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Akt2 inhibits the activation of NFAT in lymphocytes by modulating calcium release from intracellular stores

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ABSTRACT

The engagement of antigen receptors on lymphocytes leads to the activation of phospholipase C-γ, the mobilization of intracellular calcium and the activation of the NFAT transcription factor. The coupling of antigen receptors to the activation of NFAT is modulated by numerous cellular effectors including phosphoinositide 3-kinase (PI3K), which is activated following receptor cross-linking. The activation of PI3K has both positive and negative effects on the receptor-mediated activation of NFAT. An increase in the level and activity of Akt2, a target of activated PI3K, potently inhibits the subsequent activation of NFAT. In contrast, an elevation in Akt1 has no effect on signaling. Signaling pathways operating both upstream and downstream of inositol 1,4,5-trisphosphate (IP3)-stimulated calcium release from intracellular stores are unaffected by Akt2. An increase in the level of Akt2 has no significant effect on the initial amplitude, but substantially reduces the duration of calcium mobilization. The ability of Akt2 to inhibit prolonged calcium mobilization is abrogated by the administration of a cell permeable peptide that blocks the interaction between Bcl-2 and the IP3 receptor. Thus, Akt2 is a negative regulator of NFAT activation through its ability to inhibit calcium mobilization from the ER.

1. Introduction

The engagement of the B cell antigen receptor (BCR) leads to a variety of cellular outcomes depending on the context in which the signal occurs. The identity and number of transcription factors activated downstream of BCR ligation combine to determine the gene expression pattern and ultimate fate of the activated cell [1,2]. One such transcription factor that plays a critical role in the development and function of many hematopoietic cell types is the nuclear factor of activated T cells (NFAT) [3,4]. In B cells, NFAT is important for antigen receptor-induced cell proliferation in vitro, for restricting T cell-independent activation in vivo and for the formation of plasma cells in response to T cell-dependent antigens [4]. The activity of NFAT is upregulated by changes in the concentration of intracellular calcium that result from the activation of phospholipase C-γ (PLC-γ) following receptor engagement [2]. BCR ligation induces activation of a signaling cascade upstream of PLC-γ that contains the cytoplasmic protein-tyrosine kinases Lyn, Syk and Btk, the adaptor protein BLNK, and the guanine nucleotide exchange factor Vav1 [2,5–7]. Activated PLC-γ generates the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 triggers the release of calcium from intracellular stores via binding to the IP3 receptor (IP3R) on the surface of the endoplasmic reticulum. This in turn induces the opening of calcium release activated calcium (CRAC) channels on the cell surface, influx of extracellular calcium, activation of the phosphatase calcineurin, and the dephosphorylation and translocation of NFAT into the nucleus [3,8].

Multiple factors contribute to the regulation of PLC-γ and subsequent calcium signaling, including changes in the activity of phosphoinositide 3-kinase (PI3K). PI3K is also activated following clustering of the BCR [9]. Active PI3K generates phosphoinositide 3-phosphates that serve as ligands to bind proteins with pleckstrin homology (PH) domains, relocating them to the plasma membrane [10]. In B cells, the inhibition of PI3K or depletion of PI3K subunits partially inhibits calcium mobilization and NFAT activation [11–14]. However, in T cells, inhibition of PI3K has little effect on calcium mobilization. In fact, inhibition of PI3K actually enhances the TCR-mediated activation of NFAT in Jurkat T cells [15–17]. Thus, the PI3K pathway appears to have both positive and negative influences on signaling from antigen receptors to NFAT.

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Also activated downstream of PI3K is the PH domain-containing serine/threonine kinase, Akt/PKB [10,18]. Akt is important for the proliferation and survival of multiple cell types, including B cells, and is a key target of anti-cancer therapeutics [19,20]. Recently, it has been demonstrated that different Akt isoforms have both redundant and nonredundant functions in many of the systems in which Akt plays an important role [21–25]. In this study, we examined the influence of the PI3K signaling pathway on the BCR-stimulated activation of NFAT. Interestingly, we find that the PI3K-dependent inhibition of NFAT activation is mediated by Akt2. Akt2 negatively modulates NFAT activity by inhibiting calcium efflux from the ER.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies for phospho-Akt and Akt were from Cell Signaling Technology. Anti-phosphotyrosine 4G10 was from Millipore. Anti-Syk (N19) was from Santa Cruz and anti-Vav1 from Zymed Laboratories. Anti-GFP was purchased from Enzo Life Sciences and anti-GAPDH from Ambion. Horseradish peroxidase conjugated secondary antibodies were obtained from Pierce. Anti-chicken IgM for activation of DT40 cells was from Rockland Immunochemicals. Anti-CD3 was from eBioscience. Triciribine and wortmannin were purchased from Calbiochem, phorbol myristic acid and thapsigargin from Sigma, and ionomycin from Invitrogen. GFP-Trap beads were purchased from Chromotek. Small molecule inhibitors for PTP1B, TC-PTP, and SHP2 were prepared as described [26–28]. The design, synthesis and identification of the potent and selective inhibitors for Lyp and MEG2 will be described elsewhere.

2.2. Cell lines, plasmids and cell transfection

Jurkat T cells (ATCC) and Syk-deficient chicken DT40 cells [29] were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. DT40 cell media were also supplemented with 1% chicken serum. Cells were transfected at log phase density by electroporation (250 V, 975 μF) in 4 mm cuvettes using a Gene Pulser XCell (BioRad), incubated for 10 min on ice, and then allowed to recover overnight before use in experiments. Luciferase reporter plasmids pNFAT-luc and pNFκB-luc were purchased from Stratagene. Luciferase reporter plasmids pGL2-AP-1, pGL2-Basic-SP-1, and pRL-TK were from Promega. Plasmids coding for Syk or Syk(Y317F) with a GFP epitope tag at the C-terminus or a myc epitope tag at the N-terminus were described previously [30,31]. The expression plasmid for Akt2 (Akt2-flag) was a generous gift of Dr. Nagendra Prasad, Indiana University. The expression plasmid for EYFP-IP3R1 was a generous gift from Dr. Emily Taylor, University of Cambridge. The region coding for IP3R1 was amplified by PCR and subcloned into the pEGFP-C1 vector to generate EGFP-IP3R1 and the S2681A mutation was then introduced using QuickChange technology (Stratagene). Addgene was the source of expression plasmids for flag-HA-Akt1 (plasmid 9021) [32], HA-Akt1-DD (plasmid 14751) [33], Vav1-uGFP (plasmid 14557) [34], HA-GSK3β(S9A) (plasmid 14754) [35], and GCaMP3 (plasmid 22692) [36].

2.3. Luciferase reporter assays

For measurement of transcription factor activation, Syk-deficient DT40 cells were co-transfected with 20 μg of the indicated Syk-expression plasmid or empty vector, and 10 μg of the indicated luciferase reporter plasmid. In some experiments, cells were also transfected with plasmids (20 μg) encoding EGFP, Vav1-uGFP, flag-HA-Akt1, Akt2-flag, HA-GSK3β(S9A) or with empty vector. DT40 cells were stimulated by 5 μg/ml goat anti-chicken IgM antibody, unless otherwise indicated, or with a mixture of PMA (50 ng/ml) and ionomycin (1 μM) at 37 °C for 6 h. Jurkat T cells transfected with the NFAT-driven luciferase reporter plasmid were stimulated with 2 μg/ml anti-CD3 plus PMA (50 ng/ml), or with PMA plus ionomycin. For some experiments, cells were pretreated for 1 h with the indicated inhibitor or with the carrier solvent, DMSO, alone. Luciferase activity was measured in cell lysates using a luciferase assay system kit (Promega). Relative luciferase units are expressed as a fraction of that activity observed with activation by a mixture of PMA and ionomycin, unless indicated otherwise. Where indicated, values were further normalized to those obtained in cells transfected with Syk-EGFP. Data presented represent the means and standard errors of a minimum of three replicate experiments.

2.4. Cell activation assays

For the analysis of protein expression or phosphorylation, DT40 cells transfected as described above with plasmids for expression of the indicated proteins were treated with or without anti-IgM and then lysed on ice in NP40 lysis buffer (1% NP40, 150 mM NaCl, 25 mM HEPES, pH 7.5, 1 mM EDTA, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 100 μg/ml aprotinin, and 100 μg/ml leupeptin). Proteins in supernatants collected following centrifugation at 18,000×g for 5 min were separated by SDS-PAGE, transferred to PVDF membranes and analyzed by Western blotting with the indicated antibodies. Where indicated, cells were pretreated for 5 min at 37 °C with inhibitors directed against Akt (10 μM), PTP1B (200 nM), MEG2 (200 nM), TC-PTP (20 nM), SHP2 (20 nM) or Lyp (500 nM). The accumulation of inositol 1-phosphate (IP1) was detected using the IP-One ELISA kit from Cisbio Bioassays following manufacturer's instructions. Horseradish peroxidase activity was measured and standard curves were generated using a Synergy 4 plate reader and Gen5 software (BioTek). PI3K activity was measured in antiphosphotyrosine immune complexes by the in vitro phosphorylation of PI as described [37]. Phospholipids were separated by thin-layer chromatography on oxalate-activated silica gel plates.

2.5. Calcium assays

Changes in intracellular calcium levels were detected using GCaMP3 fluorescent indicator technology [36]. Syk-deficient DT40 cells were transfected as described above with plasmids encoding the GCaMP3 calcium indicator, myc-Syk, and Akt2-flag as indicated. Cells were placed in a black-walled 96-well plate and assayed for calcium flux using the plate reader. In some experiments, cells were pretreated with 20 μM TAT-IDP$_{DD/AA}$ (RKKRRQRRRGGNVYTEIK-CNSLLPLAAIVRV) [38] just prior to addition of anti-IgM. Baseline GFP fluorescence was read, cells were activated with anti-IgM, and fluorescence was monitored for 5 min. TAT-IDP$_{DD/AA}$ was synthesized using a Prelude Parallel Peptide Synthesizer (Protein Technologies, Tucson, AZ) and was purified by HPLC and verified by mass spectrometry prior to use.

2.6. Protein interaction assays

DT40 cells transiently transfected with plasmids expressing YFP-IP3R1, Akt2-Flag or Flag-HA-Akt1 were lysed in NP40 lysis buffer. Lysates were centrifuged at 18,000×g for 5 min. Supernatants were adsorbed to GFP-Trap beads and washed extensively in NP40 lysis buffer. Bound proteins were separated by SDS-PAGE and detected by Western blotting using antibodies against Akt or GFP.

3. Results

3.1. Akt2 overexpression inhibits BCR-induced NFAT activation

In DT40 B cells, signaling through the antigen receptor is coupled to the activation of multiple downstream pathways in a manner