $MgCl^2$ (buffer E). Lipid vesicles (30 μl containing 320 μM phosphatidylethanolamine, 300 μM phosphatidylserine, 140 μM phosphatidylcholine, 30 μM sphingomyelin, supplemented with 40 μM PI-4,5-P2 in buffer E) were sonicated 1 h and incubated on ice 10 min. Some aliquots were preincubated with LY294002, 0.5 μM (Calbiochem (EMD Biosciences, Inc, an Affiliate of Merck KGaA, Darmstadt, Germany)) at 37°C. The immunoprecipitates were added to the lipid mixture and incubated for 10 min at 4°C in a final volume of 40 μl. The reaction was started by adding 40 μM ATP (1 μCi of [-^{32}P]ATP in 10 μl of the assay buffer at 30°C. After 15 min, the reaction was stopped with ice-cold 150 μl HCl (1 N) on ice. The lipids were extracted by vortexing the samples with 500 μl of chloroform/methanol (1:1). After centrifugation the organic phase was washed twice with 200 μl of 1 N HCl. Phosphorylated lipids were separated by TLC, detected by autoradiography and quantified with Phosphor-Imager.

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ORIGINAL ARTICLE

The p85 regulatory subunit of PI3K mediates TSH–cAMP–PKA growth and survival signals

G De Gregorio[1], A Coppa[2], C Cosentino[3], S Ucci[1], S Messina[1], A Nicolussi[2], S D'Inzeo[2], A Di Pardo[1], EV Avvedimento[3] and A Porcellini[1,2]

[1]*Dipartimento di Patologia Molecolare IRCCS Neuromed, Località Camerelle, Pozzilli, Italy;* [2]*Dipartimento di Medicina Sperimentale, Università 'La Sapienza', Viale Regina Elena, Roma, Italy and* [3]*1 Dipartimento di Biologia e Patologia Molecolare e Cellulare, Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Università Federico II, Napoli, Italy*

Phosphatidylinositol 3-kinase (PI3K) is necessary for thyroid stimulating hormone (TSH)-induced cell cycle progression. To determine the molecular mechanism linking PI3K to TSH, we have identified a serine residue in p85α^{PI3K} phosphorylated by protein kinase A (PKA) *in vitro* and *in vivo*. Expression of an alanine mutant (p85A) abolished cyclic AMP/TSH-induced cell cycle progression and was lethal in thyroid cells (FRTL-5). The aspartic version of the p85α^{PI3K} (p85D) inhibited apoptosis following TSH withdrawal. The p85α^{PI3K} wild type not the p85A bound PKA regulatory subunit RIIβ in cells stimulated with cAMP or TSH. The binding of the aspartic version of p85α^{PI3K} to RIIβ 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. Finally, we found that the catalytic activity of PI3K was stimulated by TSH in cells expressing the wild-type p85α^{PI3K} but not in cells expressing p85A. This latter mutant did not affect the epidermal growth factor-stimulated PI3K activity. We suggest that (1) TSH–cAMP-induced PKA phosphorylates p85α^{PI3K} at serine 83, (2) phosphorylated p85α^{PI3K} binds RIIβ-PKA and targets PKAII to the membrane, and (3) PI3K activity and p21Ras binding to PI3K increase and activate PI3K downstream targets. This pathway is essential for the transmission of TSH–cAMP growth signals.
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Correspondence: Dr A Porcellini, Dipartimento di Medicina Sperimentale, Università 'La Sapienza', Viale Regina Elena, 324 00161 Roma, Italy.
E-mail: antonio.porcellini@uniroma1.it or
Professor EV Avvedimento, Dipartimento di Biologia e Patologia Cellulare e Molecolare 'L Califano', Università 'Federico II', Via S Pansini 5, 80131 Napoli, Italy.
E-mail: avvedim@unina.it
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Introduction

Stimulation of growth by cyclic AMP–protein kinase A (cAMP–PKA) in selected cell types, as in thyroid cells, is tightly dependent on Ras and Phosphatidylinositol 3-kinase (PI3K) (Kupperman *et al*., 1993; Al-Alawi *et al*., 1995; Miller *et al*., 1998). Also, cAMP–PKA are powerful survival signals in all cell types tested (Affaitati *et al*., 2003; Wang *et al*., 2005). To date, the mechanism(s) and the relevant players mediating the effects of on growth and survival are not completely known.

We have recently reported that growth of cells, NIH 3T3, normally inhibited by cAMP (Magnaldo *et al*., 1989; Chen and Iyengar 1994), can be potently stimulated by cAMP if the PKA subunit RIIβ is expressed in these cells (Porcellini *et al*., 2003). The presence of RIIβ in these lines amplified cAMP survival and transcriptional signals. We have previously shown that cAMP–PKA influence Ras signaling, by selectively stimulating the Ras–PI3K complex. Moreover, p85α^{PI3K} is an efficient PKA substrate *in vitro* (Ciullo *et al*., 2001).

We have identified a serine (83) in the p85α^{PI3K} molecule that is phosphorylated *in vivo* and *in vitro* by PKA. This phosphorylation mediates the interaction of PI3K with p21Ras and estrogen receptor α (Cosentino *et al*., 2006).

In this work, we describe the biological consequences of the expression of mutant versions of p85α^{PI3K} in FRTL-5 thyroid cells and in an engineered NIH 3T3 cell line that recapitulates the features of thyroid stimulating hormone (TSH)-dependent growth of thyroid cells. Our results demonstrate that phosphorylation of serine 83 is critical for TSH-dependent cell cycle progression and cell survival. Moreover, we found that phosphorylation of serine 83 by cAMP–PKA was essential for binding of p85α^{PI3K} to the PKA regulatory subunit RIIβ.

We conclude that, in the presence of the RIIβ-PKA subunit, abundant in cAMP-stimulated cells, p85α^{PI3K} enucleates a complex containing PKA. Phosphorylation of serine 83 in the p85α^{PI3K} molecule amplifies PKA binding to PI3K and stimulates the Ras–PI3K complex, which ultimately mediates survival and growth signals induced by TSH–cAMP. This pathway is essential for cAMP-dependent proliferation.

Results

p85 phosphorylation mediates TSH-dependent cell cycle progression and cell survival

Previous data from our laboratory have shown that the $p85\alpha^{PI3K}$ regulatory subunit can be phosphorylated by PKA *in vitro* (Ciullo *et al*., 2001). Sequence analysis of $p85\alpha^{PI3K}$ revealed a putative PKA phosphorylation site, which was conserved in mammalian $p85\alpha^{PI3K}$. To verify that this sequence was a bona fide PKA site, we have substituted this serine 83 by alanine (p85A) or aspartate (p85D) to prevent or to mimic phosphorylation of serine, respectively. The tagged wild-type recombinant protein was phosphorylated *in vivo* and *in vitro* by PKA, whereas p85A protein was not (Cosentino *et al*., 2006).

To test the biological effects of $p85\alpha^{PI3K}$ phosphorylation on thyroid cell growth, we transfected the rat thyroid cell line, FRTL-5, with p85WT, p85A and p85D expression vectors. As we did not score a significant number of stable transfectants expressing p85A, we tested the plating efficiency of cells transfected with p85A or p85D. Figure 1a shows that the expression of p85A significantly reduced the number of clones of FRTL-5 cells expressing p85A. Conversely, the number of clones was significantly higher in cells expressing p85D (Figure 1a). These data indicate that phosphorylation of serine 83 in $p85\alpha^{PI3K}$ significantly altered the growth or survival of differentiated thyroid cells. To test this notion in a defined experimental system, we used a unique cellular model that recapitulates the features of TSH-dependent growth and survival, that is, fibroblasts coexpressing the PKA regulatory subunit RIIβ and wild-type TSHR under the control of an inducible promoter (NTCRII). In this model, in the absence of RIIβ, activation of TSHR by TSH induced growth arrest in the presence of serum. In the absence of serum, these cells, stimulated with TSH, replicated their DNA but underwent apoptosis. Coexpression of RIIβ inhibited apoptosis and stimulated the growth of the cells only in the presence of TSH or cAMP (Porcellini *et al*., 2003). We have expressed p85A or p85D in these cells in the presence or absence of TSH or 8BrcAMP. Figure 1b shows that cells expressing TSHR and RIIβ, which proliferated in the presence of TSH, underwent apoptosis when starved of TSH. Expression of p85WT slightly inhibited apoptosis. Expression of p85D significantly reduced the fraction of apoptotic cells starved of TSH. On the other hand, expression of p85A significantly reduced the survival of these cells in the presence of TSH. These data indicate that phosphorylation of serine 83 amplifies the transmission of survival signals by TSH and PKA, and explain the low survival rate of thyroid cells expressing p85A (Figure 1a).

To analyse more precisely the biological effects of p85A or p85D on cycle progression and growth of TSH–RIIβ-expressing cells, we carried out a detailed cell cycle analysis by performing 5-bromodeoxyuridine (BrdU) pulse–chase labeling experiments. In low serum, TSH induced DNA synthesis and stimulated S and G2–M transition in cells expressing p85WT or control plasmids (Figure 1c). Expression of p85A resulted in a significant accumulation of cells in S phase. Note that in 270 min, control cultures exit S phase and accumulated in G2 M. Expression of p85D did not significantly alter cell cycle progression (Figure 1c). We have also tested if the expression of p85A or p85D mutants altered cell cycle progression or proliferation induced by epidermal growth factor (EGF). Supplementary Figure 1S shows that the growth rate and cell cycle progression induced by EGF or serum (not shown) were not affected by the expression of $p85\alpha^{PI3K}$ mutants.

These experiments indicate that inhibition of phosphorylation of $p85\alpha^{PI3K}$ at serine 83 slows down S–G2/M progression induced by TSH–cAMP.

Phosphorylation of serine 83 in $p85\alpha^{PI3K}$ is required for TSH-induced AKT phosphorylation

As p85A inhibited cell cycle transitions induced by TSH, we tested whether the signaling cascade triggered by TSH or cAMP was impaired. cAMP and TSH stimulated PKA, which can directly phosphorylate AKT (Sable *et al*., 1997; Filippa *et al*., 1999) and/or induce the recruitment of PI3K to the membrane (Suh *et al*., 2003). We determined the phosphorylation of AKT in cell lines expressing p85WT or p85 mutant in the presence or absence of TSH. The ratio P-AKT/total AKT, a rather accurate index of AKT activation, was determined. Cells expressing p85WT, the p85A mutants or p85D mutants were stimulated with TSH for various periods of time and tested for AKT phosphorylation. Figure 2 shows that TSH induced AKT phosphorylation and that in cells expressing p85A, AKT induction was almost completely abolished. Both FRTL-5 (Figure 2a) and NTCRII cells (Figure 2b), expressing p85A, were unable to activate AKT in the presence of TSH. The expression of p85D did not significantly affect the absolute level of AKT activation by TSH, but modified the kinetics of activation in both cell lines (Figure 2a and b). Induction of P-AKT by EGF was not affected in cells expressing both mutants (Supplementary Figure 2S). Pretreatment with a PKA inhibitor H89 (10 μM) abolished TSH-induced AKT phosphorylation (data not shown). These data indicate that serine 83 in $p85\alpha^{PI3K}$ is important for TSH induction of AKT.

Phosphorylation of serine 83 influences the formation of PKA–PI3K and Ras–PI3K complexes

The p85A mutant induced apoptosis, inhibited cell cycle progression and prevented AKT phosphorylation by TSH in thyroid and NTCRII cells. Both cell lines were exquisitely dependent on TSH and cAMP for growth. As in these cells the expression of PKA subunit RIIβ conferred TSH- and cAMP-dependent growth (Porcellini *et al*., 2003), we tested whether $p85\alpha^{PI3K}$ formed a complex with RII-PKA. The RIIβ–$p85\alpha^{PI3K}$ complex was assayed by a p85 pull-down assay, using the glutathione *S*-transferase (GST)-RIIβ fusion protein as bait (Grieco *et al*., 1996). The binding assay was carried out with extracts derived from NTCRII cells transiently transfected with the p85WT, p85A, p85D or with the

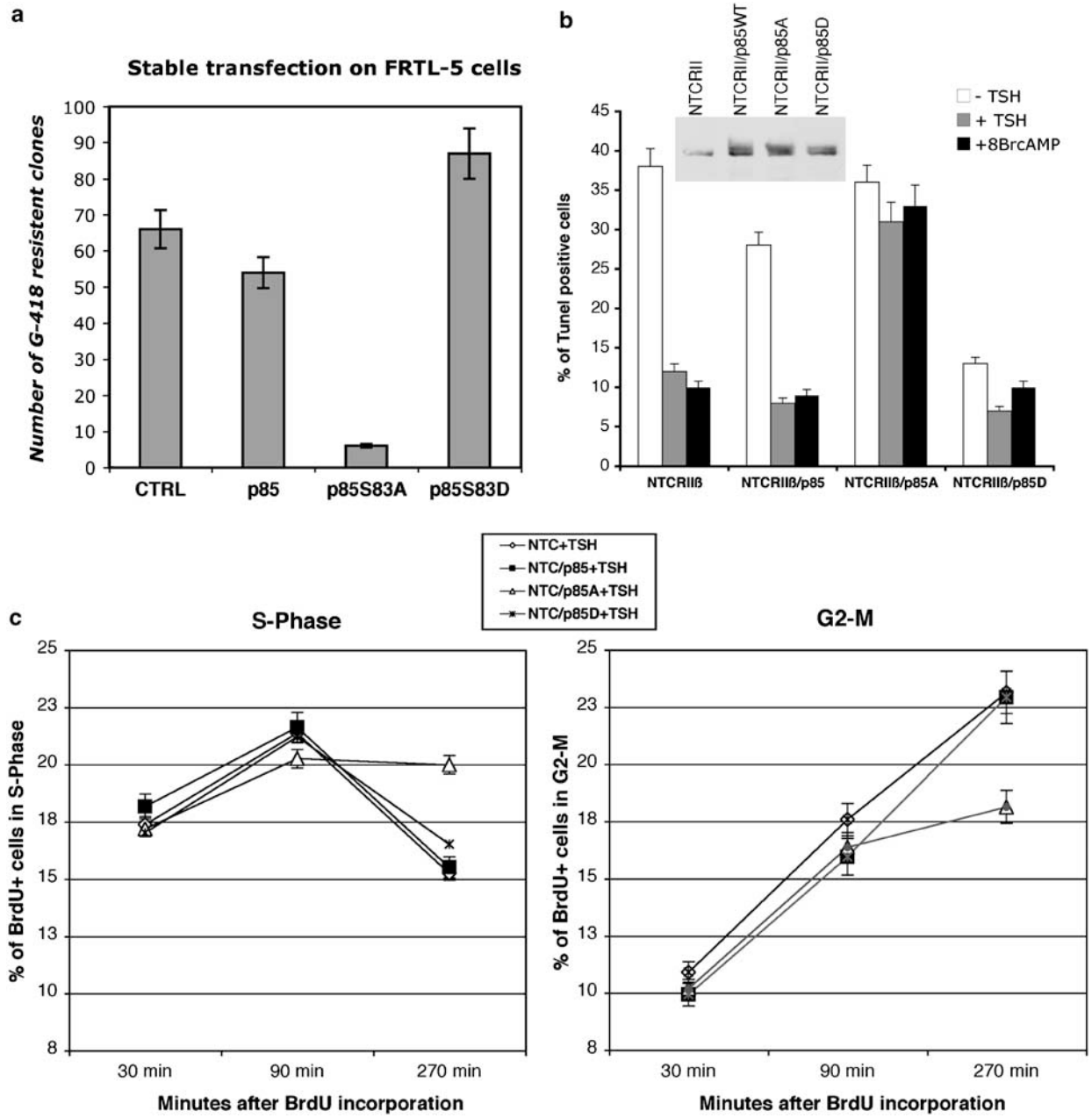

Figure 1 Phosphorylation of serine 83 of p85 mediates TSH-induced cell cycle progression and cell survival. (**a**) Plating efficiency of FRTL-5 cells expressing $p85^{PI3K}$ wild type, p85A or p85D. The ability to form G-418-resistant clones was determined by transfecting FRTL-5 cells with expression vectors encoding $p85^{PI3K}$ wild type, the A mutant version (p85A) or the D mutant version (p85D) and selecting the clones in the presence of 400 μg/ml of G-418 for 15 days. Immunoblot analysis of duplicate plates indicated comparable copy number of transfected $p85^{PI3K}$. The histogram represents the number of colonies derived from 5×10^5 transfected cells (mean of three experiments in triplicate). (**b**) Apoptosis assay of NTCRII cells transfected with cytomegalovirus (CMV) (CTRL), p85WT, p85A or p85D treated without (white bar) or with TSH (gray bar) or 8BrcAMP (black bar). Cells were transiently transfected with the constructs indicated above. At 18 h after induction with 0.5% serum in the presence of TSH or cAMP, cells were subjected to TUNEL assay as described in Materials and methods. Inset: Western blot analysis with anti-$p85^{PI3K}$ antibody to verify that expression of the constructs was comparable in the different samples. (**c**) Cell cycle progression of cells expressing p85WT, p85A or p85D. NTCRII cells were transiently transfected with the p85WT, p85A, p85D mutants or with the empty vector (control). At 24 h after transfection, the cells were serum starved for 18 h and induced with 10 mU/ml TSH in the presence of 0.5% serum. BrdU incorporation was assayed by a pulse–chase experiment. Cells were labeled for 30 min with BrdU and harvested at 0, 90 and 270 min. Cells were subjected to fluorescence-activated cell sorting (FACS) analysis for bivariate analysis of DNA content versus BrdU.

empty vector (control) and from FRTL-5 clones stably transfected with the same $p85\alpha^{PI3K}$ vectors. FRTL-5 cells were derived from the few clones expressing low levels of p85A, as shown in Figure 1a.

Figure 3a shows that phosphorylated p85WT binds the PKA type II regulatory subunit RIIβ in a TSH-dependent manner. TSH treatment (10 mU/ml) for a period of 10 min induced the formation of the complex

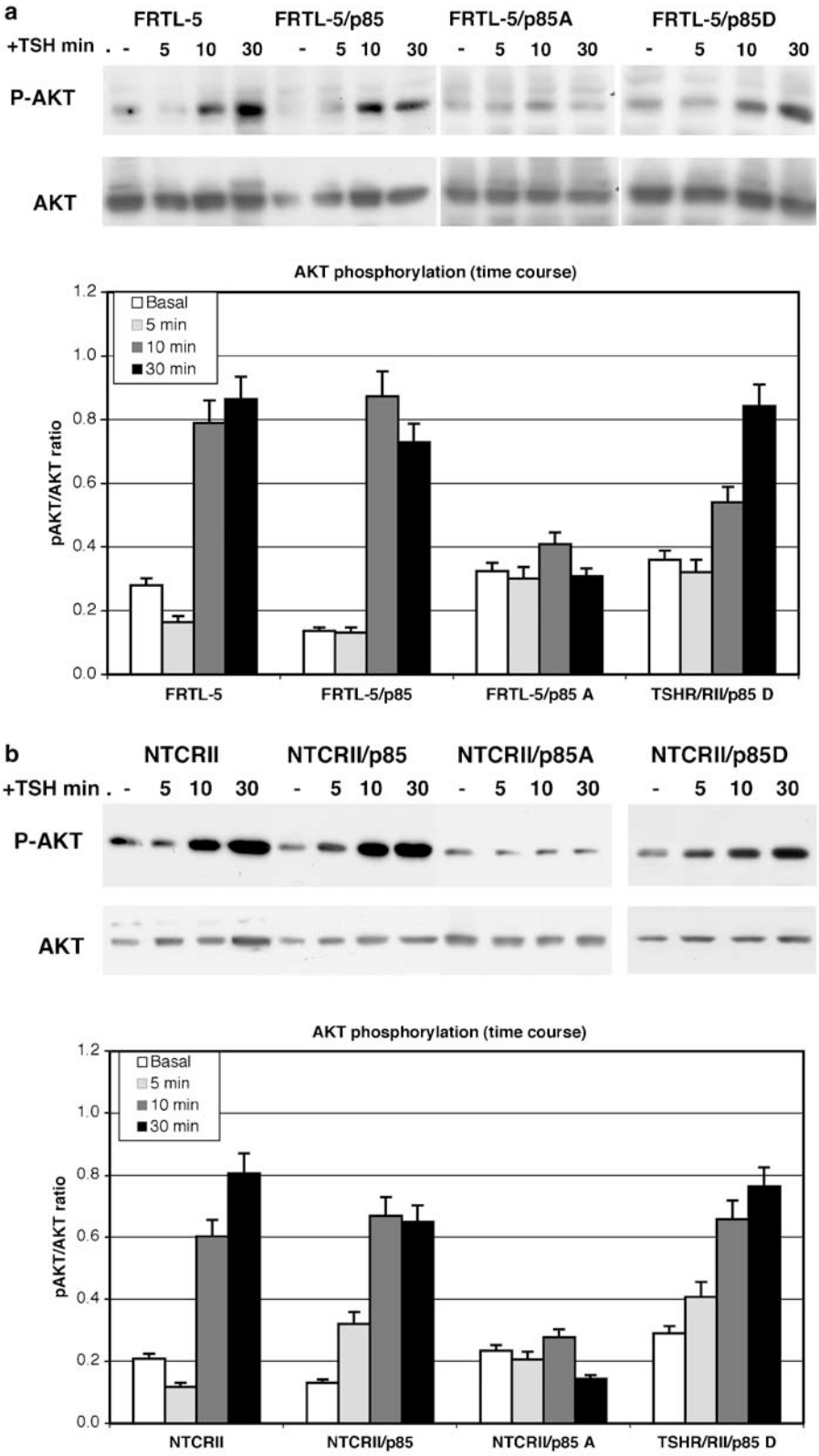

Figure 2 Phosphorylation of serine 83 is required for TSH-induced AKT phosphorylation. Time course of TSH-induced AKT phosphorylation in FRTL-5 (**a**) or NTCRII (**b**) cells expressing recombinant $p85^{PI3K}$. Representative blots of extracts derived from transiently transfected cells (NTCRII) or stable FRTL-5 clones (**b**) after 18 h of growth in 0.5% serum. TSH was added at a concentration of 10 mU/ml for the indicated time. The histogram in the lower panels shows the densitometric analysis of the P-AKT/AKT ratio relative to β-actin and normalized to the transfection efficiency, derived from three independent experiments.

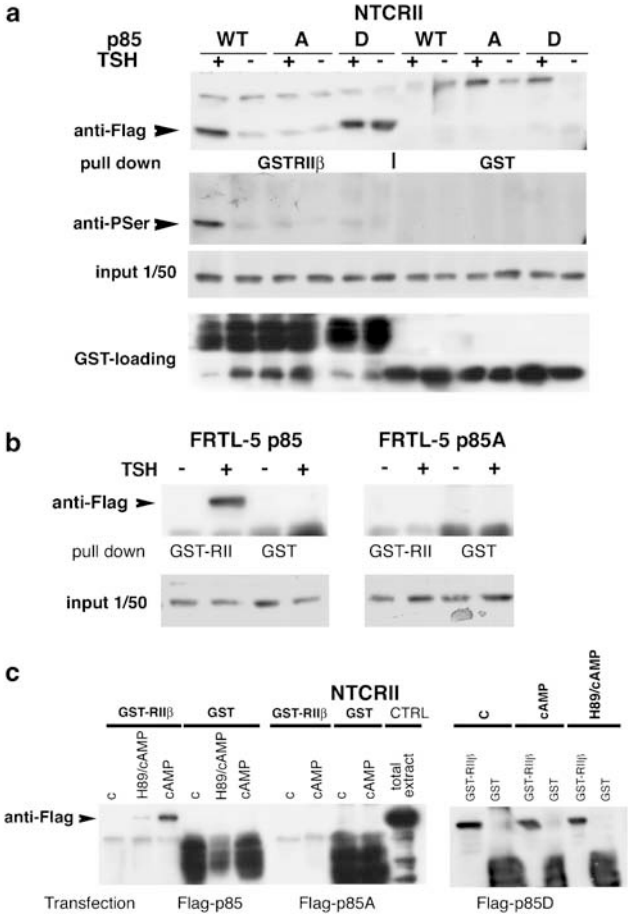

Figure 3 Phosphorylation of serine 83 in p85^{PI3K} selectively influences the formation of PKA–PI3K complex. (**a**) p85^{PI3K}–RIIβ complex formation in NTCRII cells. Pull-down experiment with GST-RIIβ challenged with extracts derived from NTCRII cells transfected with p85WT, p85A and p85D and treated with 10 mU/ml TSH for 10 min. RIIβ binding proteins were probed with anti-FLAG, anti-phosphoserine and anti-GST antibodies. (**b**) p85^{PI3K}–RIIβ complex formation in FRTL-5 cells. The same pull-down experiment described in (**a**) was carried out with extracts of stable transfected FRTL-5 cells. (**c**) p85^{PI3K} binds RIIβ in a cAMP-dependent manner in NTCRII cells. Immunoblot analysis of p85^{PI3K} bound to RIIβ in transiently transfected NTCRII cells. Cells were starved of serum for 6 h (0.5% serum) and exposed to 100 μM 8BrcAMP for 15 min with and without 10 μM H89.

in NTCRII (Figure 3a) or FRTL-5 (Figure 3b) cells expressing recombinant p85WT. In contrast, in cells expressing the p85A mutant, the formation of RIIβ–p85α^{PI3K} complex was inhibited. The binding of p85α^{PI3K} to RIIβ was observed after cAMP treatment (100 μM 8BrcAMP) (Figure 3c). Conversely, the p85D mutant was bound to RIIβ even in the absence of cAMP or TSH (Figure 3a and c). We also detected bound p85α^{PI3K} phosphorylated at serine 83 with anti-phosphoserine antibodies in p85WT, isolated from cells stimulated with TSH (Figure 3a). Most notably, the binding of p85D to RIIβ was resistant to the PKA inhibitor H89 (Figure 3c) and, as expected, it was not present in the immunoblot probed with anti-phosphoserine antibody (Figure 3a).

To demonstrate a direct interaction of RIIβ–p85α^{PI3K}, NIH 3T3 cells were transiently transfected with p85WT

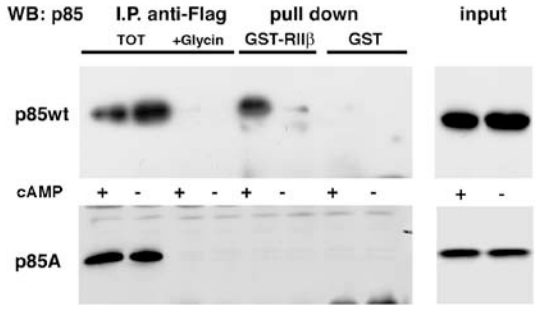

Figure 4 Purified p85 binds RIIβ *in vitro*. Exogenous p85^{PI3K} was immunoprecipitated with anti-FLAG antibody from NTCRII cells transfected with p85WT or p85A, and exposed to 100 μM 8BrcAMP for 15 min. The immunoprecipitate was extensively washed in RIPA buffer containing 1% Triton X-100 −0.1% SDS and purified by elution with 0.1 M glycine pH 3.5. Input indicates the eluted fraction; (+) glycine represents the pellet after elution. The purified p85^{PI3K} was incubated *in vitro* with GST-RIIβ in a pull-down experiment as described in Materials and methods.

or p85A and treated for 60 min with 100 μM 8BrcAMP. Exogenous p85α^{PI3K} was immunoprecipitated with anti-FLAG antibody, extensively washed in RIPA buffer containing 1% Triton X-100−0.1% sodium dodecyl sulfate (SDS) and purified by elution with 0.1 M glycine (pH 3.5). Purified p85α^{PI3K} was then incubated *in vitro* with GST-RIIβ and a pull-down experiment was repeated, as described above. Figure 4 shows that p85WT binds GST-RIIβ but not control GST. Moreover, the binding of p85α^{PI3K} to RIIβ was stimulated by pretreating the cells with cAMP (Figure 4). The p85A mutant did not bind RIIβ, both in the presence and absence of cAMP. Collectively, these data demonstrate that p85α^{PI3K} upon phosphorylation of serine 83 induced by cAMP or TSH binds PKA via the regulatory subunit RIIβ. Proximity of PKA to PI3K may favor signaling of both kinases to downstream effectors.

We have previously shown that cAMP–PKA selectively increased the association between PI3K and p21Ras (Ciullo *et al*., 2001). As this complex may mediate the cytoprotective effects of cAMP–PI3K, we set out to determine the formation of p21Ras–PI3K in cells expressing wild type or the mutant versions of p85α^{PI3K} indicated above. NTCRII cells were transfected with p85WT, p85A or p85D, and after 36 h, the cells were serum starved for 16 h before treatment with TSH for 10 min. Cell lysates were immunoprecipitated with anti-FLAG antibody and then analysed by Western blot with anti-p85α^{PI3K} and anti-Ras antibodies. In the presence of TSH, p85α^{PI3K} efficiently associated with p21Ras in p85WT-expressing cells. This association was greatly reduced in cells expressing p85A. In the absence of TSH, Ras association with p85α^{PI3K} was barely detectable in p85WT- and p85A-expressing cells. Conversely, the association of the mutant p85D to p21Ras was present in the absence of cAMP, but it was also stimulated by cAMP (Figure 5). The same results were obtained in FRTL–5-transfected clones (Figure 5).

We conclude that phosphorylation of p85α^{PI3K} at serine 83 by cAMP–PKA significantly stimulates the interaction of PI3K with PKA and p21Ras. The p85α^{PI3K}

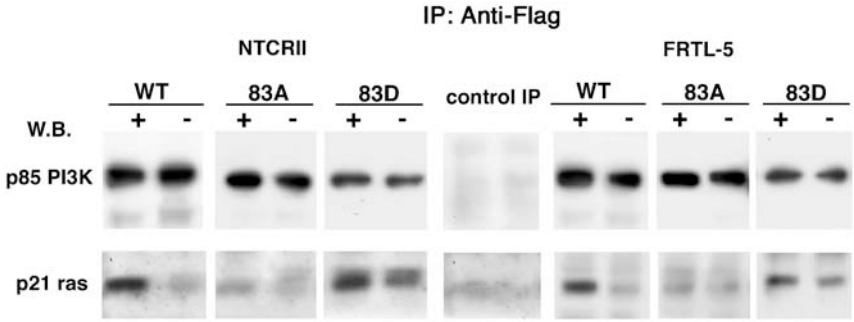

Figure 5 Phosphorylation of serine 83 selectively influences the formation of Ras–PI3K complex. The p21Ras–p85α^{PI3K} complex was assayed by immunoprecipitation with anti-FLAG antibodies and immunoblotting with anti-pan-Ras antibodies. NTCRII cells were transiently transfected with p85WT, p85A, p85D or with the empty vector. FRTL-5 cells were the stable clones indicated in Figure 1. Cells were starved of serum for 6 h (0.5% serum) and exposed to 10 mU/ml TSH for 20 min.

mutant, p85A, inhibited these interactions and downstream PI3K signaling, such as AKT phosphorylation.

To test if phosphorylation of serine 83 in p85α^{PI3K} influenced the catalytic activity of PI3K, we performed PI3K enzymatic assays on immunoprecipitated p85α^{PI3K}.

Figure 6 shows that TSH stimulated PI3K activity, although less efficiently than EGF, and that p85A inhibited TSH induction. To test if p85A inhibited PI3K activity *per se*, independent of the specific signaling pathway involved, we tested EGF-induced PI3K in cells expressing p85A or p85D. Figure 6B shows that PI3K activity induced by EGF was sensitive to the specific PI3K inhibitor LY294002 and that expression of p85A or p85D did not influence EGF-induced activity.

These data indicate that phosphorylation of serine 83 in p85α^{PI3K} selectively influences TSH- or cAMP-induced PI3K activity.

Discussion

The data presented demonstrate the importance of p85^{PI3K} in the transmission of TSH and cAMP growth and survival signals. Specifically, phosphorylation of serine 83 in the p85α^{PI3K} is essential for the binding of PI3K to PKA. We suggest that following PKA anchoring to PI3K, p21Ras associates to the complex and PI3K catalytic activity is greatly stimulated. Mutagenesis of this site profoundly and selectively impaired the transmission of TSH signals by disrupting this complex. EGF (Supplementary Figure 2S and Figure 6) or PDGF stimulation of PI3K (Cosentino *et al*., 2006) was not influenced by the substitution of serine 83 by alanine or aspartate in p85α^{PI3K}. The stimulation of PI3K catalytic activity by cAMP–TSH was evident in the absence of serum (see also, Porcellini *et al*., 2003) and was dependent on the phosphorylation of serine 83 in p85α^{PI3K}. However, the aspartic mutant of serine 83 p85α^{PI3K} replicated cAMP effects on (i) the inhibition of apoptosis by TSH starvation and (ii) the binding to PKA. It amplified cAMP effects on (i) PI3K activity, (ii) AKT phosphorylation and (iii) the efficiency of formation of the complex PI3K–p21Ras. These data indicate that serine 83 phosphorylation of p85α^{PI3K} is necessary but not sufficient to stimulate PI3K, phosphorylation of AKT and formation of the p21Ras–PI3K complex. Functional PKA is indeed required for these cAMP effects. As PKA binding to p85D is H89-resistant (Figure 3c), phosphorylation of serine 83 in p85α^{PI3K} is necessary and sufficient for PKA anchoring to PI3K.

This is a non-redundant circuit in thyroid or cells expressing TSHR and PKAIIβ, because its elimination profoundly impaired survival and growth. In contrast, in cells containing low levels of cAMP and RIIβ, the expression of p85A abolished only cAMP response, with a marginal effect on survival (data not shown; Cosentino *et al*., 2006). As for the mechanism, the data presented indicate that the serine 83 in p85α^{PI3K} is a critical site for the transmission of receptor signals to PI3K. The alanine mutant inhibits cAMP effects on PI3K activity. It is possible that a conformational change induced by phosphorylation is important for stimulation of PI3K activity. It is noteworthy that substitution of serine 83 by aspartate in p85α^{PI3K} stabilizes the complex with p110 (Cosentino *et al*., 2006).

We wish to stress that these data highlight a general role of serine 83 in the p85α^{PI3K} molecule in the transmission of cAMP signals in many cell types. We have recently found that many independent signaling pathways that stimulate cAMP converge on this site. For example, p85A disrupts the binding of PI3K to estrogen receptor and inhibits AKT activation by estrogens (Cosentino *et al*., 2006).

We suggest that phosphorylation of serine 83 by cAMP–PKA induces a conformational change in the PI3K complex, resulting in facilitated binding to receptors and to p21Ras, with stimulation of PI3K activity. We believe that this site is a nodal point where information from several receptors is channeled to PI3K.

Materials and methods

Materials and reagents

Unless otherwise specified, drugs and chemicals were obtained from Sigma Aldrich (St Louis, MO, USA) and cell culture supplies were purchased from standard suppliers, for example, Falcon, BD Biosciences, San Jose, CA, USA; GIBCO-Life

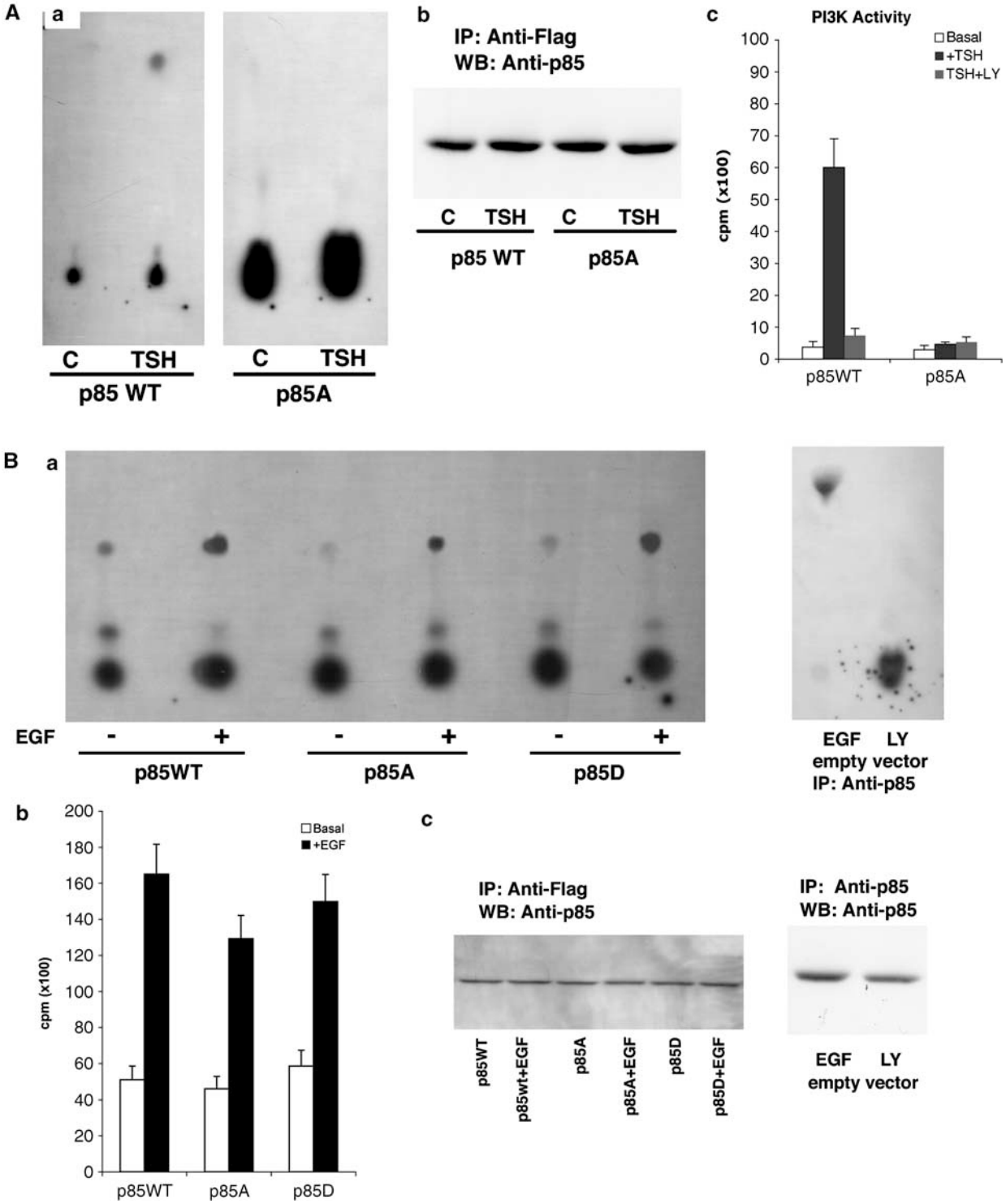

Figure 6 Selective stimulation of PI3K activity by TSH. (**A**) (a) A representative autoradiogram of the TSH-induced lipid kinase assay. NTCRII cells were transfected with p85α^{PI3K} expression vectors as indicated in Figure 1. At 48 h after transfection, cells were serum starved and maintained in low serum (0.2%) for 8 h. Cells were treated with TSH (10 mU/ml) or EGF (100 ng/ml) for 20 min. p85-FLAG was immunoprecipitated (IP) with the anti-FLAG M1 affinity gel and the immune complexes were subjected to PI3K lipid kinase assays as described in Materials and methods. (b) Immunoblot analysis indicated comparable amounts of immunoprecipitated p85-FLAG. (c) The histogram shows the densitometric analysis normalized to the IP efficiency derived from three independent experiments. (**B**) (a) A representative autoradiogram of the EGF-induced lipid kinase assay on anti-FLAG and anti-p85α^{PI3K} IP samples. The right-hand panel shows the PI3K activity derived from cells stimulated with EGF in the absence or presence of the specific PI3K inhibitor LY294002. (b) Densitometric analysis normalized to the IP efficiency from three independent experiments. (c) Immunoblot analysis indicated comparable amounts of immunoprecipitated p85α^{PI3K}.

Technologies, Invitrogen S.R.L, San Giuliano Milanese (MI), Italy, HyClone, Logan, UT, USA.

Cell lines
Mouse fibroblasts NIH 3T3 were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 2 mM glutamine (standard medium). The tTA repressor-expressing clones were grown in standard medium containing puromycin 2.5 μg/ml puromycin, 1.0 μg/ml tetracycline and 200 μg/ml geneticin (G-418). The selection and tetracycline were removed 48 h before starting the experimental procedures described below. The FRTL-5 cell line is a thyroid cell line, which has been extensively characterized with respect to thyroglobulin expression and TSH-dependent growth (Ambesi-Impiombato *et al*., 1980). Cells were grown in F12 medium with 5% calf serum and six hormones (1 mU/ml TSH, 1 μg/ml insulin, 3.6 μg/ml hydrocortisone, 5 μg/ml transferrin, 10 ng/ml somatostatin, 20 μg/ml glycyl-histidyl-lysine). FRTL-5 clone expressing the p85 variants was generated by transfecting FRTL-5 cells with the pSG5-p85 vectors. Neomycin-resistant clones were isolated and characterized for NEO and p85 expression by Western blots.

Plasmid construction
The p85α^{PI3K} plasmids have been described by Cosentino *et al*. (2006). Briefly, p85 cDNA was subcloned in pSG5 vector. FLAG sequence was added by subcloning into the *Xho*I/*Bam*HI sites of the pSG5-p85 vector a PCR fragment spanning from the *Xho*I site of $p85^{PI3K}$ at position 1014 to the end of the fragment. The vector encoding for p85-FLAG was then used as a template of PCR for site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene, Cedar Creek, TX, USA) to convert serine 83 to alanine.

Transfections
The day before transfection, cells were plated at 60% confluence in 100 mm dishes and transfected with 2 (NTCRII) or 8 (FRTL-5) μg/dish of p85WT, p85A, p85D or empty vector using 2.75 or 12.5 μl/μg DNA of Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA). Following 5 h of exposure to lipofectamine, the cells were washed and grown for 36–48 h before assays.

Cell growth analysis
Proliferation was analysed under different conditions: normal medium or low serum (0.5%) with and without 10 mU/ml TSH or 25 ng/ml EGF. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay for proliferation was performed in a 96-well flat-bottomed tissue culture tray for each time point: 1500 cells for each clone were seeded in eight replicas for each experimental point; 6 h after plating, the standard medium was removed and 100 μl of culture medium with stimuli as indicated was applied. The determinations were carried out every 24 h as follows: 0.01 ml MTT stock (5 mg/ml) was added to each well, then after 4 h of incubation at 37°C, the medium was removed and 0.1 ml isopropanol/0.04 N HCl was added. The absorbance was measured after 1 h on an enzyme-linked immunosorbent assay plate reader. We have used a Dynatech MR580 reader with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Western blot
Cells were transfected as indicated above. After 48 h, cells were exposed to 10 mU/ml TSH, 100 μM 8BrcAMP or 100 ng/ml EGF, harvested at times indicated and lysed on ice-cold RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin and pepstatin). After centrifugation at 12 000 *g*, protein concentrations were determined. Fifty micrograms of protein was subjected to 7 or 12% SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Schleicher and Schuell, Whatman plc, Brentford, Middlesex, UK, Germany). Western blot analysis was carried out by using the appropriate antibody as noted in the figures. The anti-p85 was from Upstate-Millipore (Billerica, MA, USA) (#06-497). The anti-pan-Ras antibody was from Calbiochem (affiliate of Merck KGaA, Darmstadt, Germany) (EMD Biosciences Inc., an affiliate of Merck KGaA, Darmstadt, Germany). The antibody against Akt and phospho-Akt were from Cell Signaling Technology (Beverly, MA, USA). The horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies and the ECL detection system were from Amersham Pharmacia Biotech Italia (Milano, Italy).

Pull-down and immunoprecipitation
Cells were lysed in pull-down buffer (200 mM NaCl, 50 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 10% glycerol, 1% NP-40, 10 μg/ml trypsin inhibitor, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mM NaF, 10 mM Na_3VO_4). The cell extracts (1 mg) were incubated with 1 μg of GST-HRIIβ fusion protein for 4 h at 4°C with gentle rock agitation. The pellets were washed in pull-down buffer and processed for Western blot analysis as described.

Total extracts for immunoprecipitation were prepared by incubating cells on ice with RIPA buffer containing 1 mM ethylene diaminetetraacetic acid. Cell lysates (1 mg) were incubated with 2 μg of normal mouse IgG (sc 2025) and 25 μl of protein A Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at 4°C. After centrifugation, protein lysates were incubated with 4 μg of anti-FLAG M1 affinity gel (Sigma A4596) for 4 h at 4°C on a rocking plate. The pellets were washed with RIPA buffer and processed for Western blot analysis as already described. The fusion protein was eluted by using 0.1 M glycine HCl pH 3.5 and neutralized with 1 M Tris-HCl pH 8.

Analysis of DNA content and BrdU incorporation
Cell cycle analysis was carried out by fluorocytometric absorbent cell sorter (FACS): 5×10^5 transfected cells (48 h after transfection) were plated in 60 mm dishes and grown in low-serum medium (0.5% fetal bovine serum) for 18 h and induced into the cell cycle with 0.5% serum in the presence of 10 mU/ml TSH or 100 ng/ml EGF. BrdU incorporation was assayed in a pulse–chase experiment. Cells were labeled for 30 min with BrdU to a final concentration of 20 μg/ml and harvested at 0, 90 and 270 min. After treatment, cells were fixed in 70% ice-cold ethanol for 4 h at 4°C and washed three times in phosphate-buffered saline (PBS). Cell pellet was resuspended in 0.25 ml of 1 N HCl and let to stand for 20 min at room temperature (RT). After acidic denaturation of DNA, cells were washed two times in phosphate/citric buffer (0.2 M Na_2HPO_4, pH 7.4). BrdU incorporation was revealed by anti-BrdU-FITC (Becton Dickinson, Franklin Lakes, NJ, USA) and then stained for 30 min at RT in 0.1% Triton X-100, 0.2 mg/ml DNase-free RNaseA and 20 μg/ml propidium iodide. Fluorescence was determined by using the FACScan Flow Cytometer (Becton Dickinson). Experiments were performed in triplicate. The data were acquired and analysed by CELLQuest software (Becton Dickinson) for bivariate analysis of DNA content versus BrdU and by Cell Fit Cell-Cycle Analysis Version 2 for DNA content analysis.

Apoptosis assay

5×10^5 cells were grown in 60 mm dishes. At 18 h after treatment, cells were fixed in 2% paraformaldehyde/1 × PBS for 10 min at RT and washed once in PBS + 50 mM glycine for 10 min at RT and washed again three times for 5 min in PBS. Cells were permeabilized with 0.5% Triton X-100/1 × PBS for 10 min, washed 3 × 5 min in PBS and incubated with 100 μl of 1 × TdT reaction mix. TdT-mediated dNTP nick end labeling was carried out at 37°C for 60 min using 15 U of TdT (Roche Diagnostics S.p.A, Roche Applied Science, Monza, Italy) and 2 μl of 2 mM BrdUTP. BrdUTP incorporation was revealed by anti-BrdU-FITC and then stained with propidium iodide. The data were acquired and analysed by CELLQuest software for bivariate analysis of DNA content versus BrdU. Experiments were performed in triplicate.

Lipid kinase assay

Lipid kinase activity was determined as described by Maier *et al*. (1999). Briefly, the assays were carried out in a final volume of 50 μl containing 0.1% bovine serum albumin, 1 mM ethylene glycol-bis (β-aminoethylether) tetraacetic acid, 120 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM glycerophosphate and 7 mM $MgCl_2$ (buffer E). Lipid vesicles (30 μl containing 320 μM phosphatidylethanolamine, 300 μM phosphatidylserine, 140 μM phosphatidylcholine and 30 μM sphingomyelin, supplemented with 40 μM L-α-Phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) in buffer E) were sonicated for 1 h and incubated on ice for 10 min. Some aliquots were preincubated with 0.5 μM LY294002 (Calbiochem) at 37°C. The immunoprecipitates were added to the lipid mixture and incubated for 10 min at 4°C in a final volume of 40 μl. The reaction was started by adding 40 μM ATP (1 μCi of [^{32}P]ATP in 10 μl of the assay buffer (30°C)). After 15 min, the reaction was stopped with ice-cold 150 μl of 1 N HCl on ice. The lipids were extracted by vortexing the samples with 500 μl of chloroform/methanol (1:1). After centrifugation, the organic phase was washed twice with 200 μl of 1 N HCl. Phosphorylated lipids were separated on a potassium oxalate-pretreated thin-layer chromatography plates (silica gel 60 Merck) developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60:47:11.3:2), dried, and visualized by autoradiography and quantified with Phosphor-Imager.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).