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The T cell antigen receptor activates phosphatidylinositol 3-kinase-regulated serine kinases protein kinase B and ribosomal S6 kinase 1

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Abstract The present study has explored T cell antigen receptor-regulated serine kinases in human T cells. The results identify two phosphatidylinositol 3-kinase (PI3K)-controlled serine kinases operating downstream of the T cell receptor (TCR) in primary T cells: (i) protein kinase B whose activation regulates the phosphorylation of glycogen synthase kinase 3 and (ii) ribosomal S6 kinase 1, a kinase with a critical role in the regulation of protein synthesis and cell growth. T cells express two isoforms of S6k1: a 70 kDa cytoplasmic kinase and an 85 kDa isoform that has a classic nuclear localisation. TCR ligation triggers a parallel engagement of both the 70 and 85 kDa isoforms of S6k1 in a response that requires PI3K function.

Key words: T cell receptor; Phosphatidylinositol 3-kinase; Protein kinase B; Ribosomal S6 kinase 1; Glycogen synthase kinase-3

1. Introduction

The T cell antigen receptor (TCR) coordinates activation of T cells in response to foreign antigen. It initiates intracellular signalling by recruiting and activating protein tyrosine kinases of the Src, ZAP70/Syk and Tec families [1,2]. TCR triggering regulates inositol lipid turnover and induces an elevation in intracellular concentrations of the products of phosphatidylinositol 3-kinase (PI3K), PI(3,4,5)P3 and PI(3,4P)2 [3]. The biochemistry of the mechanisms that link the TCR to PI3Ks has been the subject of investigation [4,5] but the downstream targets for the lipid products of PI3K in TCR signalling are not fully understood. Nevertheless, experiments with PI3K inhibitors indicate that PI3K-regulated signalling pathways are important for lymphocyte activation [6]. Targets for the binding of PI(3,4,5)P3 are Tec family tyrosine kinases and guanine nucleotide exchange proteins such as Vav-1 and Vav-2 [7]. There are also two serine/threonine kinases that bind PI(3,4,5)P3; the phosphoinositide-dependent protein kinase PDK-1 and protein kinase B (PKB) [8–9]. PDK1 phosphorylates key residues in the activation loops of several serine kinases including members of the protein kinase C superfamily of kinases PKC α and ζ, the ribosomal S6 kinase (S6k), S6k1 and the serine/threonine kinase PKB [10–12].

We have previously shown that ligation of the TCR complex can modulate PKB activity in the T leukaemic cell line Jurkat [13–15]. However, Jurkat cells do not express the phosphoinositide lipid 3-phosphatase PTEN and consequently they have high constitutive levels of $PI(3,4,5)P_3$ and constitutive basal activation of PI3K-dependent downstream effectors [16]. The key questions are whether TCR ligation can regulate the activity of two major PI3K-regulated serine kinase PKB and p70 S6k1 in primary T cells. The relevance of this question stems from the many significant functions ascribed to PKB and S6k1 and the importance of understanding exactly when these kinases are switched on during the life span of a T lymphocyte: for example, S6K1 is important for the regulation of protein synthesis and the control of cell growth [17,18]; PKB has been shown to play an important role in the maintenance of cell survival [19,20]. Moreover, PKB can stimulate the activity of necrosis factor κB (NFκB) transcription factors and E2F transcription factors [21,22]; the latter are important components of the mechanisms that control the mammalian cell cycle. PKB also phosphorylates and inactivates glycogen synthase kinase-3 (GSK3) [23] an enzyme which promotes the nuclear export of the nuclear factor of activated T cells (NFAT) [24], a transcription factor involved in cytokine gene induction.

In the present study we explore the ability of the TCR complex to regulate PKB and p70 S6k activity in human peripheral blood-derived T lymphocytes. The data show that ligation of the TCR induces a rapid and strong activation of PKB and a slower but strong activation of p70 S6k. These responses are PI3K-dependent revealing that PI3K couples the TCR to two biologically important serine kinases.

2. Materials and methods

2.1. Chemicals and reagents

Recombinant IL-2 was from Chiron, Emeryville, CA, USA, LY294002 from Calbiochem, Nottingham, UK, ERK2 antibodies from Transduction laboratories, UK, anti-phosphoserine 473 PKB and anti-PKB from New England Biolabs, Beverly, MA, USA. Anti-phospho GSK3α and anti-pan GSK3 were from UBI, Lake Placid, NY, USA. Rapamycin and p70S6 kinase 1 antibodies were a generous gift from G. Thomas. Horseradish peroxidase-conjugated

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Abbreviations: TCR, T cell receptor; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; S6k, ribosomal S6 kinase; GSK3, glycogen synthase kinase-3; NFAT, nuclear factor of activated T cells

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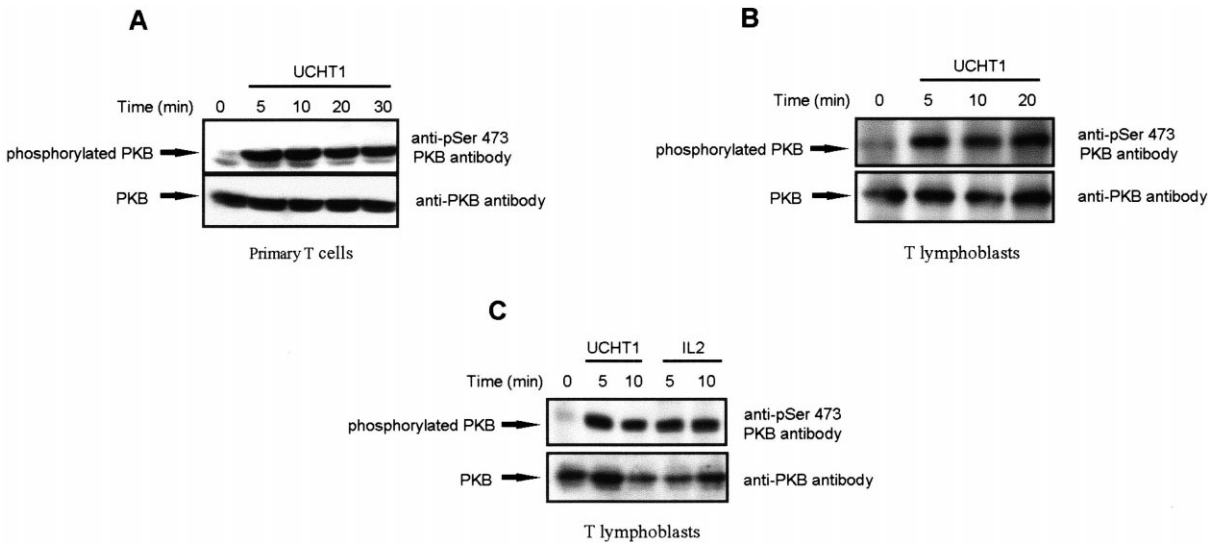

Fig. 1. The TCR activates PKB. Human primary T cells (A) and quiescent human T lymphoblasts (B,C) were stimulated or not with the anti-CD3 antibody (UCHT1, 10 μg/ml) or rIL-2 (20 ng/ml) for the indicated times. Proteins from total cell lysates were separated by 7.5% SDS–PAGE and revealed by Western blot analysis using a specific phosphoserine (Ser473) PKB antibody and a pan PKB antibody.

anti-mouse Ab and anti-rabbit Ab were from Amersham (Paris, France).

2.2. Cell culture and stimulation

T lymphocytes were prepared from peripheral blood mononuclear cells isolated from healthy donors. Human T lymphoblasts were generated as described [5] and maintained in RPMI 1640 supplemented with 10% foetal calf serum, 2 mM glutamine and IL-2 (20 ng/ml) at 37°C and 5% CO_2. T lymphoblasts were quiesced by washing three times, and replacing in RPMI 1640 with 10% serum in the absence of IL-2 for 48 h. The inhibitors LY294002 (5 μM) and rapamycin (20 ng/ml) were pre-incubated with cells for 30 min prior to TCR or IL-2 stimulation. Human T lymphocytes were stimulated via the TCR complex by treatment with 10 μg/ml of the CD3 antibody UCHT1 and by 20 ng/ml of IL-2 via the IL-2 receptor at the indicated times.

2.3. Cell extract preparation and Western blot analysis

After stimulation, cells were lysed (5×10^6 per ml) in lysing buffer described in [26]. Proteins were separated by SDS–PAGE using the following gel conditions: for PKB and GSK3, 7.5% acrylamide/0.2% bis-acrylamide; for S6 kinase, 10% acrylamide/0.1% bis-acrylamide. Proteins were transferred to PVDF membranes (Millipore) and detected by Western blot with the indicated antibodies (utilised dilutions following manufacturer's instructions for phospho PKB (1:1000), pan PKB (1:1000), phospho GSK3α (1:1000), pan GSK3 (1:1000), S6 kinase antibody (1:5000). Immunoreactive bands were visualised with the chemiluminescence Western blotting system (Amersham).

3. Results

3.1. TCR triggering activates PKB and regulates serine phosphorylation of the PKB substrate GSK3

Stimulation of PKB catalytic function requires its phosphorylation on threonine 308 and serine 473, a response mediated by a PI3K-dependent protein kinase PDK1. To examine the effects of triggering the TCR complex on the activity of PKB, primary human T lymphocytes were activated with the anti-CD3 antibody UCHT1 at different times. Total cell extracts were fractionated by SDS–PAGE and processed for Western blot analysis with a specific antisera that recognises active PKB molecules phosphorylated on serine 473. The anti-phospho PKB antibody did not react with PKB present in cell lysates from quiescent human primary T cells whereas it strongly reacted with those from cells activated by crosslinking the TCR with UCHT1 (Fig. 1A). Maximal stimulation of PKB phosphorylation was observed within 5 min of stimulation and was sustained by 30 min. A similar pattern of TCR activation of PKB was seen in quiescent human peripheral blood-derived T lymphocytes (Fig. 1B).

Previous studies have shown that PKB is activated by IL-2. We wished therefore to compare the magnitude of TCR activation of PKB in human T cells with the response to IL-2. Since primary T cells do not express IL-2 receptors, we used IL-2 receptor positive human peripheral blood-derived T lymphocytes, quiesced by cytokine deprivation and that were exposed to either IL-2 to ligate IL-2 receptor or UCHT1 to trigger the TCR complex. The experiment in Fig. 1C shows that both the TCR and IL-2 can activate PKB and moreover, the PKB response initiated by IL-2 or the TCR in human T cells are comparable.

3.2. Antigen receptor regulation of PKB is regulated by PI3K

To assess the involvement of PI3K in cellular responses we used pharmacological inhibitors of this enzyme. The data in Fig. 2A show that the PI3K inhibitor LY294002 prevents both the immediate and sustained PKB activation response seen in TCR activated cells. An endogenous substrate for PKB is serine 21 in GSK3α [23]. GSK3 proteins isolated from quiescent T cells are not reactive with an anti-Ser21 phospho specific antibody but are strongly reactive with the GSK3α present in cell lysates prepared from TCR triggered cells or from cells activated with IL-2 (Fig. 2B). LY294002 prevented TCR-induced phosphorylation of the PKB substrate GSK3α (Fig. 2B). Inhibition of PI3K also prevents IL-2-induced phosphorylation of the PKB substrate GSK3α In further experiments we explored the selectivity of LY294002 on the activation of serine kinases by antigen receptors. Antigen receptors regulate the activity of diacylglycerol-regulated kinases of the protein kinase C superfamily. In particular, a major target for antigen receptor signals is the kinase PKD/PKCμ whose activity can be readily monitored by Western blot analysis with an antibody specific for phos-

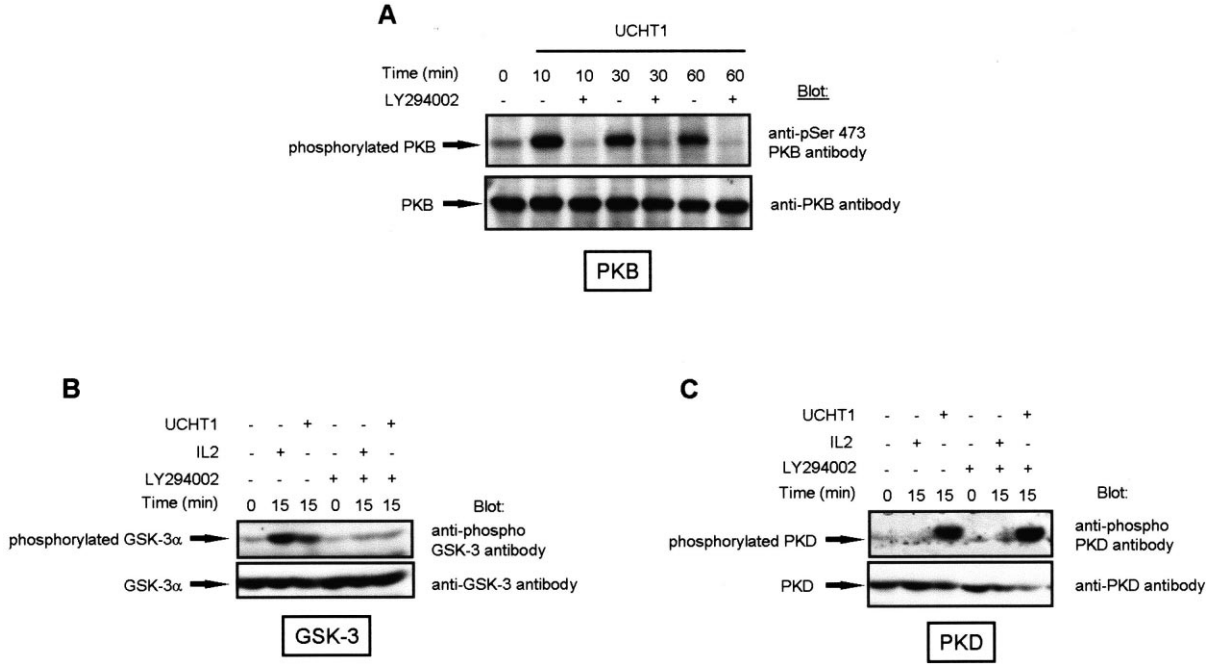

Fig. 2. PI3K inhibitors selectively block TCR activation of PKB and GSK-3α. Quiescent T blasts were stimulated or not with UCHT1 (10 μg/ml) or rIL-2 (20 ng/ml) for the indicated times following a 30-min pre-incubation in the presence or absence of PI-3 kinase inhibitor (LY294002, 5 μM) as indicated. Proteins from total cell lysates were separated on 7.5% SDS–PAGE. Blots were revealed by Western blot analysis using a specific phosphoserine (Ser473) PKB antibody and an anti-PKB antibody (A), a phosphoserine (Ser21) GSK-3α antibody and a pan GSK-3 antibody (B) and a phosphoserine (Ser916) PKD antibody and a pan PKD antibody (C).

phoserine 916 an autophosphorylation site in the carboxyterminus of this enzyme [25]. The data in Fig. 2C show that LY294002 does not prevent TCR activation of PKD.

3.3. Antigen receptor regulation of S6k1

PKB activation is mediated by the PI3K-dependent protein kinase PDK1. It was previously shown that this kinase also regulates the activity of S6k1 because it phosphorylates the critical residue serine 229 in the activation loop of the catalytic domain of the enzyme [12]. Phosphorylated and active p70 S6k1 has reduced electrophoretic mobility in SDS–PAGE gels: the inactive enzyme in T cells typically migrates as a doublet that is clearly distinct from the hyperphosphorylated active enzyme [26]. The data in Fig. 3A show that in quiescent T cells, p70S6k1 migrates predominantly as a doublet whereas in TCR activated cells four discrete phosphoforms of the enzyme can be readily discerned within 10 min of TCR ligation. TCR activation of p70S6k1 can be seen within 5 min of TCR engagement, is maximal within 10 min and is dependent on PI3K activity since it is abrogated when T cells are treated

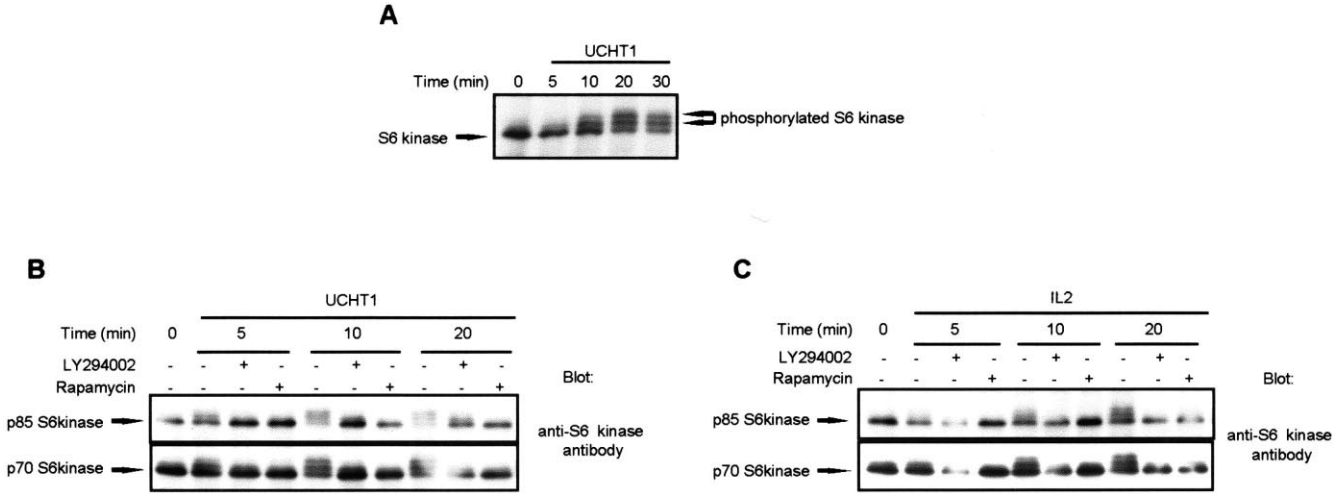

Fig. 3. The TCR regulates S6K1. Effect of pretreatment of T blasts with LY294002 or rapamycin compounds on the activation S6K1 induced by TCR crosslinking or rIL-2. Quiescent T blasts were stimulated or not with UCHT1 (10 μg/ml) (A and B) or rIL-2 (20 ng/ml) (C) for the indicated times following a 30-min preincubation in the presence or absence of LY294002 or rapamycin (20 ng/ml) as indicated. Proteins from total cell lysates were separated by SDS–PAGE (10% acrylamide/0.1% bis) to resolve the different S6k1 isoforms. The blot was revealed by Western blot analysis using specific S6k1 antisera.

with the PI3K inhibitor LY294002 (Fig. 3B). In T lymphocytes, p70S6k1 is typically thought of as an IL-2 stimulated serine kinase [27]. In the current experiment the ability of TCR ligation to activate p70S6k1 was compared with the cellular response to IL-2. The results in Fig. 3C show that the kinetics of the IL-2/S6k1 response is slightly slower than the TCR response but this is a subtle effect: the TCR response can be easily seen in cells activated for 5 min whereas 10 min is required to readily detect IL-2 activation of this enzyme. The key point is that the magnitude of the TCR/S6k1 response is comparable to the response induced by IL-2.

S6k1 is one of the major cellular targets for the immunosuppressant rapamycin and it was recognised several years ago that IL-2 activation of p70S6k was blocked by rapamycin [28]. Rapamycin is a drug that blocks T cell activation and clonal expansion in vivo and is used therapeutically as an immunosuppressant following organ transplantation. The data in Fig. 3B show that TCR activation of p70S6k1 is inhibited by rapamycin. The S6k1 antisera recognises two isoforms of S6k1; one migrating at 70 kDa and the other migrating at 85 kDa. The 85 kDa isoform of S6k1 is a splice variant that has a classic nuclear localisation sequence that restricts its expression to the nuclear compartment. Interestingly, the data in Fig. 3A show that TCR engagement triggers a parallel engagement of both the cytosolic p70 and the nuclear p85 S6k1 isoforms in T cells. Moreover stimulation of both isoforms is sensitive to inhibition by the PI3K inhibitor Ly294002 and is sensitive to rapamycin.

4. Discussion

The present study has explored the ability of the T cell antigen receptor to regulate the activity of two PI3K-controlled serine kinases PKB/Akt and S6k1. Biochemical links between the TCR and PI3K have been recognised for many years [4–6] but downstream targets for PI3K in the context of antigen receptor signalling in T cells have not been well defined. Importantly the present experiments have been performed in primary peripheral blood-derived human T cells rather than in transformed cell lines. The data show that triggering of the TCR complex causes a rapid and sustained activation of AKT/PKB and S6k1. These responses were dependent on the activity of PI3K. The present data show also that the TCR can induce phosphorylation on serine 21 of GSK3α, an endogenous substrate of PKB. The phosphorylation of GSK3 on serine 21 inactivates this enzyme. GSK3 was identified initially as a regulator of glycogen metabolism but it is now recognised that it has broader functions in the control of transcription factors: interactions between GSK3 and β-catenin regulate activity of the T cell factor-lymphoid enhancer factor (Tcf-Lef) [29]. GSK3 can also control the nuclear export of NFAT; transcription factors involved in cytokine gene induction [24]. The phosphorylation and inactivation of GSK3 mediated by the TCR/PKB axis would favour nuclear retention of NFAT in the nucleus and increase its transcriptional activity.

The second PI3K-regulated serine kinase shown herein to be activated by the TCR is S6k1. One interesting feature about TCR regulation of S6k1 is that TCR engagement triggers a parallel engagement of both the 70 kDa and 85 kDa isoforms of this enzyme. The 85 kDa isoform of S6k1 has a classic nuclear localisation sequence restricting its expression to the nuclear compartment [30]. PI3K-dependent activation of p85 S6k1 by the TCR is thus a clear indication that PI3K can couple the TCR to serine kinases in the nucleus. S6 kinases are well documented as targets for cytokine signalling in lymphocytes but have not been recognised as a target for antigen receptor signalling possibly because many studies of TCR signal transduction are performed on transformed cell lines either T leukaemic cells lines or T cell hybridomas. These are invaluable models for antigen receptor regulation of tyrosine kinases but they cause problems in the analysis of S6k regulation. For example, Jurkat cells one of the most frequently used model cell systems for exploration of TCR signal transduction have basely high constitutively active S6k that cannot be further activated by any exogenous stimuli. The functional relevance of S6k1 regulation by the TCR stems from the critical role that this kinase has in the regulation of protein synthesis and cell growth. For example, S6k1 has been shown to regulate the activity of E2F transcription factors in T cells [26]. E2Fs regulation play an important role in controlling ordered progression through the cell cycle. The role of S6k as an essential regulator of cell growth and cell size has been well documented in lower eukaryotes such as *Drosophila* [18]. Primary T cells are extremely small, metabolically inactive cells and an increase in protein synthesis and protein turnover is essential for the cell growth that enables T cells to reach the necessary size for optimum function and proliferation. The ability of the TCR to activate S6k1 shows that an evolutionarily conserved pathway for the regulation of cell growth is under the control of the specific immune system in T lymphocytes. S6k1 control enables the antigen receptor to initiate the changes in protein synthesis and cell growth that will allow the T cell to develop its functional programme.

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