Cell. Mol. Life Sci. (2011) 68:3065–3079
DOI 10.1007/s00018-010-0606-1

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Biased binding of class IA phosphatidyl inositol 3-kinase subunits to inducible costimulator (CD278)

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Received: 15 April 2010 / Revised: 12 November 2010 / Accepted: 26 November 2010 / Published online: 28 December 2010

Abstract To better understand T lymphocyte costimulation by inducible costimulator (ICOS; H4; CD278), we analyzed proteins binding to ICOS peptides phosphorylated at the Y_{191}MFM motif. Phosphorylated ICOS binds class IA phosphatidyl inositol 3-kinase (PI3-K) p85α, p50-55α and p85β regulatory subunits and p110α, p110δ and p110β catalytic subunits. Intriguingly, T cells expressed high levels of both p110α or p110δ catalytic subunits, yet ICOS peptides, cell surface ICOS or PI3-kinase class IA regulatory subunits preferentially coprecipitated p110α catalytic subunits. Silencing p110α or p110δ partially inhibited Akt/PKB activation induced by anti-CD3 plus anti-ICOS antibodies. However, silencing p110α enhanced and silencing p110δ inhibited Erk activation. Both p110α- and p110δ-specific inhibitors blocked cytokine secretion induced by TCR/CD3 activation with or without ICOS costimulus, but only p110α inhibitors blocked ICOS-induced cell elongation. Thus, p110α and p110δ are essential to optimal T cell activation, but their abundance and activity differentially tune up distinct ICOS signaling pathways.

Keywords PI3-Kinase · Inducible costimulator · ICOS · T lymphocyte

Introduction

The inducible costimulator (ICOS, CD278) is a costimulatory molecule of T lymphocytes with sequence and functional homology to CD28. Whereas CD28 is expressed by most resting $CD4^+$ T cells and is considered the main costimulatory molecule for these cells to initiate immune responses [1, 2], ICOS is typically expressed at high levels by activated T lymphocytes [3–6], contributing to the differentiation and effector functions of $CD4^+$ T cells in normal and pathological conditions. Neither CD28 nor ICOS encodes intrinsic enzymatic activity, so that their functional distinctions in terms of signaling derive from their ability to bind enzymes or adaptor proteins in the cytoplasm.

Both CD28 and ICOS have intracytoplasmic sequence motifs (YxxM) able to bind the regulatory subunits of class IA PI3-kinases upon Tyr phosphorylation [7–13]. However, CD28 and ICOS costimulation has different signaling outcomes [13–15]. This is due, on one hand, to the ability of the YMNM motif in CD28, but not the YMFM motif in ICOS, to bind the adaptor Grb2. Besides, additional proline-rich and Tyr-containing motifs present in CD28 can bind the Grb-2/GADS adaptor proteins, the Itk and Lck kinases [16–18], or PI3-kinase complexes having p85β regulatory subunits [19]. On the other hand, it has been

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Electronic supplementary material The online version of this article (doi:10.1007/s00018-010-0606-1) contains supplementary material, which is available to authorized users.

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reported that ICOS binds the p55 or p50 isoforms of the regulatory PI3-kinase α subunits in activated human T cells better and has a stronger associated PI3-kinase activity [11, 13, 20].

PI3-kinases comprise at least three different classes (class I–III) based on substrate specificity and regulation, playing important roles in many cell functions [21–25]. Class I PI3-kinases are heterodimers of one regulatory and one catalytic subunit. They are further divided into class IA or class IB, depending on their regulatory subunits. Class IB PI3-kinases contain p110γ subunits associated to either p101 or p84/87^{PIKAB} regulatory subunits; they are typically activated upon recruitment to cell surface G-protein coupled receptors (GPCR) and are mainly, but not exclusively, expressed by cells of hematopoietic origin.

Class IA PI3-kinases are heterodimers containing p110α, p110β or p110δ catalytic subunits bound to one of five regulatory subunit isoforms [22–24, 26]. These are p85α, p55α and p50α, produced by alternative splicing of the *pik3r1* gene product, plus p85β and p55γ encoded by the genes *pik3r2* and *pik3r3*, respectively. They all share a C terminal region with one region involved in binding to the p110 catalytic subunits flanked by two conserved SH2 domains that bind Tyr-phosphorylated YxxM motifs typically present in activated growth factor receptors or their substrates. Lymphocytes express the ubiquitous p110α and p110β catalytic subunits, plus the p110δ highly expressed in hematopoietic cells. The α and β regulatory subunits are the main regulatory subunits in lymphocytes, with p55γ levels being low in lymphoid cells [27, 28]. Although there seems to be a great deal of redundancy among PI3-kinase subunits, subunit-specific functions have also been determined (see, i.e., [29–34]); these might stem from their relative abundance in particular cells or tissues, but also by preferential binding to specific molecules. In the case of the catalytic subunits, their selective binding to other membrane-bound proteins like Ras isoforms [35–37] or GPCR [38, 39] has been proposed as the possible mechanism for selective activation [26].

This work was initially aimed at further defining ICOS functions by searching for molecules specifically bound by the cytoplasmic tail of ICOS. We used Tyr-phosphorylated or -unphosphorylated synthetic peptides of the cytoplasmic tail of mouse ICOS to "pull-down" molecules from cell lysates. The polypeptides specifically bound to the cytoplasmic domain of ICOS included different regulatory and catalytic subunits of class IA PI3-kinase. Our data show that both p110α and p110δ catalytic units of class I PI3-kinases were expressed at high levels in T lymphocytes, yet p110α catalytic subunits were preferentially bound by ICOS, reflecting the level of association of p110α to the regulatory subunits of PI3-kinase in T lymphocytes. Whereas we herein confirm the importance of the PI3-kinase p110δ catalytic subunits in T cell activation, we also find an essential role for the p110α catalytic subunits of PI3-kinase to TCR/CD3 activation and ICOS costimulation, as shown by means of siRNA silencing and pharmacological inhibition of different catalytic subunits of PI3-kinases, and a preferential role of p110α catalytic subunits in PI3-kinase-mediated, ICOS-induced changes in T cell shape.

Materials and methods

Antibodies and other reagents

Monoclonal antibodies used were anti-mouse/human ICOS (CD278) C398.4A [3, 4] anti-mouse CD3 YCD3-1 [40] and anti-mouse CD11b M1/70 [41]. They were purified by protein A- or protein G-affinity chromatography from hybridoma supernatants. Recombinant mouse ICOS ligand-Fc chimera (rmB7-H2/Fc) was from R&D. Anti-phosphotyrosine monoclonal antibody PY-20 was obtained from GE Healthcare. Rabbit polyclonal anti-PI3K p85 (UBI 06-195) and anti-ERK antibodies were from Upstate Biotechnology. Rabbit anti-PI3K p110α (H-201), anti-PI3K p110β, (S-19), anti-PI3K p110δ (H-219), anti-Akt1/2 (H-136) and goat anti-PI3K p55γ (N-13) were from Santa Cruz Biotechnology. The mouse monoclonal anti-PI3 K p110α (19/PI3 K p110α) was from BD Biosciences; rabbit anti-p110α monoclonal antibody C73F8 was ref. 4249 from Cell Signaling Technology. Rabbit anti-ZAP-70 antibody was obtained and purified, as described in [10]. Rabbit antibody specific for dually phosphorylated ERK (Anti-Active MAPK ref. V6671) was from Promega; rabbit anti-phospho-Akt(Ser473) antibody was from Cell Signaling Technology (no. 9271). Streptavidin Sepharose and horseradish peroxidase-coupled Protein A were from Sigma. Protein A-Sepharose was from GE Healthcare. Protein kinase B (Akt) inhibitor IV and Akt inhibitor V (Triciribine), PI3-Kα inhibitor IV, PI3-Kα inhibitor VIII (PIK-75), PI3-Kβ inhibitor VI (TGX-221), PI3-Kγ inhibitor, PI3-Kγ inhibitor II and Lck inhibitor II were from Calbiochem; the PI3-kinase inhibitor LY 294002 was from Sigma. PI3-Kδ inhibitor IC87114 was purchased from Symansis Pty. (New Zealand).

Cells and cell lines

The SR.D10 (D10) line [42, 43] subcloned from the D10.G4.1 CD4$^+$ T cell line [44] and clone H4$^-$.A5 (an ICOS$^-$ mutant of SR.D10) were used. Irradiation, selection for loss of ICOS expression and cloning of H4$^-$.A5 cells were performed, as described [43]. Selection of CD4$^+$ T cells from BALB/c mice and generation of CD4$^+$ T cell

blasts by Concanavalin A activation of $CD4^+$ T cells were performed, as described previously [10]. Blasts used were ≥90% $CD4^+$ and ≥80% $ICOS^+$, as determined by flow cytometry.

"Pull-down" with peptides and protein analysis

Biotinylated mouse ICOS and CD28 peptides, phosphorylated or not at precise Tyr residues, were synthesized and purified at the protein chemistry service of the CIB, CSIC. The following peptides were used: ICOS: GSSVHDPNSEY$_{191}$MFMAAVNTNKKSRLAGVT-Biotin; ICOSpTyr: GSSVHDPNSEpY$_{191}$MFMAAVNTNKKSRLAGVT-Biotin; sICOSpTyr: VHDPNSEpY$_{191}$MFMAAVNTNK-Biotin; CD 28pTyr: RLLQSDpY$_{189}$MNMTPRRPGLTRKPYQPYAPAR-Biotin. For proteomic analysis, 4–8 × 10^8 cells were lysed at 1–2 × 10^7 cells/ml in CHAPS lysis buffer (10 mM CHAPS in 50 mM Tris/HCl, 150 mM NaCl, pH 7.6, containing 1 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and 1 mM $NaVO_4$). After centrifugation, post-nuclear lysates were pre-cleared by rotation (1 h at 4°C) with Streptavidin-Sepharose (100 μl of beads) and centrifuged. Pull-down was then carried out using biotinylated peptides (100 μg) previously incubated with Streptavidin-Sepharose (100 μl of beads/pull-down) and washed with lysis buffer to remove unbound peptide. The peptide-containing beads were incubated with the pre-cleared lysate for 2–4 h at 4°C with rotation. The beads were then washed four times with cold washing buffer (2 mM CHAPS in 50 mM Tris/HCl, 150 mM NaCl, pH 7.6, containing 1 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM $NaVO_4$) and once with distilled water. Two-dimensional separation (IEF-SDS PAGE) of the bound proteins was performed by IEF in pH 3–10 non-linear 7-cm strips (Bio-Rad, Hercules, CA) followed by SDS PAGE in 10% acrylamide gels, as described [45, 46]. Spots of interest from SYPRO-stained SDS-PAGE gels were excised automatically in a ProteomeWorks Plus Spot Cutter System (Bio-Rad, Hercules, CA), deposited in 96-well polypropylene reaction plates and digested with trypsin using a DigestPro MS (Intavis AG, Cologne, Germany). MS and MS/MS analysis of tryptic peptides was performed with an Autoflex Smartbeam TOF/TOF (Bruker Daltonics, Bremen, Germany) spectrometer equipped with a LIFT ion selector and a reflectron ion reflector. Data collection was performed in fully automated, fuzzy-logic mode. Analysis of mass data was performed using flexAnalysis 3.0 software (Bruker-Daltonics, Bremen, Germany). MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker-Daltonics, Bremen, Germany) to search a non-redundant protein database from the National Center for Biotechnology Information (Bethesda, MD) using MASCOT software (Matrix Science, London, UK).

Pull-down assays for immunoblotting were carried out as above, except that 2–4 × 10^7 cells and 15–20 μg of peptide bound to 15–20 μl of packed Streptavidin-Sepharose beads were used for each determination, and the final wash in water was omitted. The samples were instead extracted with SDS-PAGE sample buffer.

Immunoprecipitation of PI3-kinase subunits was performed, as described previously [45, 46], except that 15–20 μl of Protein A-Sepharose beads was used to bind the precipitating antibodies (5 μg/determination). For immunoprecipitation of surface ICOS, D10 cells (2 × 10^8 cells/point, 2 × 10^7/ml) were incubated with anti-ICOS antibody (10 μg/ml) for 15 min and washed. Generation of pervanadate and pervanadate activation of cells were carried out, as described in detail in [45, 46]. After 5 min incubation at 37°C, the cells were lysed, pre-cleared with normal mouse Ig coupled to Sepharose and the antibody bound to surface ICOS immunoprecipitated by adding Protein A-Sepharose.

Cell activation and ELISA for IL-4

Cells were activated with antibody-coated latex beads for the times indicated in each case, as described in detail previously [10]. For the assays in the presence of inhibitors, the cells were kept in medium containing the inhibitor for 10 min before activation. IL-4 in the supernatants of cells activated for 4 h was determined by capture ELISA, as described [10].

Elongation assay

Glass coverslips of 10-mm diameter were placed into 24-well tissue-culture plates and incubated overnight at 4°C with 0.3 ml of anti-ICOS antibody, rmB7-H2/Fc chimera, anti-CD3 antibody or poly-L-lysine (10 μg/ml in PBS). The coverslips were washed with PBS, and 250,000–300,000 D10 cells were added in 0.5 ml of 10 mM HEPES, 0.1% glucose PBS, pH 7.2. After a brief centrifugation, the cells were incubated for 20–40 min at 37°C, washed with PBS, and fixed for 5 min with 0.5 ml 4% formaldehyde warmed at 37°C. After washing with PBS, cells were permeabilized for 5 min with PBS/0.1% saponin. Then, the cells were stained for 30 min with 50 nM FITC-phalloidin (Sigma) or AlexaFluor-568-phalloidin (Molecular Probes) in PBS/0.1% saponin. After further washing with PBS/0.1% saponin, the cells were washed once with PBS and the coverslips mounted on VECTASTAIN (Vector). Cell images were acquired at room temperature at a magnification of 40 using an Axioplan Universal microscope (Carl Zeiss, Jena, Germany) and a Leica DFC 350 FX CCD camera. The images

were recorded on a disk and analyzed using Adobe Photoshop 6.0 (Adobe). Quantification of elongation was performed using ImageJ 1.38× public domain software (National Institutes of Health). For each cell, the elongation index was calculated as the ratio of the longest axis to the longest segment normal to the first one. A cell with a regular shape would yield an elongation index of 1, with the value increasing as the cell elongates. In each experiment, 10–20 cells/field in six different fields were quantified.

Immunoblot

For immunoblotting of cell lysates, 10–20 µl of lysate (equivalent to 2–4 × 10^5 cells) was mixed V:V with 2× reducing SDS Laemmli sample buffer. Immunoprecipitates were extracted with 1× reducing sample buffer. The samples were boiled for 1 min and separated by SDS-PAGE in 10% acrylamide gels. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore) and were then blocked for 2 h with PBS/0.1% Tween (PBST) containing 3% non-fat dry milk. After washing with PBST, the membranes were incubated overnight with the antibodies in PBST/0.2% gelatin/0.1% NaN_3. They were washed with PBST and incubated for 1 h at room temperature with HRP-coupled Protein A in PBST-containing 3% non-fat dry milk. After further washing, blots were visualized using the Supersignal West Pico chemiluminiscence substrate (Pierce).

For detection of phosphotyrosine, phospho-Erk or phospho-Akt, blot membranes were blocked using Tris-buffered saline/0.1% Tween (TBST), 2% non-fat dry milk, 0.5% gelatin and 200 mM sodium orthovanadate. They were washed with TBST and incubated with antibodies in TBST/0.2% gelatin/0.1% NaN3. After washing with TBST, the membranes were incubated for 1 h at room temperature with HRP-coupled Protein A in TBST/2% non-fat dry milk/0.5% gelatin and washed with TBST. Visualization was carried out as described above. Quantification of individual protein bands was performed with the Image J software (Image J 1.38x; National Institutes of Health).

Quantitative real-time PCR

Total RNA was extracted with the RNeasy kit (Qiagen, Hildesheim, Germany), and cDNAs were generated from 2 µg RNA in the presence of random hexamer primers with the SuperScriptRNaseH-Reverse Transcriptase kit (Invitrogen). Gene expression levels were determined by real-time PCR by means of LightCycler FastStartDNA Master SYBR Green I (Roche) containing 2 µl cDNA and 500 nM primer. The primers used were: mouse β actin, CTAAGGC CAACCGTGAAAAG (forward), ACCAGAGGCATACA GGGACA (reverse); PI3-kinase regulatory subunit p85α, GAATGTTCGACTCTATACAGAACACAA (forward), CATCTCCAAGTCCACTGACG (reverse); PI3-kinase regulatory subunit p85β, CCCTTGGATGGATCTTCTGA (forward), GGGTCAGGTGGGGAGAAC (reverse); PI3-kinase catalytic subunit p110α, GACAAGAACAAG GGCGAGAT (forward), CAGTACCCAGCGCAGGAC (reverse); PI3-kinase catalytic subunit p110β, AGAAGC TGGCTTGGACCTG (forward), CAGAGCGATCTCC TGTTGCT (reverse); PI3-kinase catalytic subunit p110γ1, AACAATCTCCGCCCACAA (forward), CACTCCAGAC TTTCCTTTATCTTCTT (reverse); PI3-kinase catalytic subunit p110δ, AGCTGCTCCAAAGATATCCAGT (forward), TGCTTTAGCGCCTCTTCCT (reverse). Real-time PCR data were analyzed by the comparative cycle threshold (CT) method with the Lightcycler Software version 5 included with the Lightcycler® System (Roche Diagnostics Inc.) and normalized to internal controls (mouse β-actin).

siRNA knockdown

siRNA oligonucleotides targeting the mouse p110α catalytic subunit (Silencer® Select siRNA s71604; sense sequence GACACUACUGCGUAACUAUtt, antisense sequence AUAGUUACGCAGUAGUGUctg), the p110δ catalytic subunit (Silencer® Select siRNA s71605; sense sequence GGAUGAAGCUGGUUGUUCtt, antisense sequence UGA ACAACCAGCUUCAUCCgc) or control siRNA oligonucleotides (Silencer® Select Negative Control #1 siRNA), all from Applied Biosystems, Ambion, were used.

D10 cells (5 × 10^6/100 µl) were transfected with siRNA or control oligonucleotides (1 µM, p110α; 2 µM, p110δ) using program T-20 of Nucleofector (Amaxa Biosystems) and incubated for a further 40 h in Click's EHAA medium supplemented with 10% heat-inactivated FCS (culture medium) containing 5 U/ml mouse IL-2, 10 U/ml mouse IL-4 and 25 pg/ml mouse IL-1α [10]. Then, viable cells were counted, washed, filtered through a 30-µm mesh and used for activation with antibody-coupled beads or lysed.

Results

Analysis of the proteins bound to pYMFM-ICOS peptide: regulatory PI3-kinase subunits

To identify proteins specifically bound by the cytoplasmic tail of ICOS upon phosphorylation of its YMFM motif, we performed proteomic analysis of the proteins bound in pull-down assays using one biotinylated peptide spanning 29 residues of the ICOS cytoplasmic tail (ICOS peptide, see "Methods") or the same peptide phosphorylated in Tyr_{191} corresponding to the YMFM motif (ICOSpTyr peptide). Once bound to streptavidin agarose, the peptides were

incubated with lysates of the mouse $CD4^+$ T cell line D10 or an ICOS-negative mutant ($H4^-$.A5) of D10 with similar results.

Different PI3-kinase subunits were consistently and specifically detected in the pull-downs of the Tyr-phosphorylated ICOS peptide (Table 1). These included three different regulatory subunits of class IA PI3-kinases (p85α; p85β; p50-55α) and the p110α catalytic subunit. The abundance of each subunit in the pull-downs was then compared to their abundance in the cells determined by RTqPCR in D10 cells, the $H4^-$.A5 mutant or normal spleen $CD4^+$ T lymphocytes (Fig. 1a). Concerning regulatory subunits, the RTqPCR results show that the mRNA for PI3-kinase α regulatory subunits is far more abundant than that of β subunits. This is in contrast with the proteomic data from pull-down assays, where, according to protein staining, p85β accounted for up to 30% of the regulatory subunits detected (data not shown), indicating a higher than expected binding of these subunits.

Since the primers used for RTqPCR did not distinguish among the different isoforms of regulatory α subunits, we could not determine their abundance based on the mRNA data. However, protein staining of pull-downs indicated that regulatory p50-55α accounted for about 30% of the co-precipitated regulatory subunits (data not shown). The regulatory PI3-kinase α subunits bound to the phosphorylated ICOS peptide were further analyzed by immunoblotting using antibodies against regulatory PI3-kinase that are known to recognize the different α isoforms (p85α; p50-55α) as well as p85β [19] (Fig. 1b, see also Fig. 2). According to this analysis, the phosphorylated ICOS peptide preferentially bound the p50-55 isoforms of the regulatory subunits, as judged by comparing the OD of each band in the pull-down with the corresponding band in the cell lysates. These data are in agreement with previous data showing that, in activated human T lymphocytes, ICOS preferentially recruits PI3-kinase p50-55α [11].

Analysis of the proteins bound to pYMFM-ICOS peptide: preferential co-precipitation of PI3-kinase 110α catalytic subunits

We did not find PI3-kinase class IA catalytic subunits other than p110α in our proteomic search (Table 1). Since it is well established that lymphocytes express high levels of the p110δ catalytic subunit [47], we looked for the possibility of p110δ being expressed at low levels in the cells used. RTqPCR analysis of different catalytic units shows that the mRNA for both p110α and p110δ class IA PI-3-kinase catalytic subunits was expressed at relatively much higher levels than catalytic p110β subunits in the different types of cells analyzed, including normal spleen $CD4^+$ T lymphocytes (Fig. 1a). Taking these data into account, both the p110α and p110δ catalytic subunits should be expected in the pull-down proteome.

The catalytic subunits of PI-3-kinase recruited by the phosphorylated ICOS peptide were further analyzed by immunoblotting using subunit-specific antibodies (Fig. 1b). We found that the primary antibodies against the the anti-p110β and anti-p110δ subunits were better than the polyclonal or monoclonal anti-p110α antibodies used, as judged by comparing the OD in cell lysate blots at a fixed exposure time with the abundance of each subunit expected from the RTqPCR data. To obviate these differences, we used the OD of cell lysates as a reference to equalize the results in the pull-down (Fig. 1b).

Once corrected for antibody quality, we observed that the levels of p110α catalytic subunit co-precipitated in ICOSpTyr pull-downs were comparatively much higher than those of p110β or p110δ, which agreed with p110β and p110δ being undetected in the proteomic analysis. Although the low binding of p110β was expected considering the RTqPCR data, that of p110δ was not.

To further determine whether the differences between p110α and p110δ were peculiar to the peptide used, we compared the PI3-kinase subunits specifically bound by the 29-residue Tyr-phosphorylated ICOS peptide (ICOSpTyr)

Table 1 Proteins identified in pull-down assays of Tyr-phosphorylated ICOS peptides

Apparent MW of bound protein, kDa	Protein Matched[a]	Accession Code	Mascot score
110	PI3-kinase catalytic subunit, p110α	gil6679317	152
85	PI3-kinase regulatory subunit, p85α	gil129388	164
83	PI3-kinase regulatory subunit, polypeptide 2, p85β	gil6679321	164
55	PI3-kinase regulatory polypeptide 1, isoform 1, p55α	gil68299809	151
50	PI3-kinase regulatory polypeptide 1, isoform 1, p55α	gil68299809	209

[a] All protein matches are from *Mus musculus*

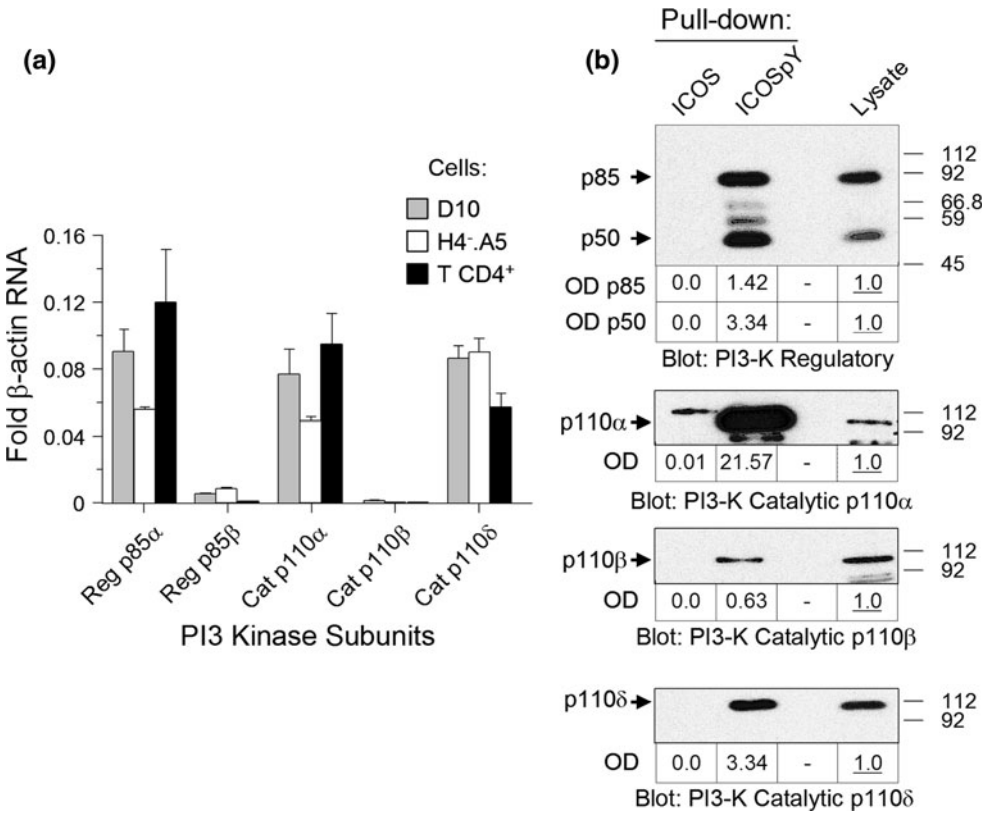

Fig. 1 Abundance of PI3-kinase class IA subunits in cells and in pull-down assays using ICOS peptides. **a** Relative levels of mRNA for the alpha and beta regulatory subunits (Reg p85α; Reg p85β) or the alpha, beta and delta catalytic subunits (Cat p110α; Cat p110β; Cat p110δ) of PI3-kinase, as determined by quantitative real-time PCR in cells of the D10 and H4^{-}.A5 CD4^{+} T cell lines or in CD4^{+} T lymphocytes from the spleen. Mean ± SEM of triplicate determinations of one representative experiment of three performed. **b** Comparison of relative PI3-kinase regulatory and catalytic subunit abundance in the cell lysate of D10 H4^{-}.A5 cells or in pull-downs of pTyr-phosphorylated- (ICOSpY) or non-phosphorylated (ICOS) ICOS peptides, as determined by immunoblot with antibodies specific for PI3-kinase regulatory subunits or the catalytic p110α, p110β and p110δ subunits. Data from one representative experiment of the five performed. The OD values for the immunoblots of regulatory or catalytic PI3-kinase subunits in peptide pull-downs were equalized considering the OD in cell lysates as 1 (*underlined*)

with a shorter (18 residue) version of the same peptide (sICOSpTyr) or a Tyr-phosphorylated peptide (CD28pTyr) corresponding to part of the cytoplasmic tail of CD28 (Fig. 2). Similar results were obtained in all the phosphorylated peptides, with clearly more p110α catalytic subunit bound than p110δ (Fig. 2).

These results broadened and more precisely defined the PI3-kinase subunit binding to phosphorylated ICOS previously detected by co-immunoprecipitation or by the yeast three-hybrid method [9–12].

Relative abundance of PI3-kinase subunits in ICOS immunoprecipitates

Then, we determined whether the differences in the association of PI3-kinase catalytic subunits could be found in immunoprecipitates of the ICOS molecule expressed by T cells as they were in peptide pull-downs. To this end, D10 cells were treated or not for 5 min with the phosphatase inhibitor pervanadate, which produced Tyr phosphorylation in many cell substrates (Fig. 3a). Then, ICOS was immunoprecipitated with specific antibodies. In terms of p110α and p110δ coprecipitation, ICOS immunoprecipitates from pervanadate-treated cells yielded results similar to those of pull-down assays with phosphorylated peptides, namely p110α was present at higher levels than expected compared to p110δ based on their relative abundance in the lysates (Fig. 3b; see also Fig. S2). Concerning regulatory subunits, the p50-55 isoforms were preferentially co-precipitated with ICOS, as shown by the relative abundance in immunoprecipitates and cell lysates (Fig. 3b) and in agreement with data from activated human T cells [11].

Differential binding of PI3-kinase catalytic subunits to ICOS reflects their binding to PI3-kinase regulatory subunits

We examined whether the bias towards p110α association in ICOS peptide pull-downs or ICOS immunoprecipitation was due to a biased binding of these catalytic subunits to

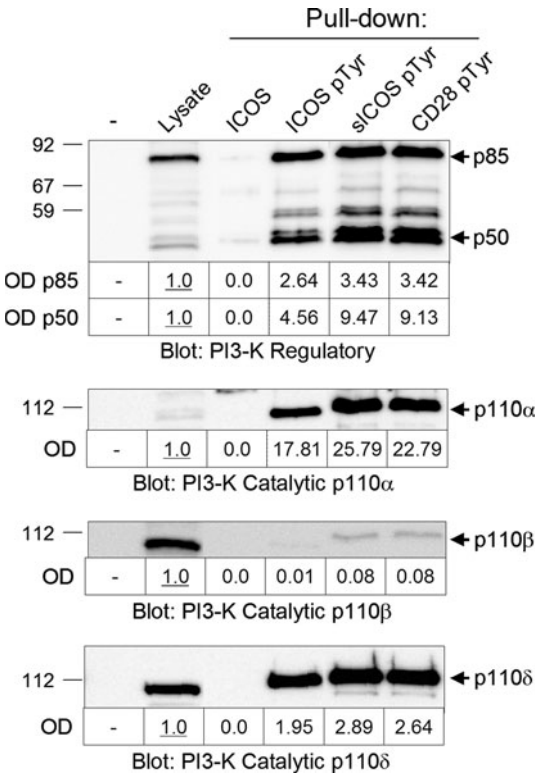

Fig. 2 Comparison of class IA PI3-kinase subunits associated with phosphorylated (ICOSpTyr; sICOSpTyr) or unphosphorylated (ICOS) ICOS peptides, or one phosphorylated CD28 peptide (CD28pTyr) in pull-down assays of D10 lysates, as determined by immunoblot with subunit-specific antibodies. Data from one representative experiment of three performed with similar results. The OD values for the immunoblots of regulatory or catalytic PI3-kinase subunits in peptide pull-downs were equalized considering the OD value of each subunit in cell lysates as 1 (*underlined*)

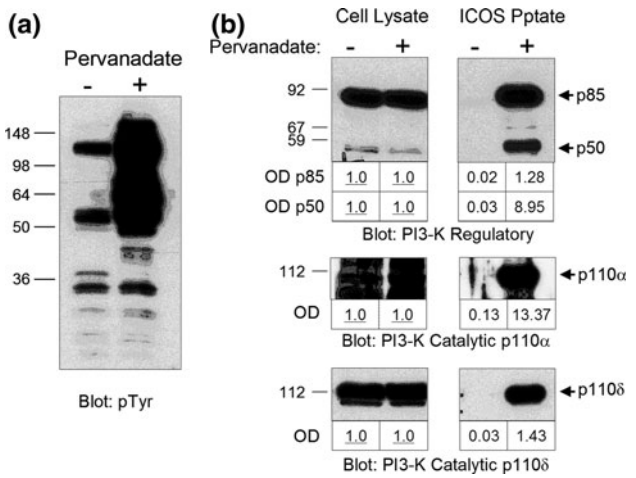

Fig. 3 Class IA PI3-kinase subunits associated with immunoprecipitates of cell surface ICOS before or after induction of intracellular protein phosphorylation with the phosphatase inhibitor pervanadate. **a** Comparison of pTyr phosphorylation in lysates of D10 cells treated (+) or not (−) with pervanadate for 5 min, as determined by immunoblot with the anti-pTyr antibody. **b** Class IA PI3-kinase subunits co-precipitated with ICOS immunoprecipitates of D10 cells treated (+) or not (−) with pervanadate for 5 min were determined by immunoblot with subunit-specific antibodies. The OD values for the immunoblots of regulatory or catalytic PI3-kinase subunits in ICOS immunoprecipitates were equalized considering the OD value of each subunit in cell lysates as 1 (*underlined*). Data from one representative experiment of four performed

the regulatory PI3-kinase subunits. Indeed, immunoprecipitation of PI3-kinase with antibodies recognizing the p85 and p50 PI3-kinase regulatory subunits shows that p110α co-precipitation is comparatively better than that of p110δ (Fig. 4a), similar to the results obtained in pull-downs of phosphorylated ICOS peptides or in ICOS immunoprecipitates (Figs. 1, 2, 3).

Since these results were obtained using the D10 cell line, we also analyzed PI3-kinase immunoprecipitates from normal ones, using equal numbers of freshly isolated $CD4^+$ T lymphocytes or $CD4^+$ T cell blasts. On a per cell basis, $CD4^+$ blast lysates had a higher content of all PI3-kinase subunits than spleen $CD4^+$ T cells, but the relative amount of each subunit was roughly the same as in $CD4^+$ T cells. Immunoprecipitation of PI3-kinase regulatory subunits from these cells shows co-immunoprecipitation of both p110α and p110δ catalytic subunits (Fig. 4b, Fig. S1a). However, whereas the p110δ catalytic subunits (and the p85 regulatory subunits) were readily detectable in the cell lysates, p110α catalytic subunits were detected as faint bands, highlighting the higher levels of co-precipitated p110α. These differences were detected regardless of whether using monoclonal (Fig. 4b, see also Fig. S2b) or polyclonal anti-p110α antibodies (Fig. S1a). This again indicates a preferential binding of p110α over p110δ in the PI3-kinase complexes. Interestingly, lower relative levels of p50–p55 PI3-kinase regulatory subunits were detected in the mouse $CD4^+$ T cell blasts compared to normal $CD4^+$ T lymphocytes (Fig. S1b), yet the preferential association of p110α to the regulatory subunits was maintained.

The different association of p110α and p110δ to PI3-kinase regulatory subunits was surprising. We hypothesized that it could be due to low protein levels of p110δ protein, despite the data on mRNA abundance indicating roughly equal levels for p110α and p110δ catalytic subunits (Fig. 1). Alternatively, a fraction of p110δ might be acting independently of and be unavailable for co-precipitation with the regulatory subunits of class IA PI3-kinases. To sort out these possibilities, lysates of the D10 cell line were depleted of the regulatory subunits of class IA PI3-kinases by three successive immunoprecipitations with specific antibodies (immunodepletions 1, 2 and 3 of PI3-K R in Fig. 5a) or depleted of the unrelated cytoplasmic protein ZAP-70 (C immunodepletions 1, 2 and 3 in Fig. 5a). The immunoprecipitation of regulatory PI3-kinase effectively removed these subunits, as shown by the very

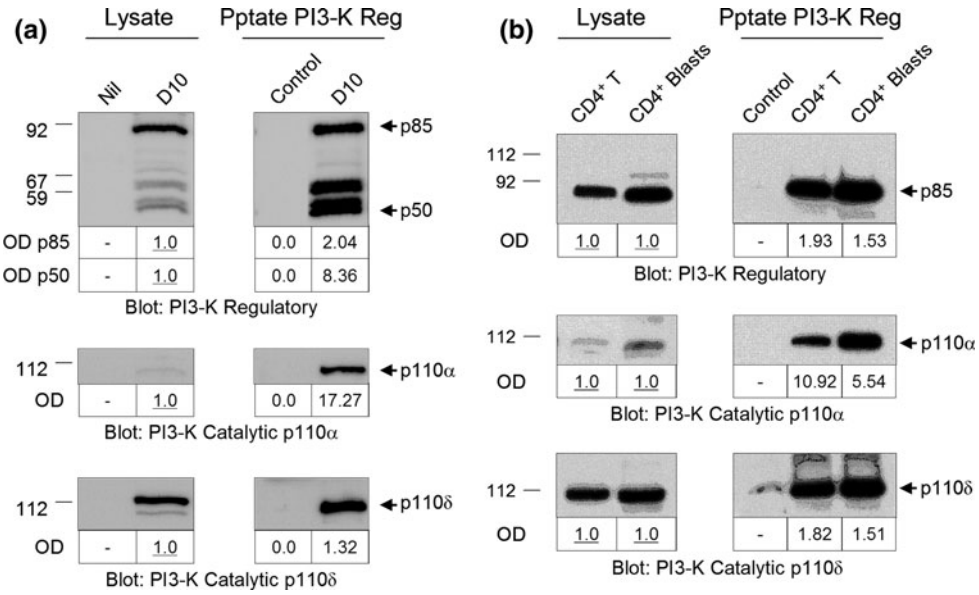

Fig. 4 Class IA PI3-kinase catalytic subunit abundance in immunoprecipitates of PI3-kinase regulatory subunits (Pptate PI3-K Reg) compared to their abundance in: **a** lysates from D10 cells; **b** lysates of $CD4^+$ spleen T lymphocytes or $CD4^+$ blasts, determined by immunoblot with subunit-specific antibodies. Data from one representative experiment of three (**a**) or two (**b**) performed. The OD values for the immunoblots of regulatory or catalytic PI3-kinase subunits in immunoprecipitates or peptide pull-downs were equalized considering the OD value of each subunit in cell lysates as 1 (*underlined*)

low or negligible levels of p85, p50 and p55 subunits remaining in the third regulatory PI3-kinase immunoprecipitates. Then, the lysates obtained after control or PI3-kinase depletion were divided and immunoprecipitated with antibodies specific for the p110α or the p110δ catalytic subunits (Fig. 5b). Most p110α catalytic subunits were bound to the regulatory subunits, as shown by the low p110α levels that could be immunoprecipitated in lysates depleted of PI3-kinase regulatory subunits, as compared to p110α immunoprecipitates from control lysates (Fig. 5b). In contrast, p110δ was still abundant after depletion of PI3-kinase regulatory subunits (Fig. 5b). This fact plus the low levels of PI3-kinase regulatory subunits found in p110δ immunoprecipitates from lysates previously depleted of regulatory subunits indicates that, indeed, there is a fraction of p110δ catalytic subunits that is not bound to the regulatory subunits in these lysates. (Fig. 5b).

Effect of PI3-kinase isoform-specific siRNA or inhibitors on ICOS costimulation

To confirm the importance of PI3Kα and PI3Kδ in ICOS costimulation, D10 cells were transfected with subunit-specific siRNA. Transfection of p110α siRNA produced a clear and specific inhibition of PI3Kα levels when compared to cells transfected with control siRNA (Fig. 6a). PI3Kβ levels were slightly enhanced, possibly to compensate for the loss of p110α, whereas p110δ levels remained essentially unchanged (Fig. 6a). We then analyzed early downstream signals (Akt, Erk) previously known to be the targets for ICOS costimulation [10]. Our results show that transfection of PI3 Kα siRNA significantly inhibited the early phosphorylation of protein kinase B (Akt) induced by anti-CD3 plus anti-ICOS antibodies (Fig. 6b) at times previously determined to be optimal for early Akt phosphorylation, confirming the importance of p110α in ICOS costimulation. Intriguingly, the activation of the MAPK Erk was not inhibited, but markedly enhanced in PI3K p110α-silenced cells (Fig. 6b). This was specific to Erk, as activation of the MAPK p38 was also inhibited in p110α-silenced cells (data not shown). Interestingly, strong siRNA-mediated silencing of p110α produced only a partial inhibition of p110α binding to phosphorylated ICOS, which was not fully compensated by the enhanced binding of p110δ or p110β (Fig. S2). This might explain the partial inhibition of Akt activation observed in Fig. 6b.

Since previous data have shown an essential role of the p110δ catalytic subunit in T cell activation, we also checked for the effect of p110δ silencing. Expression of p110δ was clearly inhibited upon specific siRNA transfection (Fig. 6c), whereas both p110α and p110β were moderately enhanced (Fig. 6c). When the transfected cells were activated by combined anti-CD3 and anti-ICOS antibodies, early phosphorylation of Akt was inhibited (Fig. 6d). Unlike what happened in p110α silencing, Erk activation was inhibited by p110δ siRNA (Fig. 6d). Since the regulatory subunit levels were not affected in either case, the different effects of p110α or p110δ siRNA on Erk activation are likely due to the altered ratios of the catalytic subunits. Intriguingly, cytokine (IL-4) secretion was not inhibited in p110α- or p110δ-siRNA transfected cells (data not shown). Transfection of both PI3 Kα and PI3 Kδ siRNA was also performed, which produced decreased levels of both p110α and p110δ catalytic subunits, whereas the level of the regulatory p85α and p50α PI3-kinase subunits was unchanged (Fig. S3). It also augmented levels of p110β protein about two-fold, but since the levels of this

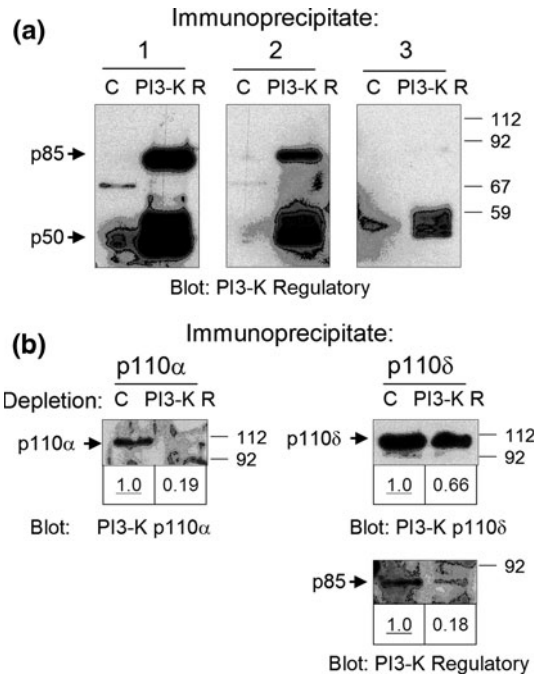

Fig. 5 Removal of PI3-kinase regulatory subunits efficiently removes p110α, but not p110δ catalytic subunits in D10 cells. **a** Cell lysates of D10 cells were subjected to three sequential immunoprecipitation steps with a control antibody (anti-ZAP-70; C) or with antibodies specific for PI3-kinase regulatory subunits (PI3-K R; p85) to deplete PI3-kinase regulatory subunits and the associated catalytic subunits. Immunoblot of the immunoprecipitates with anti-p85 antibody shows the presence of residual regulatory subunits in the third p85 immunoprecipitation, as compared to the first immunoprecipitate. **b** Lysates from **a**, pre-cleared (PI3-K R) or not **c** with anti-PI3-K regulatory antibodies, were divided and immunoprecipitated with anti-p110α or anti-p110δ antibodies. The catalytic subunits present in each immunoprecipitate were then detected by immunoblot with anti-p110α- (*left*) or anti-p110δ-specific antibodies (*right*), as shown in the figure. PI3-kinase regulatory subunits present in p110δ immunoprecipitates were also determined by immunoblot (*right, lower panel*). The OD of immunoprecipitates in the control, undepleted lysates, was taken as a reference in each case and its value considered as 1. Data from one representative experiment of three performed

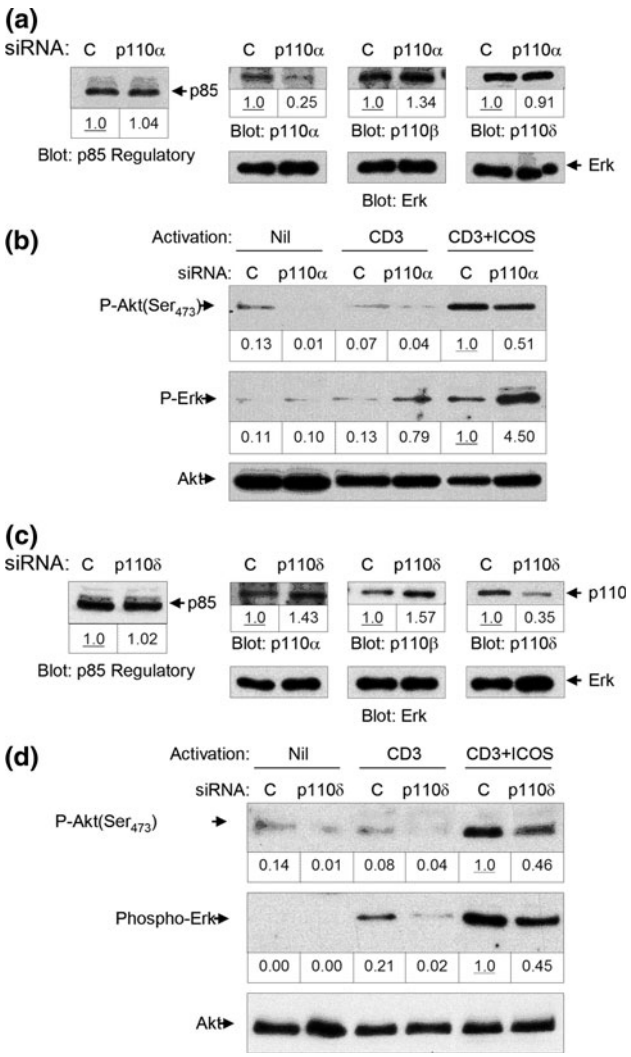

Fig. 6 Effect of siRNA interference of PI3-kinase catalytic subunits on ICOS function. **a**, **c** Cell lysates of D10 cells trasfected with control siRNA (C) or siRNA specific for p110α (p110α) (**a**) or p110δ (p110δ) (**c**) were checked by immunoblot with antibodies specific for regulatory and catalytic class IA PI3-kinase subunits. As a reference, the OD value in control cell lysates was considered as 1 (*underlined*). Immunoblot with antibodies against an unrelated protein (Erk) was used as a control for protein load. Data from one representative experiment of two performed with similar results. **b**, **d** D10 cells trasfected with control (C), p110α-specific (p110α) (**b**) or p110δ-specific (p110δ) (**d**) siRNA were activated for 10 min at 37°C with polystyrene beads coated with anti-CD3 antibody (0.5 μg/ml) plus a control antibody (CD3), anti-CD3 plus anti-ICOS (5 μg/ml, CD3 + ICOS) or uncoated (Nil). The reaction was stopped, the cells lysed and the lysates blotted with antibodies specific for activated (phosphorylated) Akt [P-Akt(Ser_{473})] or Erk (P-Erk), as indicated. Protein loads were corrected using anti-Akt antibody as reference. In each case, the OD value in lysates of control cells activated with anti-CD3 plus anti-ICOS antibodies was considered as 1 (*underlined*)

subunit are low, it is likely that this cannot fully compensate for the loss of catalytic subunits. CD3- or CD3 plus ICOS-mediated activation of both Akt and Erk phosphorylation is inhibited in the silenced cells, indicating that the effect of p110δ silencing is dominant over that of p110α (Fig. S3).

Different PI3-kinase inhibitors were used to further assess the importance of the PI3-kinase catalytic subunits in ICOS costimulus. ICOS-expressing cells were activated for 4–5 h with polystyrene beads coated with anti-CD3 antibodies plus anti-ICOS or control antibodies, and the culture supernatants were then analyzed for cytokine (IL-4) content (Fig. 7a). Broad PI3-kinase inhibitors like LY294002 strongly inhibited TCR/CD3 activation and ICOS costimulation of IL-4 secretion (Fig. 7a), as expected from previous data in the same cell line [10]. Interestingly, both the PI3 Kα-specific inhibitor PIK-75 and the PI3Kδ inhibitor IC87114 inhibited CD3 activation and ICOS

costimulation in this system (Fig. 7a). In contrast, inhibitors specific for PI3-kinase IA PI3Kβ (TGX221) or the PI3-kinase IB PI3Kγ did not significantly inhibit ICOS costimulation in D10 cells (Fig. 7a, Fig. S4a) or in ICOS$^+$ blasts of normal spleen CD4$^+$ T cells (Fig. S4b). Inhibitors of the Akt (PKB) kinase, one major target downstream of PI3-kinases, clearly inhibited cytokine secretion (Fig. S4a,b).

Early Akt and Erk phosphorylation in the presence of isoform-specific inhibitors was also analyzed (Fig. 7b). The PI3 Kδ inhibitor IC87114 strongly inhibited CD3- or CD3 plus ICOS-induced Akt phosphorylation, whereas Erk

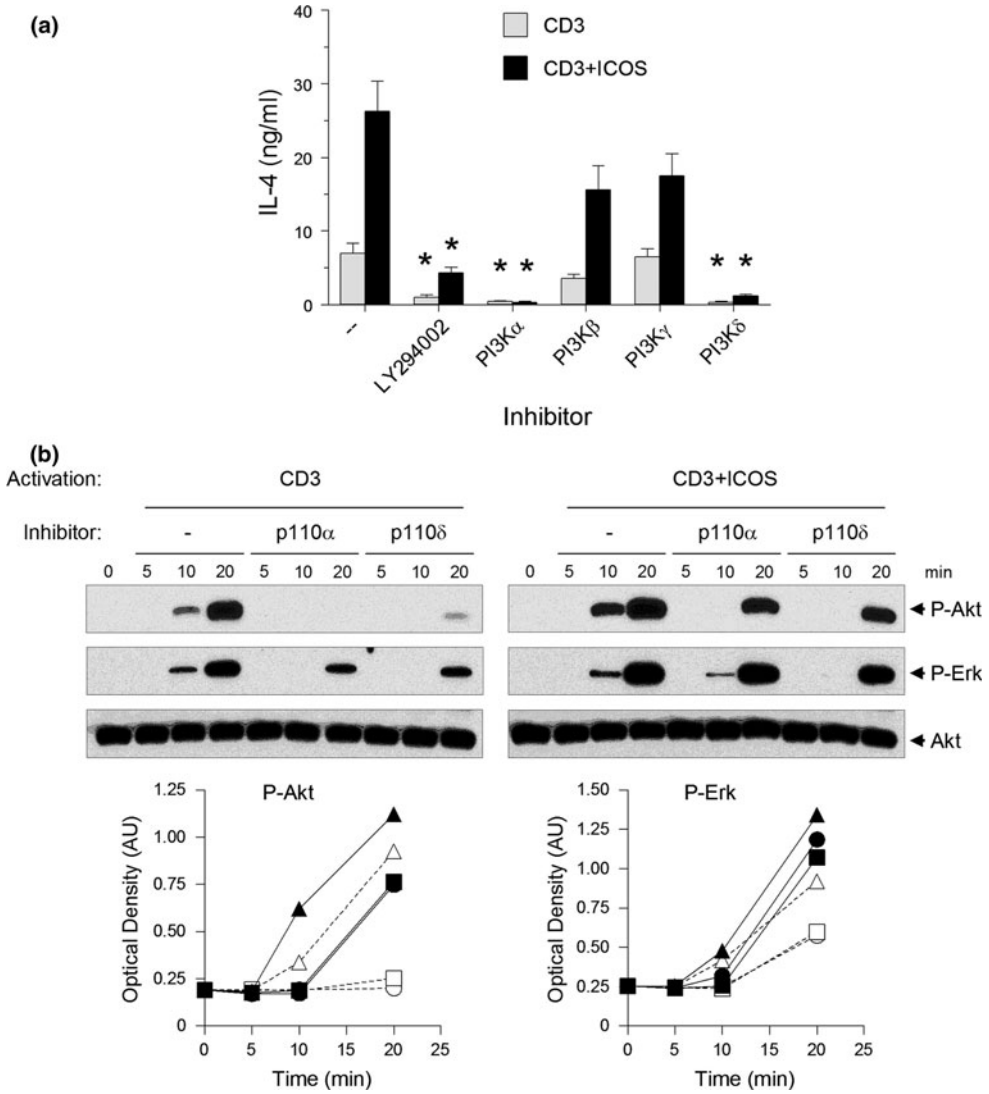

Fig. 7 Effect of PI3-kinase subunit-specific inhibitors on ICOS costimulation. **a** IL-4 content in supernatants of D10 cells activated with antibody-coated polystyrene beads coated with anti-CD3 antibody (1 μg/ml) plus a control antibody (CD3, *grey bars*), or anti-CD3 plus anti-ICOS (5 μg/ml, CD3 + ICOS, *black bars*) in the absence (−) or the presence of PI3-kinase inhibitor LY294002 (20 μM), or inhibitors specific for PI3-kinase p110α (PIK-75, 1 μM), p110β (TGX-221, 1 μM), p110γ (PI3-Kγ inhibitor II, 12 μM) or p110δ (IC87114, 5 μM). After 4 h incubation, the supernatants were collected and IL-4 determined by ELISA. Mean ± SEM of triplicate determinations from one experiment of two performed with similar results. Asterisks indicate significant ($p < 0.05$) differences with control CD3− or CD3 + ICOS-activated cultures, as determined by the two-tailed Student's *t* test. **b** D10 cells were activated with polystyrene beads coated with anti-CD3 antibody (0.5 μg/ml) plus a control antibody (CD3) or anti-CD3 plus anti-ICOS (5 μg/ml, CD3 + ICOS) for the times indicated, in the absence (−) or the presence of specific p110α (PIK-75, 1 μM) or p110δ (IC87114, 5 μM) PI3K inhibitors. The reaction was stopped, the cells lysed and the lysates blotted with antibodies specific for activated (phosphorylated) Akt (P-Akt) or Erk (P-Erk), as indicated in the *upper panel*. After correcting for the protein loads using anti-Akt antibody blots, the OD values for P-Akt and P-Erk were plotted, as shown in the *lower panel*. *Open symbols* cells activated with anti-CD3; *closed symbols* cells activated with anti-CD3 plus anti-ICOS. *Triangles* no inhibitor, *circles* p110α inhibitor, *squares* p110δ inhibitor. *AU* arbitrary units. Data from one experiment of two with similar results

phosphorylation was more resistant to inhibition (Fig 7b). This agrees with previous data on the effects of p110δ inhibition, loss or inactivation on Akt and Erk phosphorylation [48–50]. Like IC87114, the PI3 Kα-specific inhibitor PIK-75 effectively inhibited Akt phosphorylation, and, unlike the results of p110α silencing, Erk phosphorylation was also moderately inhibited.

Taken together, these data suggest that loss of PI3-kinase α or δ expression in the silenced cells induces a misbalanced PI3-kinase function. This leads to altered activation of downstream pathways. This is not found using subunit-specific inhibitors where subunit activity, not abundance, is changed.

PI3-kinase p110α-specific, but not p110δ-specific inhibitors block cell shape changes induced by ICOS alone

The data presented hitherto show that both p110α and p110δ can modify ICOS costimulation of TCR/CD3 stimuli. Previous results show that ICOS ligation, without any additional stimulus, induces elongation and actin cytoskeleton changes in T lymphoblasts in a Src kinase- and PI3-kinase dependent fashion [51, 52]. Indeed, ICOS ligands like anti-ICOS antibodies or B7-H2/Fc chimeras induced D10 cell elongation (Fig. 8, Fig. S4). This was not observed using other activating stimuli like anti-CD3 or phorbol esters that enhanced cell spreading (Fig. S4). So, we used this model to study the role of the different PI3-kinase catalytic subunits in signaling by ICOS alone. As shown in Fig. 8, the PI3 K inhibitor LY294002 or the PI3Kα inhibitor PIK-75 suppressed ICOS-induced D10 elongation, whereas other PI3-kinase inhibitors, including the PI3Kδ inhibitor IC87114, or the Akt inhibitor triciribine had no significant effects. One Lck inhibitor also inhibited cell elongation, in agreement with Lck being the main Src family kinase in D10 cells (Fig. 8a).

Discussion

PI3-kinase subunits in phosphorylated ICOS and in immunoprecipitates

We have analyzed the nature of PI-3-kinase subunits able to bind to ICOS using proteomic and immunoblot analysis of proteins in pull-down assays with ICOS peptides or in ICOS immunoprecipitates. Concerning the regulatory subunits, we found a higher relative binding of p50-55α regulatory isoforms over p85 isoforms to ICOSpTyr sequences that was expected based on recent data of PI3-kinase binding to ICOS upon activation [11]. Our analysis of the PI3-kinase catalytic subunits bound to phosphorylated ICOS peptides, or to ICOS immunoprecipitates from activated cells, shows a previously unnoticed preferential co-precipitation of p110α over the other class IA catalytic subunits (p110δ or p110β) present in lymphocytes. This was unexpected, as p110α and p110δ—but not p110β—catalytic subunit mRNAs were found at roughly similar levels in $CD4^+$ T cells or in the different T cell lines used.

Interestingly, the analysis of the catalytic subunits found in immunoprecipitates of class IA regulatory subunits also detected a preferential binding of p110α subunits over p110δ or p110β. This was consistently found in all the $CD4^+$ T cells and T cell lines analyzed, including normal spleen cell $CD4^+$ T lymphocytes and $CD4^+$ blasts. These results indicated that it was the regulatory-catalytic subunit complexes present in the cells rather than the sequence of ICOS that were responsible for the high abundance of p110α associated to ICOS. This was also shown by the similar results obtained using a shorter variant of the ICOS cytoplasmic sequence or a cytoplasmic CD28 peptide.

Pairing of distinct PI3-kinase class IA catalytic and regulatory subunits

Surprisingly, immunodepletion of regulatory subunits efficiently depleted p110α, but left significant amounts of p110δ catalytic subunits still available for immunoprecipitation in the lysates. This was unexpected, as the current paradigm holds, on the one hand, that class IA catalytic subunits are unstable and are degraded in the absence of regulatory ones, and on the other hand, that there is no evidence for a preferential pairing between distinct PI3-kinase catalytic and regulatory subunits (see [23, 24, 53] for recent reviews). The possibility that our results are due to the antibody used not recognizing regulatory subunits like p85β or p55γ, allowing for remaining non-precleared PI3 K complexes, is unlikely, as these subunits are expressed at low levels in lymphoid cells compared to the α subunits (Fig. 1a; [19, 27]), and the antibody used for immunodepletion is not isoform-specific. Furthermore, we were unable to detect p55γ subunits by immunoblot in p55γ-specific immunoprecipitates (J.M. Rojo et al., data not shown), in agreement with published data [47]. Secondly, if this were true, it would mean that there is a hitherto unnoticed preference for p85β–p110δ or p55γ–p110δ subunit pairing.

There is the possibility that p110α is detected better because the interaction between p110δ and the PI3-kinase regulatory subunits is weaker than that of p110α subunits. Although this possibility should be explored, it should also be noted that the abundance of mRNA for regulatory and catalytic subunits shown in Fig. 1a indicates a clear excess of the catalytic subunits over the regulatory ones.

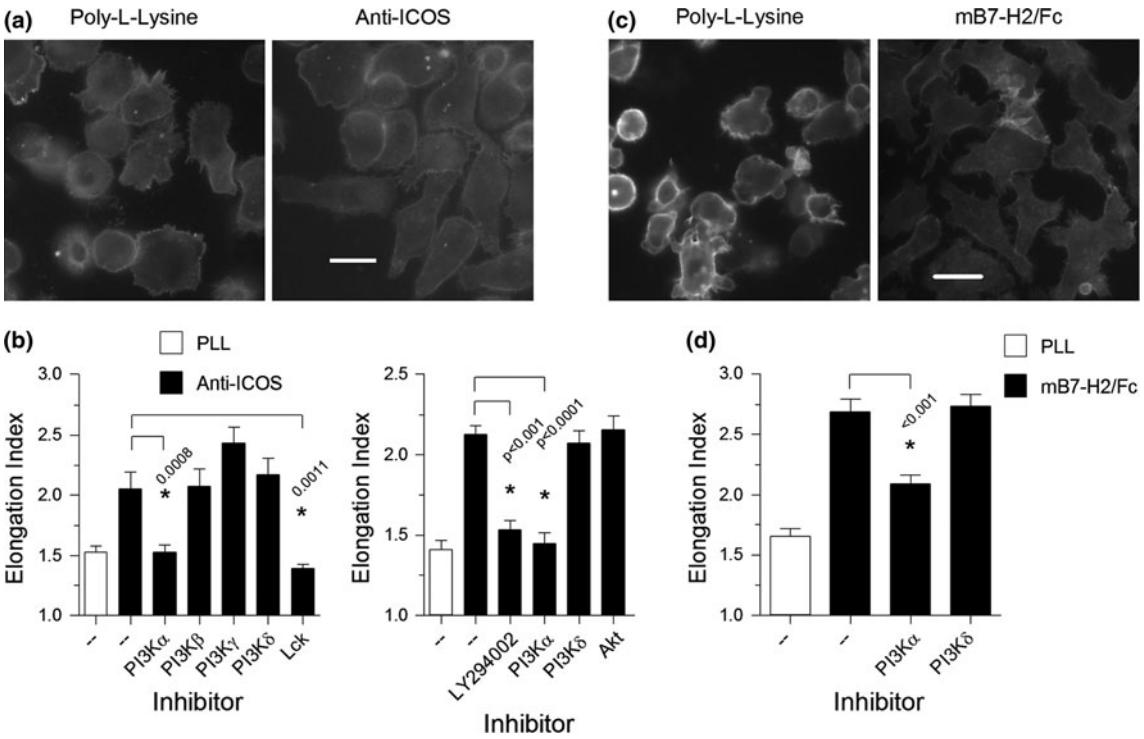

Fig. 8 Effect of PI3-kinase, Akt and Lck inhibitors on ICOS-induced cell elongation. **a** Ligation of ICOS with specific antibodies attached to coverslips induces elongation of D10 cells and reorganization of actin cytoskeleton, as shown in phalloidin-stained cells (*bar length* is 10 μm). **b** Graphs showing the effect of PI3-kinase, Akt and Lck inhibitors on D10 elongation, as determined by elongation index. The concentration of PI3-kinase inhibitors was as in Fig. 7a; Akt inhibitor triciribine was 5 μM; Lck inhibitor II was used at 200 nM. Mean ± SEM of values from one experiment of five performed with similar results. Significant differences ($p < 0.05$) with ICOS-activated cells, as determined by the two-tailed Student's *t* test, are indicated. **c**, **d** Elongation of D10 cells and reorganization of the actin cytoskeleton is efficiently induced by B7-H2/Fc chimeras of the ICOS ligand, as shown in phalloidin-stained cells (*bar length*, 10 μm). **d** Inhibition of the p110α PI3-kinase catalytic subunit, but not inhibition of the p110δ subunit, significantly reduces the elongation of D10 cells induced by the ICOS ligand chimera, as determined by elongation index. PI3-kinase inhibitors as in (**a**). Mean ± SEM of elongation index from one experiment of two performed with similar results. Significant differences ($p < 0.05$) with ICOS-activated cells, as determined by the two-tailed Student's *t* test, are indicated

PI3-kinase regulatory polypeptides stabilize the catalytic subunits and enhance their half-life, but they are not essential to their enzyme activity and actually inhibit their lipid kinase activity [54]. In fact, recruiting regulatory subunits by phosphorylated peptides does rescue the inhibited activity of the catalytic subunits [54]. The p110*β* and p110*δ* catalytic polypeptides can be recruited to the membrane and activated by G protein-coupled receptors or the small GTPase TC21, respectively [37, 38]. Although the role of regulatory subunits in the binding to these proteins has not been determined, some results shown by Delgado et al. are compatible with part of the cell's p110*δ* being bound to TC21 independently of regulatory subunits [37].

In a situation of excess of the catalytic over the regulatory subunits, it is conceivable that, however short-lived, there is a fraction of "free" catalytic subunits not bound to regulatory subunits. Since both p110α and p110*δ* mRNA occur at roughly the same level (Fig. 1), the fact that the free subunits are mainly p110*δ* might indicate a preferential binding of p110α to the regulatory subunits in these cells. Furthermore, we observed no significant changes in the level of regulatory subunits upon silencing of the p110α and/or p110*δ* catalytic polypeptides (Fig. 6, Fig. S3). The level of the scarce p110*β* subunits was modestly enhanced, but this probably did not compensate for the loss of the far more abundant and functionally relevant p110α and p110*δ* subunits. It follows then that either there was a fraction of free catalytic subunits before p110α silencing or that there was a fraction of free regulatory subunits after it.

Functional importance of p110α to TCR activation and ICOS costimulation

The relevance of p110α PI3-kinase to TCR activation and ICOS costimulation was further emphasized by our analysis of cytokine secretion in the presence of class-specific PI3-kinase inhibitors and of ICOS signaling in the presence of p110α siRNA. Both approaches coincided in

demonstrating a major role of the catalytic p110α in ICOS costimulus. This was not surprising, as p110α and p110δ are the main class IA catalytic subunits used in the cells, and p110α was preferentially bound to the regulatory subunits and ICOS. Furthermore, and in full agreement with the data on catalytic subunit binding to ICOS, cell elongation induced by ICOS ligands was blocked by p110α, but not p11δ inhibitors.

Elucidating the role of the ubiquitously expressed p110α and p110β in T cell function has been hampered by the embryonic lethality of mice deficient for these isoforms [55, 56]. In these conditions, the interest has focused on the role in thymus cell differentiation, T cell activation, differentiation and trafficking, or the impact in autoimmune diseases of the class IA p110δ and class IB p110γ catalytic subunits, whose expression is particularly high in leukocytes (reviewed in [22–24]).

Initial experiments using $p110\delta^{-/-}$ mice detected impaired B cell receptor, but not T cell receptor signaling [57]. In contrast, Okkenhaug et al., using mice carrying a loss of function point mutation ($110\delta^{D910A/D910A}$), observed reduced TCR-dependent proliferation in vitro and in vivo, and altered Th1 and Th2 differentiation linked to inhibited phosphorylation of Akt and Foxo, two PI3-kinase downstream targets [50]. Besides, one specific inhibitor for p110δ blocks T lymphocyte proliferation and cytokine production by CD3 and CD3 plus CD28 activation [58]. In agreement with these data, we found that inhibition of p110δ significantly inhibited the activation of IL-4 secretion induced by anti-CD3 or anti-CD3 plus anti-ICOS. The p110δ catalytic subunit is recruited to the TCR complex through the small GTPase TC21, participating in homeostatic proliferation and T cell survival [37]; this phenomenon might be behind some of the functional and survival defects of $CD4^+CD25^+Foxp3^+$ regulatory T cells found in $110\delta^{D910A/D910A}$ mice [59].

These data on p110δ point to a different effect of deletion ($p110\delta^{-/-}$) or silencing versus impaired function (i.e., in $110\delta^{D910A/D910A}$ mice) or pharmacological inhibition. Unexpectedly, we found that, like p110δ siRNA, p110α siRNA partially inhibited the early activation of Akt and that, like p110δ-specific inhibitors, p110α inhibitors blocked both CD3- and CD3/ICOS-mediated cytokine secretion. This suggests that in normal circumstances both p110α and p110δ fulfill distinct, non-redundant roles in TCR/CD3 activation and costimulation. Yet, if the catalytic subunits are not inhibited but removed, i.e., by siRNA, the remaining subunits can replace at least some of their functions, perhaps because low levels of active Akt are compatible with efficient T cell activation [60].

How p110δ as well as p110α affects CD3 activation and ICOS costimulation is not clear, but the fact that both p110δ and p110α silencing partially inhibit Akt, and the blocking of cytokine secretion by Akt inhibitors points to PDK1/Akt as key players in our system. Akt1 can promote NF-AT activation through phosphorylation of the NF-AT-binding Homer proteins [61]. We have observed that NF-AT activation is essential to IL-4 secretion in the short-term activation of D10 cells, as shown by the blocking effect of cyclosporin [10], and blocking PKA, another PDK1/Akt target capable of activating NF-AT [62], partially inhibits IL-4 secretion (J.M. Rojo et al., unpublished).

Taken together, our results point to class IA PI3-kinases as major players in ICOS costimulation. CD28 has a YMNM cytoplasmic motif that binds distinct adapter protein or enzymes as well as PI3-kinases [2, 63], whereas so far only PI3-kinases have been found associated to ICOS (this work and [9–13]). Leaving their expression patterns and ligands aside, differences between CD28 and ICOS include one residue in the YMxM motif (YMFM in ICOS) having important functional consequences in terms of costimulation [13, 14]. Besides, CD28 has a C-terminal $AAY_{216}RP$ motif that is absent in ICOS; phosphorylation of this motif allows the binding of PI3-kinase p85β regulatory subunits [19].

Our results also show that there can be a preference in the association of p110α catalytic subunits to ICOS as a consequence of their preferential association to the regulatory subunits. Furthermore, our results suggest that a fraction of the p110δ catalytic subunits might be functioning independently of their binding to PI3-kinase regulatory subunits.

Acknowledgments The skillful technical assistance of Maria Luisa del Pozo and Marta Blanco-Berrocal is gratefully acknowledged. Y.Y.A. is the recipient of a Predoctoral Fellowship of the "Junta de Ampliación de Estudios" (JAE) Program (C.S.I.C., Ministerio de Ciencia e Innovación, Spain). P.P. is a Tenured Sciencist of C.S.I.C. at the Centro Nacional de Microbiología, I.S.: Carlos III. This work was supported by grants PI070620 and PI070484 (Fondo de Investigación Sanitaria, Ministerio de Ciencia e Innovación, Spain) and by AIRC (Milan) (to U.D.).

Conflict of interest The authors declare no financial conflict of interest.

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