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Research paper

Type II phosphatidylinositol 4-kinase β is an integral signaling component of early T cell activation mechanisms

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ABSTRACT

The early signaling events in T cell activation through CD3 receptor include a rapid change in intra cellular free calcium concentration and reorganization of actin cytoskeleton. Phosphatidylinositol 4-kinases (PtdIns 4-kinases) are implicated as key components in these early signaling events. The role of type II PtdIns 4-kinase β in CD3 receptor signaling was investigated with the help of short hairpin RNA sequences. Cross-linking of CD3 receptors on Jurkat T Cells with monoclonal antibodies showed an early increase in type II PtdIns 4-kinase activity and co-localization of type II PtdIns 4-kinase β with CD3 ζ. Transfection of Jurkat T Cells with shRNAs inhibited CD3 receptor mediated type II PtdIns 4-kinase activation with a concomitant reduction in intra cellular calcium release, suggesting a role for type II PtdIns 4-kinase β in CD3 receptor signal transduction. Knock-down of type II PtdIns 4-kinase β with shRNAs also correlated with a decrease in PtdIns 4-kinase activity in cytoskeleton fractions and reduced adhesion to matrigel surfaces. These results indicate that type II PtdIns 4-kinase β is a key component in early T cell activation signaling cascades.

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1. Introduction

Occupancy of T cell receptor with MHC–peptide complex leads to rapid turn over of phosphatidylinositol 4,5 bis phosphate (PtdIns 4,5P_2) pools and a concomitant release of intra cellular free calcium concentration [1–5]. In addition to their role in calcium signaling, PtdIns 4,5P_2 pools are shown to regulate actin polymerization [6–8] and there by suggested to play a role in T cell adhesion, motility and immune synapse formation. Following hydrolysis, receptor sensitive PtdIns 4,5P_2 pool is rapidly resynthesized by sequential phosphorylation of phosphatidylinositol by PtdIns 4-kinases and PtdIns 4*P* 5-kinases [9–12]. Presence of multiple forms of PtdIns 4-kinases (type II PtdIns 4-kinase α and β, type III PtdIns 4-kinase α and β) and lack of selective inhibitors to these molecular forms make it difficult to assess the individual contribution of these species in T cell activation mechanisms. While type III PtdIns 4-kinases are suggested to play a role in Golgi functions and vesicular trafficking [13–16] very little is known about physiological roles of type II PtdIns 4-kinases. A few reports suggest that immunoinhibition of type II PtdIns 4-kinases led to a reduction in respiratory burst of human neutrophils stimulated with fMLP [17]. Similarly, inhibition of type II PtdIns 4-kinases α and β showed a reduction in the entry of Lysteria into cells [18]. Type II PtdIns 4-kinase α was shown to be involved in endocytosis of EGF receptors and in Golgi functions [19–21]. Type II PtdIns 4-kinase β was shown to be cytosolic and moves to plasma membrane in response to growth factor stimuli, suggesting that it plays a role in membrane signaling events [22].

Activation of T cells through Concanavalin A or through CD3 receptor cross linking had shown an early increase in type II PtdIns 4 kinase activity, presumably to replenish the rapidly diminishing PtdIns 4,5P_2 pools [23Fernandis, 1998 #910, 24]. Concomitantly an increase in type II PtdIns 4-kinase activity was also seen in actin cytoskeletal fractions [25]. These studies suggest that the molecular species of type II PtdIns 4-kinase that participates in early T cell activation is a potential candidate for developing knowledge based immunomodulators. Growth factor receptor mediated regulation and membrane association suggest that type PtdIns 4-kinase β may be the molecular species involved in early T cell signaling. This

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hypothesis was addressed in the present manuscript with the help of short interfering RNA sequences of type II PtdIns 4-kinase β and the results provide evidence that type II PtdIns 4-kinase β is indeed an integral part of CD3 receptor signaling cascades.

2. Materials and methods

2.1. Cells and antibodies

Jurkat T cell line was purchased from National Cell Repository, National Center for Cell Sciences, Pune, India. Jurkat T cells were grown in RPMI 1640 supplemented with 50 μM β-mercaptoethanol and 10% fetal bovine serum. Monoclonal anti-ζ antibodies, anti HA antibodies, anti actin antibodies and polyclonal anti rabbit IgG conjugated with alkaline phosphatase were from Santa Cruz Biotechnology, USA. Monoclonal anti-human CD3 was from R&D systems. Mouse IgG conjugated with alexa and rabbit anti-mouse antibodies were from Sigma, MO, USA. Polyclonal anti-type II PtdIns 4-kinase β antibodies were raised in rabbits using purified recombinant human type II PtdIns 4-kinase β. These antibodies were further purified on type II PtdIns 4-kinase β affinity column.

2.2. Reagents

Phosphatidylinositol (PtdIns) and MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) were from Sigma, MO, USA. Protein A agarose beads were from Banglore Genei Pvt Ltd, Bangalore, India. Nitrocellulose sheets were from Life Technologies Inc., USA. Fluo3/AM and Ionomycin were from Boehringer Mannheim, Germany. Matrigel coated plates were from Sigma, MO, USA. Endotoxin free plasmid isolation kits were from Qiagen, Maryland, USA. RPMI (Roswell Park Memorial Institute) 1640 was from Gibco BRL, Invitrogen. Gamma ^{32}P labeled ATP was from Board for Radiation and Isotope Technology, Mumbai, India. HA and GFP tagged Type II PtdIns 4-kinase β constructs were generous gift from Tamas Balla, NIH, USA. All other chemicals were of analytical grade.

2.3. Design and construction of short hairpin RNA (shRNA) plasmids

Short hairpin RNA sequences were generated against human type II PtdIns 4-kinase β (Accession No: AY065990) from two different regions using online software tools Genscript siRNA Target Finder. These sequences were absent in type II PtdIns 4-kinase α. The sequences selected were: 1). 5′ GTGCCTATCTAGTGGACAACA 3′, 2). 5′ GGCTTGTCACTGAGACATTTA 3′. Using these sequences primer pairs were synthesized commercially with a 9 nucleotide loop sequences. These sequences were cloned into pMU6 vector with deleted GFP sequences.

2.4. Transfection of Jurkat T Cells

Jurkat T cells in the mid-log growth phase (cell density of 3×10^5 per mL) were centrifuged at 200×*g* for 10 min. Cell density was adjusted to (1×10^7) in 0.4 mL of serum free RPMI medium and 30 μg of plasmid DNA was added and incubated on ice for 10 min. Cells were electroporated at 240 V and 960 μF. Pulsed cells were kept on ice for 10 min. Transfected cells were grown in RPMI containing 10% fetal calf serum in a humidified 5% CO_2 incubator for 36 h. Expression of type II PtdIns 4-kinase β was assayed on immunoblots or observed under fluorescence microscope.

2.5. Analysis of CD3 receptor expression on Jurkat T cells

Jurkat T cells in the mid-log growth phase were transfected with shRNAs as written in methods. Post transfection cells were harvested and washed with Phosphate Buffer saline (PBS) pH 7.4. Jurkat T cells were incubated with anti-Fc receptor (CD16/32) Abs for 5 min at room temperature, followed by staining with anti-CD3 and FITC conjugated secondary antibodies. All the antibodies binding was done in staining buffer (PBS pH 7.4 and 2% serum). Data were analyzed with help of CellQuest.

2.6. Stimulation of Jurkat T Cells with anti-CD3 antibodies

Jurkat T cells (5×10^6) were suspended in 0.2 mL serum free RPMI. Cells were incubated with anti-CD3 antibody (2 μg) for 10 min at 4 °C. CD3 receptors were crosslinked with rabbit anti-mouse antibodies for 3 min at 37 °C. Cell stimulation was stopped with 1 mL of ice cold RPMI. Cells were centrifuged and lysed in 0.2 mL lysis buffer (25 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, 0.25 mM sodium orthovanadate, 1 mM PMSF and 10 μg/mL benzamidine) followed by centrifugation at 13,000×*g* for 30 min. Cell lysates were used for immunoprecipitation.

For immunoprecipitation studies, 1 μg of relevant antibody (anti ζ/anti actin) was added to the Jurkat T cell lysates and incubated on ice for 2 h. At the end of this incubation, 0.02 mL of protein A agarose beads were added and further incubated on ice for 1 h with intermittent shaking. The beads were washed with lysis buffer three times and assayed for PtdIns 4-kinase activity.

2.7. Isolation of cytoskeletal fraction

Jurkat T cells (5×10^6) were suspended in 0.2 mL RPMI 1640 medium and were stimulated with anti-CD3 for 3 min as described above. The stimulation was terminated with 1 mL of ice-cold RPMI medium. Cells were lysed in ice-cold lysis buffer (0.02 mL) and centrifuged at 367×*g* to remove nuclei and cell debris. Cytoskeleton was obtained as detergent insoluble pellet from post nuclear supernatant. The post nuclear supernatant was centrifuged at 40,000×*g* for 40 min [26]. Cytoskeletal fractions were washed thrice with lysis buffer and assayed for PtdIns 4-kinase activity.

2.8. PtdIns 4-kinase assay

PtdIns 4-kinase assay was carried out in a final volume of 0.05 mL containing 50 mM Tris (pH 7.6), 10 mM $MgCl_2$,0.25 mM EGTA, 0.1 mM sodium orthovanadate, 20 μg/mL PtdIns, 100 μM (γ-^{32}P) ATP (200–300 cpm/*p*mole) and 0.3% Triton X-100 [24]. The reaction was initiated with the addition of γ-^{32}P ATP and incubated at room temperature (~25 °C) for 6 min. The reaction was terminated with 0.05 mL of 12 N HCl. Phospholipids were extracted with 0.5 mL of chloroform:methanol:water (15:15:5 v/v). The organic phase was washed with 0.1 mL of methanol:1 N HCl (1:1 v/v) and applied on Merck Silica gel thin layer chromatography plates pretreated with 60 mM EDTA, 2% sodium potassium tartarate in 50% ethanol (pH 8.0). The chromatograms were developed with chloroform:methanol:ammonium hydroxide:water (90:90:7:20 v/v). Labeled phospholipids were visualized by autoradiography and quantified by scintillation counting as described [27,28].

2.9. Cell adhesion assay

Cell adhesion assays were carried out on Matrigel coated surfaces. Matrigel (10 μg/mL) was coated onto 96 well flat bottomed tissue culture plates at 37 °C for overnight. These wells were blocked with 1% bovine serum albumin (BSA) in phosphate buffer Saline (PBS pH 7.4) at