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Stable Activation of Phosphatidylinositol 3-Kinase in the T Cell Immunological Synapse Stimulates Akt Signaling to FoxO1 Nuclear Exclusion and Cell Growth Control

Stéphanie Fabre,* Valérie Lang,* Julie Harriague,† Aude Jobart,* Terry G. Unterman,‡ Alain Trautmann,* and Georges Bismuth2*

We have previously reported at the single cell level that PI3K is activated after conjugate formation between T lymphocytes and APCs. However, in contrast to cells exposed to an asymmetrical signal that usually increase 3′-phosphoinositides (3′-PI) transiently in the region of the activated receptors, T cells contacting APC accumulate 3′-PI across their whole plasma membrane far beyond the region of the immunological synapse (IS). Importantly, this effect is maintained over time, for hours, and although PI3K-dependent pathways translate in various cell types extracellular stimuli into a wide range of biological events, in primary T cells this stability is mostly required for cell division induced by Ag. Using imaging methodologies, the present article elucidates the molecular mechanisms responsible for this particular functioning of the PI3K pathway in primary human T lymphocytes interacting with APCs, especially with dendritic cells. The results reveal that the IS unremittingly recruits PI3K to maintain high 3′-PI levels in T cells through phosphotyrosine-dependent mechanisms, suggesting a major participation of class Ia PI3K. This persistent activation of PI3K results in the Akt-dependent sequestration of the FoxO transcription factor, FoxO1, outside the nucleus of T cells interacting with APCs. Using an active form of FoxO1, we demonstrate that this compartmentalization process can affect T cell growth after Ag recognition. We conclude that the need for sustained PI3K signaling within the consolidated IS is probably an undemanding tactic used by primary T cells critical for initiating cell cycle progression through the prolonged inactivation of FoxO1, one important factor that can control cell quiescence.

Antigen-specific activation of T lymphocytes by APC is associated with T cell polarization toward the APC and the formation of a highly specialized junction, the immunological synapse (IS), characterized by a distinct segregation of Ag receptors and adhesion and signaling molecules (1–4). These phenomena require extensive remodeling of the T cell cytoskeleton and are associated with a tyrosine phosphorylation cascade that can activate numerous cellular effectors. Among these, PI3Ks are key enzymes, the function of which in the immune system has been highlighted over the past few years, primarily in studies using a genetic approach (5–9). However, direct demonstration that PI3K is activated in resting T cells during IS formation has only been recently reported (10, 11).

The PI3K family consists of three main classes of enzymes, I, II, and III, that phosphorylate membrane phosphatidylinositol (PI3Ks) (PtdIns), PtdIns 4-phosphate, or PtdIns 4,5-bisphosphate (PtdIns(4,5)P₂) on the D3 position of the inositol ring. Class I PI3Ks, the best studied, are activated by extracellular stimuli, and class IA PI3Ks are recruited downstream of receptors associated with tyrosine kinases such as the BCR and the TCR for Ag, cytokine receptors, or costimulatory molecules, whereas class IB PI3Ks act downstream of G-protein-coupled receptors such as chemokine receptors (7, 9, 12). Class IA PI3Ks are heterodimeric enzymes constituted by a regulatory subunit, generally p85α, and a p110 catalytic subunit in tight interaction. The Src homology 2 domains of the regulatory subunit are recruited on the consensus motif YxxM phosphorylated upon tyrosine kinase-coupled receptor engagement (13), thereby conveying the p110 subunit close to the plasma membrane to catalyze the formation of PtdIns(3,4)P₂ and PtdIns 3,4,5-trisphosphate (PtdIns(3,4,5)P₃), two phosphoinositides that are almost undetectable in resting cells. This process allows the recruitment and activation of effector proteins containing a pleckstrin homology (PH) domain specific for these 3′-phosphoinositides (3′-PI) (14), including the serine-threonine kinase Akt, which can operate fused with the GFP as a probe to dynamically follow 3′-PI in living cells.

This strategy was successfully used to reveal the existence of an intense and sustained production of 3′-PI after conjugate formation between a T cell and an APC in both human and murine primary T cell systems (10, 11). In the presence of Ag, GFP-Akt-PH accumulated to the T cell plasma membrane rapidly upon contact initiation. Moreover, 3′-PI was increased for hours (11), a result confirmed in a more recent study (15), showing that its activation

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Abbreviations used in this paper: IS, immunological synapse; CFP, cyan fluorescent protein; DC, dendritic cell; FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; PH, pleckstrin homology; 3′-PI, 3′-phosphoinositide; PtdIns, phosphatidylinositol; PtdIns(4,5)P₂, PtdIns 4,5-bisphosphate; PtdIns(3,4,5)P₃, PtdIns 3,4,5-trisphosphate; pTyr, phosphorytrosine; SAG, superantigen; YFP, yellow fluorescent protein.
by Ag was a long-lasting process representing a striking feature of PI3K metabolism in T cells. It was also found that PtdIns(3,4,5)P₃ accumulated not only at the contact zone, but also in the plasma membrane outside the IS when T cell-APC contact continued, suggesting that PI3K activation may be not restricted to the IS. This is in contrast with the current view of 3'-Pi accumulation established in different cellular models, such as fibroblasts or neutrophils, where the regulation of PI3K has been extensively studied, and this process is thought to be limited to the activated pole of the cell (16–19). Interestingly, it also appeared that this prolonged PI3K activation was essential for T cell proliferation, but not for IS formation and stability or for early signals triggered by the T cell/ APC contact, including increased phosphorytrosine (pTyr) accumulation and calcium responses (10, 11).

Based on these observations, we determined the mechanisms underlying the persistent accumulation of PtdIns(3,4,5)P₃ observed in T cells contacting APCs, and how the asymmetrical activation of the T cell through the IS may account for the increase in PtdIns(3,4,5)P₃ in the whole plasma membrane. We also asked whether any downstream signaling pathway of critical importance for cell division is activated by Ag in T cells that requires this sustained PI3K metabolism. The results of this study reveal that the IS unremittingly recruits PI3K to maintain high PtdIns(3,4,5)P₃ levels in T cells, and that this sustained activation of PI3K metabolism results in the sequestration of FoxO transcription factors outside the nucleus, a compartmentalization process important for T cells to undergo cell growth after Ag recognition.

Materials and Methods

Cells

Human T lymphocytes were isolated from blood donors by Ficol density gradient centrifugation, followed by negative depletion on magnetic beads (T cell negative isolation kit; Dynal Biotech). Peripheral blood monocytes, purified with the negative monocyte isolation kit (Dynal Biotech), were derived into dendritic cells (DCs) in RPMI 1640 (Biochrom), 10% FCS in the presence of 20 ng/ml IL-4 (PeproTech), and 800 U/ml GM-CSF (Leucomax; Novartis/Shering-Plough) during 6 days before maturation with 5 µg/ml LPS (Sigma-Aldrich). The Raji B cell line was cultured in RPMI 1640, 10% FCS, 50 µM penicillin, and 50 µg/ml streptomycin. T8.1 is a murine T cell hybridoma expressing a human-mouse chimeric TCR specific for the tetanus toxin peptide 110.1.134 restricted to HLA-DRB1*1102. They were cultured in DMEM (Invitrogen Life Technologies), 10% FCS, 50 µM penicillin, 50 µg/ml streptomycin, 50 µM 2-ME (complete medium) supplemented with 400 nM methotrexate, and 1 mg/ml G-418. A 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-ME (complete medium) supplemented with PBS-4% paraformaldehyde. T8.1 clone stably expressing the fusion protein GFP-Akt-PH was cultured in complete medium supplemented with 1 µM puromycin. Murine fibroblasts L625.7 expressing human MHC class II molecules were used as APC and maintained in complete medium containing 250 µg/ml G-418.

Plasmid constructs

GFP-Akt-PH was a gift from Dr. T. Meyer (Stanford University School of Medicine, Stanford, CA). Cyan fluorescent protein (CFP)-Akt-PH was obtained by subcloning Akt-PH into the pECEPP-C1 fusion vector (BD Clontech) as an EcoRI restriction enzyme fragment. p85α DNA was obtained by PCR from a construct provided by Dr. E. Howard-Cofield (University of Kent, Canterbury, U.K.) and cloned into pCR2.1-TOPO (Invitrogen Life Technologies). p85α was then subcloned into the pECEPP-C1 and the pYFP fusion vectors (BD Clontech), as an XbaI restriction enzyme fragment, resulting in expression of fusion proteins with GFP or yellow fluorescent protein (YFP) being N-terminal. FoxO1-GFP and the triple mutant T24A/S256A/S319A FoxO1-GFP constructs were previously described (20).

Cell transfection and flow cytometric analysis

Freshly prepared human primary T cells were transfected with Nucleofector technology (Amaxa) according to the manufacturer’s instructions with 5 µg of the indicated construct. Expression levels were controlled by FACS analysis (FACScan cytometer; BD Biosciences). For measurements of cell apoptosis, transfected T cells were grown in complete medium in 96-well, round-bottom plates (2 × 10⁵ cells/well) and analyzed after 12 or 36 h of culture at 37°C. Cells were washed once in PBS-2% FCS, then in PBS alone and resuspended in 150 µl of annexin V binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂). Cells were labeled for 15 min at room temperature with 5 µl of annexin V-PE (BD Pharmingen) and subjected to FACS analysis.

For activation assays, 24 h after transfection with the indicated constructs, T cells were cultured as described above in the presence of 0.5 × 10⁶ irradiated Raji B cells (10,000 rad) with or without superantigen (SAg) at 20 ng/ml. After 6 or 24 h of culture, CD69 expression was measured by FACS with a PE-conjugated CD69 Ab (BD Pharmingen). Cell growth and nucleic acid contents were measured after 48 or 72 h of activation under the culture conditions described above by FACS analysis of unlabeled cell suspensions or after overnight incorporation of propidium iodide (10 µg/ml) in cells fixed with PBS-1% paraformaldehyde (Sigma-Aldrich), respectively.

Fluorescence imaging

DCs or Raji B cells were plated on polylsyline-coated glass coverslips mounted on 30-mm petri dishes and incubated for 30 min at 37°C in RPMI 1640 in the presence of recombinant staphylococcal enterotoxin E, staphylococcal enterotoxin A, staphylococcal enterotoxin B, and staphylococcal enterotoxin C3 (Toxin Technology) SAg at 200 ng/ml each. Transfected T cells were then added to APCs and incubated at 37°C in RPMI 1640–0.5% FCS for various times. Conjugates were fixed for 10 min at room temperature, incubated in PBS-4% paraformaldehyde (PFA) and washed twice in PBS-4% PFA before incubation at 4°C in PBS. Studies with conjugates were performed on an Eclipse TE3000 inverted microscope (Nikon) with a ×60 oil objective. Images were collected with a cooled CCD camera (CoolSNAPfx; Roper Scientific) and the Metavue Imaging system (Universal Imaging), then analyzed with MetaMorph software (Universal Imaging).

For immunofluorescent staining, paraformaldehyde-fixed cells were incubated for 20 min in PBS-0.1 M glycine and permeabilized in PBS-0.1% Triton X-100 for 10 min at room temperature. After three washes in PBS, they were incubated in PBS containing 0.2% BSA (Sigma-Aldrich) for 45 min. Cells were then stained with anti-phosphotyrosine 4G10 Ab (Upstate Biotechnology), followed by incubation with Rhodamine Red-X-conjugated secondary Ab (Jackson ImmunoResearch Laboratories) at 4°C for 30 min. After three washes in PBS-0.2% BSA and one in PBS, coverslips were finally mounted in Mowiol (Sigma-Aldrich). Quantitative analysis of fluorescence intensity was performed on 12-bit images after background subtraction using the MetaMorph software.

Video imaging

All time-lapse experiments were conducted using Raji B cells as surrogate APCs because of the higher frequency of contacts they formed with resting human T cells compared with DCs in live video-recording experiments. Thirty minutes before the experiment, Raji B cells were plated on glass coverslips mounted on 30-mm petri dishes and incubated at 37°C in presence of a mixture of SAg (recombinant staphylococcal enterotoxin E, staphylococcal enterotoxin A, staphylococcal enterotoxin B, and staphylococcal enterotoxin C3; 200 ng/ml each). Transfected T cells were then added to the APCs in a final volume of 200 µl. Transmitted light and GFP fluorescence images were acquired sequentially using a ×20 objective and an ×40 oil objective and the Metafluor imaging system (Universal Imaging). Videos were made with Premiere software (Adobe Systems).

Photobleaching measurements

T8.1 GFP-Akt-PH/L625.7 conjugates formed in presence of Ag (1 µg/ml peptide 110.434 restricted to HLA-DRB1*1102) were analyzed after 1 h of interaction. Fluorescence recovery after photobleaching (FRAP) analysis was conducted on a Leica TCS SP2 AOBS confocal laser-scanning microscope equipped with a thermostated chamber at 37°C, using the 488 nm line of a 20-mW argon laser and ×63 oil objective. Prebleaching and recovery images were collected at 208-ms time intervals scanning a single Z-section of the whole cell at 10% laser transmission. Photobleaching was conducted at 100% laser transmission for three iterations on a small membrane region at the rear of the cell. Quantitative analysis was performed on the resulting 12-bit images using MetaMorph Imaging software. The average fluorescence images were acquired sequentially using a ×20 or a ×40 oil objective and the Metafluor imaging system (Universal Imaging). Videos were made with Premiere software (Adobe Systems).
within a small membrane region outside the IS, at the rear of the cell, or within the IS. Between the bleaching periods, the cell was imaged with low intensity light. Practically, we recorded one 8-bit image every 2.012 s at 7% laser transmission, in turn with 1.006-s photobleaching pulses at 100% laser transmission. The Metamorph software was then used to measure the loss in fluorescence intensity as a function of time in different membrane regions of the T cell adjacent to the bleached one or in the whole T cell.

Results

PI3K recruitment to the IS induced by Ag recognition

The spatio-temporal localization of PI3K during T cell-Ag recognition and its relationship to the distribution of PtdIns(3,4,5)P3 outside the IS observed in living T cells interacting with APCs have not been previously examined. To assess PI3K localization during this process, we performed dynamic analyses using a GFP fusion protein containing p85α, the main regulatory subunit of the class IA PI3K expressed in T cells. In unstimulated human peripheral blood T cells, this probe has a diffuse cytoplasmic distribution, with some cells showing additionally a few hot spots of fluorescence at the plasma membrane (Fig. 1A). Major changes occurred in T cells contacting Ag-pulsed APCs. Fig. 1B shows a series of images extracted from a video (supplemental Video 1) in which an IS is formed between a T cell and an APC (in this case a Raji B cell). Rapidly, within a few seconds after the initial contact, the probe began to accumulate in the IS. In <1 min, GFP-p85α was largely removed from the cytoplasm. Identical results were observed with Ag-pulsed DCs as APCs. Fig. 1C shows several fixed T-DC conjugates analyzed after 20 min of interaction, illustrating the marked and exclusive redistribution of GFP-p85α to the IS, a situation found with almost all conjugates.

Sustained 3'-PI metabolism in T cells contacting DCs correlates with prolonged PI3K recruitment and phosphoryrosine accumulation at the IS

PI3K metabolism is activated for hours in murine T cells contacting APCs, as measured by GFP-Akt-PH accumulation at the plasma membrane (10, 11). Whether this biological phenomenon is also true for human T cells has not been examined. Mainly, its relationship with a prolonged PI3K recruitment to the IS is unknown. To explore this, resting human T cells were transfected with GFP-p85α and incubated several hours with Ag-pulsed DCs. The cellular localization of the probe was then examined on fixed conjugates. The same cells were analyzed for their pTyr contents. As shown in Fig. 2A, GFP-p85α was persistently recruited to the IS, and the probe never accumulated outside the contact zone. T cells with GFP-p85α relocalized to the IS were also strongly labeled by the pTyr Ab at the different time points studied. This pattern of costaining was observed in ~100% of cells showing a significant GFP-p85α relocalization at the synaptic region. Moreover, a high correlation between GFP-p85α and pTyr fluorescence levels was found (Fig. 2B), making clear the relationship between the two processes. This superimposed and restricted labeling at the synapse highly contrasts with the very homogeneous distribution of GFP-Akt-PH at the T cell plasma membrane within and outside the contact zone in these long-lasting conjugates (Fig. 2C).

Quantitative analyses also were conducted to follow the evolution of pTyr in T-DC synapses after various periods of incubation. The number of conjugates with increased pTyr was found to be rather stable (Fig. 3A). In these conjugates, pTyr decreased throughout the experiment, but never vanished (Fig. 3B). Similar results were obtained with the T8.1 hybridoma T cell interacting with MHC class II-expressing fibroblast APCs presenting a tetanus toxin peptide (not shown) and previously used to demonstrate sustained 3'-PI metabolism upon Ag recognition in T cells (10).

IS is the major site of 3'-PI production in T cells

As stated above (see introduction), T cells contain various PI3Ks that can be activated by phosphotyrosine-dependent and -independent mechanisms, and a deficiency in p110γ, the catalytic subunit of class IB PI3K activated by G protein-coupled receptors, has been shown to affect the proliferative response of murine T cells induced by anti-CD3 Ab (21). Thus, showing a parallel accumulation of pTyr-dependent class IA PI3Ks members such as p85α and pTyr within the IS does not exclude the recruitment of PI3Ks from other classes outside this particular location to activate 3’-PI synthesis. It has been reported that when overexpressed in cells, p85α inhibits pTyr-associated PI3K activity (22). One proposed mechanism is that the occupation of phosphorylation sites by the monomeric p85α form competitively limits interaction with the active heterodimer. We thus reasoned that if 3’-PI synthesis induced by Ag recognition originated from the synapse only, especially through the pTyr accumulated within this site, overexpression of p85α should inhibit the increase in 3’-PI in the whole cell. To evaluate this, we expressed a YFP-p85α molecule in resting T cells and analyzed the plasma membrane accumulation of a co-transfected CFP-Akt-PH probe after Ag recognition. Fig. 4 shows various 2-h conjugates from the same preparation with T cells expressing significant amounts of CFP-Akt-PH, but different levels of YFP-p85α. The results showed that translocation of Akt-PH to the plasma membrane was strongly impaired in the presence of a high level of YFP-p85α. This clearly suggests that the IS is the main, if not the exclusive, source of 3’-PI continuously synthesized through a pTyr-dependent process in T cells contacting APCs.

To support this finding, a different strategy was used based on photobleaching methodologies. We assumed that if PtdIns(3,4,5)P3, was primarily produced at the IS, its rapid diffusion from this place to the rest of the plasma membrane should be detectable. To explore this possibility, we first examined fluorescence recovery of membrane GFP-Akt-PH after photobleaching. Experiments were performed with T8.1 hybridoma T cells stably expressing GFP-Akt-PH (10), whose size makes laser beam focusing easier to perform than in resting T cells and also exhibits a sustained accumulation of GFP-Akt-PH in the entire plasma membrane (see Fig. 5A). After 1 h of interaction, several T8.1-APC conjugates were selected on which a small membrane region located at the rear of the T cell (outlined box in Fig. 5A) was photobleached for 624 ms, resulting in 70% probe fluorescence extinction. The average fluorescence intensity in the bleached area was then quantified on whole T cell images collected at different time intervals at low laser power. We observed that membrane fluorescence of GFP-Akt-PH was very rapidly restored after photobleaching with a time constant (τr) of 3.1 ± 0.80 s (n = 11; Fig. 5B). These data allow us to estimate a value for the PtdIns(3,4,5)P3 diffusion coefficient of ~0.92 ± 0.37 μm²/s, which is of the same order as the value recently determined using a different strategy in a fibroblast T cell model (0.37 ± 0.25 μm²/s) (23).

FLIP is the decrease/disappearance of fluorescence in a defined region adjacent to a repetitively bleached region in living cells; as a result, this technique can indicate whether a molecule is rapidly exchanged between separate cell compartments or various regions of a given compartment, like the plasma membrane. We therefore used this method to test whether labeled phospholipids diffusing through the bleached area in the FRAP experiments come from the pool accumulated at the IS. A typical experiment is shown in Fig. 5C, in which is shown a rapid darkening of the cell in and outside...
the IS by ~70 s of repeated bleaching of a small distinct membrane area (white box) at the rear of the cell. In different T cells making conjugates, fluorescence intensities were measured in separate regions of the cell membrane, including the synapse, while repetitively bleaching (Fig. 5D, left panel). As a control, membrane fluorescence was measured in unbleached cells of the same field. A strong decrease in fluorescence significantly greater than the normal signal attenuation due to the prolonged illumination of the preparation was observed. The delayed decrease in fluorescence in the region of the synapse at early time points during bleaching may be due to a rapid association of newly synthesized PtdIns(3,4,5)P₃ with some residual free cytoplasmic probe balancing the extinction rate of labeled PtdIns(3,4,5)P₃. These results indicate that GFP-Akt-PH is free to diffuse throughout the whole plasma membrane, including the IS. They also further demonstrate that a polarized production of PtdIns(3,4,5)P₃ in the IS can easily account for its accumulation outside. To investigate more directly the role played by the IS as a source of 3'-PI, we followed the whole cell FLIP while photobleaching a small area within or outside the synapse. The decrease in cell fluorescence was much more rapid and pronounced when the IS was bleached (Fig. 5D, right panel). These results are not formal proof that the IS is the main source of PtdIns(3,4,5)P₃, but collectively with our PI3K relocalization studies they strongly suggest that these phospholipids are produced only in this region of the cell contacting the APC.

Sustained activation of 3'-PI metabolism in T cells promotes prolonged nuclear exclusion of FoxO1

After conjugate formation, even after several hours of contact, PI3K inhibitors strongly alter Ag-induced T cell proliferation (11). Hence, we speculated that one function of the sustained 3'-PI production in T cells might be the prolonged control of one of the various targets downstream of PI3K that are thought to affect T cell cycle progression. In a variety of cell systems, Akt promotes the nuclear exclusion of FoxO proteins (20, 24, 25), a family of transcription factors whose activity in unstimulated cells promotes cell cycle arrest and apoptosis (26–29). In agreement, recent work has indicated that FoxO3a deficiency leads to spontaneous lymphoproliferation in murine T cells (30). FoxO1 is the most abundant FoxO family member in human T cells (31). We therefore thought it of particular interest to assess its cellular trafficking in resting T cells and its role in the context of synapse formation and sustained PI3K signaling after Ag recognition.

Experiments were performed with the wild-type molecule (FoxO1-GFP) and a constitutively active FoxO1 mutant (FoxO1 AAA-GFP), where three Akt-phosphorylation sites (Thr24, Ser256, and Ser319) were mutated into Ala. These sites are highly conserved in other FoxO proteins and are required for their nuclear exclusion and suppression of FoxO function (20). An empty GFP vector was used in parallel. Human resting T cells were transfected with the different constructs and controlled for similar GFP expression levels by FACS 24 h later (not shown). At this time, depending on the experiments, transfection efficiency was ~50–60% that of viable cells, but fluorescence quickly decreased (not

FIGURE 1. Synaptic recruitment of p85α PI3K after Ag recognition. A, GFP-p85α distribution in unstimulated human T cells. B, In a time-lapse imaging experiment, GFP-p85α-transfected resting human T cells were added to Raji B cells previously pulsed with SAg (0.2 μg/ml). Images were extracted from a video (supplemental Video 1) showing the formation of a typical conjugate. C, Differential interference contrast (DIC) and fluorescence images of GFP-p85α-transfected T cell-DC conjugates after 20 min of interaction. DCs were previously pulsed with SAg (0.2 μg/ml).
We first controlled, with annexin V, T cell apoptosis induced by FoxO1 overexpression. As shown in Fig. 6A, a small but significantly increased staining compared with the GFP empty vector was observed with the mutant and to a lesser extent with the wild-type FoxO1 molecule 36 h posttransfection. We next followed the impact of FoxO1 overexpression on CD69 expression, a usual parameter for monitoring activation of primary T cells early after Ag recognition (Fig. 6B). Only the mutant slightly impaired CD69 expression after 6 and 24 h of coculture with APCs, but not enough to suggest that the level of FoxO1 activity is critical in the initial steps of T cell activation. We then assessed the impact of FoxO1 overexpression on T cell growth induced by Ag recognition. The increase in T cell size was measured on GFP-positive and negative viable T cells within the forward and side scatter lymphocyte gate delineated on the whole cell population after a 2-day culture with APCs (Fig. 6C). The results showed that cells transfected with FoxO1 AAA-GFP were much smaller than FoxO1-GFP- or GFP-expressing cells. No such difference was seen within the GFP-negative cell populations. The nucleic acid content measured with an intercalating dye was also strongly reduced in cells expressing the constitutively active mutant (Fig. 6D), suggesting that the decrease in cell size is because FoxO1 AAA-GFP-expressing cells are in a different T cell cycle phase than those expressing empty GFP or FoxO1-GFP. Intriguingly, we repeatedly found a partial, but significant, alteration of these biological parameters in the fluorescent-negative cell population gated from FoxO1 AAA-GFP-transfected samples. This might be indicative of an indirect suppressive effect of FoxO1 AAA-expressing cells on the whole T cell population through an as yet unknown mechanism. However, we could not exclude that some cells initially transfected no longer expressed the transgene at this stage of the assay.

We next monitored the localization of FoxO1 in unstimulated T cells and in T cells contacting Ag-pulsed DCs. The predominant nuclear localization of FoxO1-GFP was obvious in unstimulated T cells (shown). We first controlled, with annexin V, T cell apoptosis induced by FoxO1 overexpression. As shown in Fig. 6A, a small but significantly increased staining compared with the GFP empty vector was observed with the mutant and to a lesser extent with the wild-type FoxO1 molecule 36 h posttransfection. We next followed the impact of FoxO1 overexpression on CD69 expression, a usual parameter for monitoring activation of primary T cells early after Ag recognition (Fig. 6B). Only the mutant slightly impaired CD69 expression after 6 and 24 h of coculture with APCs, but not enough to suggest that the level of FoxO1 activity is critical in the initial steps of T cell activation. We then assessed the impact of FoxO1 overexpression on T cell growth induced by Ag recognition. The increase in T cell size was measured on GFP-positive and negative viable T cells within the forward and side scatter lymphocyte gate delineated on the whole cell population after a 2-day culture with APCs (Fig. 6C). The results showed that cells transfected with FoxO1 AAA-GFP were much smaller than FoxO1-GFP- or GFP-expressing cells. No such difference was seen within the GFP-negative cell populations. The nucleic acid content measured with an intercalating dye was also strongly reduced in cells expressing the constitutively active mutant (Fig. 6D), suggesting that the decrease in cell size is because FoxO1 AAA-GFP-expressing cells are in a different T cell cycle phase than those expressing empty GFP or FoxO1-GFP. Intriguingly, we repeatedly found a partial, but significant, alteration of these biological parameters in the fluorescent-negative cell population gated from FoxO1 AAA-GFP-transfected samples. This might be indicative of an indirect suppressive effect of FoxO1 AAA-expressing cells on the whole T cell population through an as yet unknown mechanism. However, we could not exclude that some cells initially transfected no longer expressed the transgene at this stage of the assay.
cells (Fig. 7A). After Ag recognition, FoxO1-GFP was totally excluded from the nucleus, a feature found in ~80% of transfected T cells with extensive contacts with DCs. This is illustrated in Fig. 7B, left panel, which shows several T-DC conjugates analyzed after 20 min of interaction. It should be noted that many cells exhibited a higher accumulation in discrete regions of the cytoplasm, but not systematically and not always in the synaptic region. This nuclear exclusion was never observed with the FoxO1 AAA-GFP mutant that inhibits cell proliferation (Fig. 7B, right panel). Shown in Fig. 7C are sequential images extracted from a time-lapse experiment (supplemental Video 2), indicating additionally that translocation of FoxO1 is not immediate after the beginning of the contact, in contrast to the translocation of Akt, which usually starts concurrently with contact establishment (10). Moreover, it can be seen that the redistribution of FoxO1 is not polarized at any time point. Prolonged incubations were next performed to analyze the localization of FoxO1 after several hours of interaction between T cells and DCs. A similar translocation of FoxO1-GFP was observed, as illustrated in the conjugates shown in Fig. 7D, left panel, and also in the quantitative analysis in Fig. 7D, right panel, demonstrating that the very large proportion of T cells with nuclear exclusion of FoxO1-GFP was fully maintained over time. The PI3K inhibitor wortmannin added on preformed T-DC conjugates antagonized this sustained cytoplasmic retention of FoxO1 (Fig. 7E). Together, these findings demonstrate that in T cells contacting APCs, FoxO1 is strongly and enduringly unbalanced toward the cytoplasm downstream of PI3K and Akt.

FIGURE 3. Sustained pTyr levels at the synapse between human T cells and DCs. A, Quantitative analysis showing the percentage of T cell-DC conjugates with increased pTyr at the synapse relative to the total number of conjugates after different periods of interaction. Data represent the mean ± SD of three independent experiments with >100 cells analyzed in each. B, Distribution of pTyr levels in T cells interacting with DCs for different periods of incubation (n = 80). The pTyr level is shown as the average fluorescence intensity within the whole T cell, quantified using Metamorph software. Polynomial curves fitting these distributions also are shown.

FIGURE 4. p85α PI3-K overexpression inhibits 3'-PI synthesis. Human resting T cells were cotransfected with CFP-Akt-PH and YFP-p85α, and fluorescence was analyzed on fixed conjugates after 2 h of interaction with Raji B cells in the presence of SAg (0.2 μg/ml). Three typical conjugates from the same cell preparation with T cells expressing different levels of YFP-p85α are shown.

Discussion

Until recently, limited information has been available regarding the role of PI3K in T lymphocytes after IS formation. Recent studies using imaging approaches have revealed that this specific cell-to-cell interaction is a powerful trigger for PI3K activation (10, 11). These studies also revealed that, unlike other systems, continuous accumulation of 3'-PI is required for Ag-induced proliferation of T cells. Thus, understanding how this prolonged accumulation of PtdIns(3,4,5)P3 is implemented by the IS and how it can result in T cell proliferation is of critical importance.

3'-PI content increases immediately at the IS after Ag recognition in T cells contacting APCs (10, 11). Subsequently, 3'-PIs rapidly accumulate far beyond the contact zone. Because PI3K recruitment at the plasma membrane is essential to catalyze the phosphorylation of membrane phosphoinositides (8, 13), we examined the trafficking of one of its main regulatory subunits, p85α, during conjugate formation. Our results show that PI3K recruitment during T cell Ag recognition is a highly polarized process and is localized primarily to the region of the IS. This was found at the earliest time points of conjugate formation, in agreement with our previous observations that a GFP-Akt-PH probe begins to translocate to the T cell plasma membrane at the onset of the response in the small contact area of the T cell with the APC (10). In this study we found that p85α remained strictly concentrated in this membrane region when the contact continues. A role for the IS in maintaining the 3'-PI signal for hours in murine T cells has been reported (11). Continuous activation is probably involved, because destabilizing preformed T cell-APC conjugates with an MHC class II-peptide specific Ab reverses the membrane localization of GFP-Akt PH (15). We found that persistent activation also takes place at the T-APC synapse in human T cells, illustrated by the sustained increase in pTyr that occurs at the contact zone. Although the specific mechanisms accounting for this accumulation of pTyr remain to be clarified, this is likely to explain the prolonged localization of GFP-p85α at this site. It is worth noting that incubating...
preformed conjugates with the Src kinase inhibitor PP2 efficiently reversed both pTyr labeling and GFP-Akt-PH translocation (not shown). We do not believe that the SAg presentation to human T cells used in our study explains this continued signaling, because sustained pTyr has already been shown in a human CD4⁺ T cell clone activated by a nominal Ag (32). Moreover, increased pTyr and 3'-Pi lipid synthesis are seen with the T8.1 hybridoma murine T cell model specific for a given peptide (not shown). This suggests that human and murine T cells may use similar signaling strategies to maintain sustained changes in 3'-Pi metabolism. In this case, one should expect to find persistent changes pTyr accumulation in the two separate murine TCR transgenic models in which sustained PI3K activation has been observed (11, 15). This will require additional experiments, but it will be interesting to test this hypothesis with various agonist peptide systems, because pTyr increased only transiently in another TCR transgenic T cell model (33).

FIGURE 5. Rapid diffusion of 3'-Pi from the synapse in the whole plasma membrane. A, Representative images showing a typical FRAP experiment on GFP-Akt-PH in T8.1 murine T cells after 1 h of contact with L625.7 fibroblasts loaded with specific Ag. The T cell was imaged at regular intervals before and after a 624-ms bleach pulse at maximum laser power focused on a small region at the rear of the cell (outlined box). t = 0 s corresponds to the beginning of the recovery period. B, Fluorescence intensity, normalized as a percentage of the initial fluorescence, from quantitative FRAP analysis was plotted against time. Mean values for 11 representative cells from two independent experiments are shown. C, FLIP experiments were performed on GFP-Akt-PH T8.1 cells after 1 h of contact with Ag-loaded L625.7 fibroblasts. Cells were repeatedly bleached in the indicated zone (white box) and imaged between consecutive bleach pulses. A detail of the bleached area with higher magnification is shown in the right corner of each panel. D, Left panel, Time course of mean fluorescence intensities measured in eight separate cells in bleached areas and in regions of equal area located within the synapse and on the side of the T cells, as illustrated by the blue and red boxes in C. Fluorescence in neighboring unbleached cells was plotted as a control. Right panel, In this representative experiment, independent cells with similar fluorescence levels and contacting L625.7 APCs were repeatedly photobleached within a small membrane region outside the IS, at the rear of the cell, or within the IS. The loss of fluorescence intensity as a function of time in the whole T cell was then plotted. In the two panels, fluorescence intensities were normalized as a percentage of the initial fluorescence.
activation (34, 35), and a compensatory role for p85β has been discussed (36). Although our results do not resolve this issue, the finding that competing for PI3K pTyr-binding sites within the IS inhibits GFP-Akt-PH membrane recruitment (Fig. 4) supports the concept that the binding of class IA PI3K to pTyr contained within the IS plays an important role in the activation of 3'-PI metabolism in T cells. Together with the photobleaching data, our experiments indicate that the broad distribution of GFP-Akt-PH in the whole plasma membrane is due to continuous spreading of PtdIns(3,4,5)P₃ out of the IS, the major site of PtdIns(3,4,5)P₃ production in T cells contacting APCs. This conclusion is supported by the disappearance of membrane GFP-Akt-PH in the whole cell after a few photobleaching iterations given within the synapse (Fig. 5D).

Maintaining PI3K in an active state is required to induce T cell proliferation in response to Ag stimulation (11). Several 3'-PI-dependent pathways that control cell division have been described.
FIGURE 7. Ag-induced, PI3K-dependent, nuclear exclusion of FoxO1 in T cells. A, FoxO1-GFP distribution in resting human T cells. B, Distribution of FoxO1-GFP or FoxO1 AAA-GFP mutant in human T cells after 30 min of interaction with SAg (0.2 μg/ml)-pulsed DCs. C, Left, In a time-lapse imaging experiment, FoxO1-GFP-transfected cells were added to SAg-pulsed (0.2 μg/ml) Raji B cells. Images were extracted from a video (supplemental Video 2) showing the formation of a typical conjugate. Right, Fluorescence intensities across the black line (shown in the corresponding images) are plotted as line intensity histograms after two-dimensional deconvolution on 12-bit images. D, Left, DIC and FoxO1-GFP fluorescence images of T-DC conjugates after 4 h of interaction. Right, Quantitative analysis showing the percentage of T cells in conjugates with a cytoplasmic or a nuclear localization of FoxO1-GFP after different periods of incubation. Results are for >100 cells analyzed from randomly acquired fields. E, Left, DIC and FoxO1-GFP fluorescence images of Ag-induced T-DC conjugates after 4 h of interaction. Wortmannin (100 nM) was added during the last 2 h of contact. Right, Quantitative analysis showing the percentage of T cells with a cytoplasmic or a nuclear localization of FoxO1-GFP with or without wortmannin. Results are shown for >100 cells in each group analyzed from randomly acquired fields.
Recruitment and activation of PI3K, thereby suppressing the function of FoxO proteins, resulting in T cell proliferation. Interestingly, it has recently been reported that sustained Akt signaling induced by late-acting and inducible costimulatory molecules also regulates long term T cell survival for several days after Ag encounter (47). Thus, the need for sustained TCR and/or costimulatory signaling(s) is probably a straightforward means used by T cells and APCs to maintain continuous activation of PI3K-dependent metabolic cascades to shape the different steps of the immune response. 

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Disclosures

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References


