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Antigen receptor regulation of phosphoinositide-dependent kinase 1 pathways during thymocyte development

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Abstract Phosphoinositide-dependent kinase 1 (PDK1) is essential for T cell development but little is know about the stimuli that regulate PDK1 signaling in vivo. The thymus contains a heterogeneous mixture of cells at different stages of development making it difficult to use biochemical techniques to examine the activity of PDK1 pathways as thymocytes develop in situ. Herein, we use a single cell assay to quantify activation of the PDK1 target kinase ribosomal S6 kinase 1 (S6K1) in different murine thymocyte subsets immediately ex vivo. This technique allows an assessment of S6K1 activation as thymocytes respond to developmental stimuli in vivo. These studies reveal that only a small percentage of thymocytes show evidence for activation of PDK1 mediated signaling in situ. The thymic subpopulations that contain active PDK1/S6K1 are those known to be responding to signaling by the pre T cell receptor and the mature alpha/beta T cell antigen receptor (TCR). Moreover, loss of antigen receptor signaling in T cell progenitors that cannot rearrange their TCR beta locus prevents in vivo activation of S6K1. The present data identifying antigen receptor signaling as a key activator of PDK1 mediated signaling afford a molecular explanation for the important role of this molecule in T cells.

Keywords: Thymocyte; Kinase; Signal transduction; T cell receptor; Pre T cell receptor; PDK1; S6K1; Thymus; TCR signaling

1. Introduction

T lymphocyte differentiation, proliferation and maturation in the thymus are fundamental processes in the formation of the adaptive immune system. Thymocyte development is controlled by cytokines, chemokines, the pre T cell antigen receptor (preTCR) and the mature T cell antigen receptor (TCR) complex which engage signal transduction pathways mediated by tyrosine kinases, adapters and GTPases [1–4]. Serine kinases are also crucial for T cell development as judged by the impact of deleting phosphoinositide-dependent kinase 1 (PDK1) in T cell precursors [5]. PDK1 phosphorylates a key 'T' loop site within the catalytic domain of AGC kinases including diacylglycerol regulated kinases of the protein kinase C (PKC) family and phosphatidyl inositol-3 kinase (PI3K)-controlled serine kinases such as Akt (protein kinase B, PKB) and the 70 kDa ribosomal S6 kinase 1 (S6K1) [6,7]. A key step in T cell development is characterised by the co-expression of the receptors for major histocompatibility complex (MHC) molecules, CD4 and CD8. Conditional gene deletion of PDK1 in T cell precursors prevents T cell differentiation to the CD4/8 double positive stage of T cell development and also has a role in regulating the size or mass of T cell progenitors [5].

Despite the importance of PDK1 in the thymus, nothing is known about the regulation of PDK1 signaling during thymocyte development. In this respect, in vitro studies in mature peripheral T cells have revealed that PDK1-mediated signal transduction pathways can be triggered by a variety of stimuli including antigen receptors and costimulators, as well as chemokines and cytokines [8–10]. Although thymocyte development is regulated by the antigen receptor and signals from stromal cells stimulated via Notch and/or cytokines such as Interleukin 7 (IL-7) [11–13], little is known about the relative contribution of these signals to the activation of PDK1-mediated pathways in thymocytes in vivo. However, it has recently been reported that expression of a constitutively active PKB can overcome the requirement for Notch in the survival and differentiation signals associated with β-selection [14].

The thymus contains a heterogeneous mixture of cells at different stages of development and thus the analysis of signal transduction pathways induced in situ in these different subsets requires a sensitive and quantitative single cell assay. One such assay has been described that uses flow cytometry to quantify phosphorylation of the ribosomal S6 subunit on S235/236 in single cells [5]. The phosphorylation of S6 is mediated by S6K1 which must itself be phosphorylated by PDK1 at its T loop site to become activated [15–17]. S6K1 has an additional requirement for PDK1 function as its activation is dependent on PKB, another PDK1 substrate, that regulates S6K1 via modulation of Tsc-1/2 function [18–20]. S6 phosphorylation thus requires the coordinate activation of two PDK1 controlled serine kinases and provides a very sensitive measure for PDK1 function because it quantifies phosphorylation of a downstream target of this pathway at a point where there has been considerable signal amplification. Importantly, the ability of S6 phosphorylation to report PDK1 activity has been verified in PDK1 null embryonic stem cells and thymocytes [5,16].

The present results establish that that there is dynamic regulation of S6 phosphorylation in developing thymocytes and that the preTCR and the mature TCR are potent in vivo regulators of this signal response during thymocyte development.

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2. Results

2.1. Activation of PDK1/S6K1signaling by the preTCR

The progression of T cells through the different stages of intrathymic differentiation may be tracked by the sequential pattern of expression of a number of surface markers including CD44, CD25, CD4 and CD8 [2,21]. Early T cell progenitors are negative for both CD4 and CD8 (termed double negative, DN). The earliest of these DN thymocytes are $CD44^{+}CD25^{-}$ (termed DN1); they then go on to acquire CD25 (DN2) where they become committed to the T-cell lineage and begin to rearrange T-cell receptor β-loci. The cells then downregulate the expression of CD44 to become DN3 thymocytes and continue β-chain rearrangements to completion. At the DN3 pre-T cell stage, cells that have successfully rearranged their TCRβ-locus express a functional receptor complex known as the preTCR complex, which comprises the preTCRα chain, the TCRβ subunit and the CD3 antigen. Relocation of the preTCR to the plasma membrane induces DN3s to proliferate rapidly, downregulate CD25 and differentiate into DN4 cells [22].

To explore the role of the preTCR in regulating PDK1 signaling a flow cytometric based assay was used to quantify S6 phosphorylation in ex vivo DN3 and DN4 thymocytes from wild type C57/B6 mice. Immediately ex vivo DN3 cells were heterogeneous for phosphoS6, the majority of cells were phosphoS6^{low} but a significant percentage of cells, approximately 20%, were phosphoS6^{high}. DN4 thymocytes were primarily phosphoS6^{high} (Fig. 1a). Treatment of cells with rapamycin, which inhibits the activity of mTOR, is known to rapidly reverse S6 phosphorylation. The data in Fig. 1a show that phosphoS6 staining in DN3 and DN4 thymocytes was lost when cells were treated with rapamycin. To estimate the maximal potential for S6 phosphorylation, thymocytes were activated pharmacologically with phorbol 12,13-dibutyrate (PDBu), which activates S6K1 via stimulation of Protein Kinase C serine kinases. Stimulation for 30 min with PDBu induced S6 phosphorylation in the phosphoS6^{low} population (Fig. 1a). Moreover, levels of S6 phosphorylation in phosphoS6^{high} cells were similar to those in PDBu activated cells. The requirement of PDK1 for S6K1 activation has been reported previously [5,16]. Fig. 1b shows that DN4 thymocytes from mice with a T cell specific gene deletion of PDK1($LckCre^{+}Pdpk1^{-/-}$) had no basal level of S6 phosphorylation, although these cells were able to respond to pharmacological stimuli and induce S6 phosphorylation in response to PDBu.

The DN3 thymocyte subpopulation can be subdivided into cells that have not yet completed TCRβ locus rearrangements and those that express a functional TCRβ subunit that allows surface expression and signaling of the preTCR complex [23]. Only cells with a functional preTCR transit to the DN4 stage. To examine if preTCR signaling plays a role in regulating S6 phosphorylation in DN3 thymocytes we determined if those DN3 cells with high phosphoS6 correspond to those that express TCRβ subunits. The most sensitive way to assess TCRβ expression in DN subpopulations is to use intracellular (ic) staining protocols. Analysis of icTCRβ expression revealed a small but discernable subpopulation of DN3 cells that expressed icTCRβ, while the majority of DN4 cells expressed icTCRβ (Fig. 2a). Simultaneous analysis of icTCRβ expression and S6 phosphorylation showed that DN3 thymocytes that express icTCRβ were generally phosphoS6^{high} whereas DN3 thymocytes that were icTCRβ null were uniformly phosphoS6^{low} (Fig. 2b). Thus there was a correlation between the expression of the TCRβ and the induction of S6 phosphorylation. To test

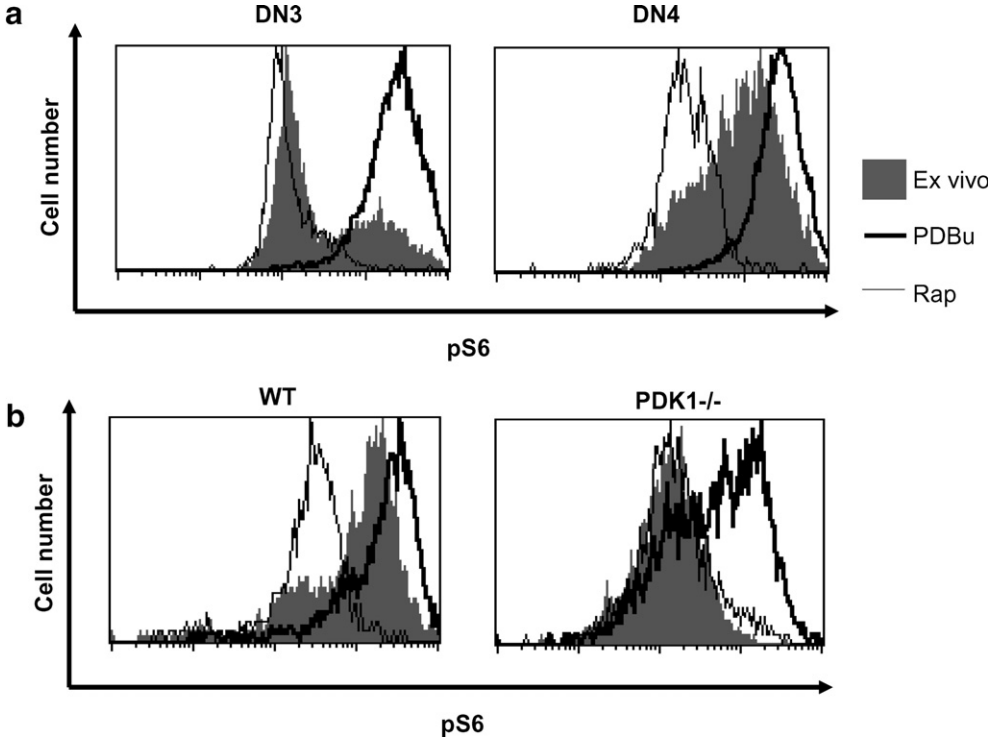

Fig. 1. S6 phosphorylation in DN subsets. Histograms show S6 phosphorylation in (a) WT DN3 (left) and DN4 (right) thymocytes and (b) DN4 thymocytes from WT (left) and $LckCre^{+}Pdpk1^{-/-}$ (right) mice. S6 phosphorylation was measured either immediately ex vivo (filled) or after treatment for 30 min with 20 nM Rapamycin (fine line) or 4 nM PDBu (bold line).