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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, potentially 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.

Keywords

Akt; NFAT; Bcl-2; IP3 receptor; calcium mobilization; B cell signaling

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Conflict of interest

The authors declare no conflict of interest

Author contribution

VAM and RLG designed the research; VAM performed the research; WHW generated the IP3R vectors; ANL and LLP synthesized and characterized the peptide inhibitor; YH, SZ and ZYZ designed and synthesized the phosphatase inhibitors; VAM and RLG wrote the manuscript.

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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 also is 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.

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 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 IU/ml penicillin G, and 100 μ g/ml streptomycin. DT40 cell media was also supplemented with 1% chicken serum. Cells were transfected at log phase density by electroporation (250V, 975 μ F) 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 pNF κ B-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-GSK3 β (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 μ g of the indicated Syk-expression plasmid or empty vector, and 10 μ g of the indicated luciferase reporter plasmid. In some experiments, cells were also transfected with plasmids (20 μ g) encoding EGFP, Vav1-uGFP, flag-HA-Akt1, Akt2-flag, HA-GSK3 β (S9A) or with empty vector. DT40 cells were stimulated by 5 μ g/ml goat anti-chicken IgM antibody, unless otherwise indicated, or with a mixture of PMA (50 ng/ml) and ionomycin (1 μ M) at 37°C for 6 h. Jurkat T cells transfected with the NFAT-driven luciferase reporter plasmid were stimulated with 2 μ g/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 μ g/ml aprotinin, and 100 μ g/ml leupeptin). Proteins in supernatants collected following centrifugation at 18,000 $\times g$ 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 μ M), 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 μ M TAT-IDP_{DD/AA} (RKKRRQRRRGGNVYTEIKCNLLPLAAIVRV) [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 $18,000 \times g$ 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 that is dependent on the expression of the Syk protein-tyrosine kinase [39]. For example, the engagement of the BCR leads to the activation of PLC- γ , the mobilization of calcium and the activation of NFAT, and also to the activation of PI3K and its downstream effector, Akt. To monitor NFAT activation, we transfected Syk-deficient DT40 cells with or without plasmids directing the expression of Syk-EGFP along with an NFAT-driven luciferase reporter plasmid. Crosslinking of the BCR with antibodies against surface IgM failed to lead to the activation of NFAT in the Syk-deficient cells as expected, but signaling was restored by the expression of Syk-EGFP (Fig. 1A). To explore a role for the PI3K pathway in NFAT activation, we pretreated Syk-EGFP-expressing cells with 100 nM wortmannin, an irreversible PI3K inhibitor [40]. Wortmannin caused a decrease in the BCR-stimulated activation of NFAT by approximately 50% (Fig. 1A). Thus, the overall effect of the activation of PI3K in DT40 cells was to enhance signaling from the BCR to NFAT.

This inhibitory effect of wortmannin on NFAT signaling that we observed in DT40 cells is in contrast to what is seen in Jurkat T cells in which treatment with wortmannin enhances rather than inhibits the activation of NFAT [15, 16]. One major difference between Jurkat and DT40 cells is the presence or absence of certain phosphoinositide phosphatases. Jurkat cells lack both SHIP-1 and PTEN [41, 42]. Thus, levels of PIP3 are elevated and Akt is constitutively active in these cells. To examine this in DT40 cells, which retain the expression of SHIP-1 [43], we monitored the BCR-stimulated phosphorylation of Akt, a commonly used marker of the activation of both PI3K and Akt. In unstimulated cells,

phospho-Akt levels were low as determined by Western blotting (Fig. 1B). Crosslinking of the BCR led to the phosphorylation of Akt in cells expressing Syk-EGFP. We verified the receptor-mediated activation of PI3K using a lipid kinase assay of immune complexes isolated with antibodies against phosphotyrosine. PI3K activity was low in unstimulated cells, but was enhanced following receptor clustering in Syk-EGFP-expressing cells (Fig. 1C). Cells treated with the tyrosine phosphatase inhibitor, pervanadate, were used as a positive control.

These results suggested the possibility that a high level of Akt activity such as that found in Jurkat cells, but not DT40 cells, might be inhibitory to the receptor-mediated activation of NFAT. To determine if this was the case, we sought to artificially elevate the level and activity of Akt in DT40 cells. In many cancer cells, the activity of Akt is elevated due to overexpression [44]. Syk-deficient DT40 B cells transfected to express Syk-EGFP along with the NFAT-luciferase reporter plasmid were co-transfected with or without a plasmid coding for flag-tagged Akt2 to elevate the level of the kinase. The exogenous expression of Akt2-flag increased the overall level of Akt and phospho-Akt in the cells as determined by Western blotting. The over-expression of Akt2 reduced NFAT activity by >50% (Fig. 2A). As a control, another set of cells were transfected with a Vav1-uGFP expression plasmid. Vav1, like Akt, possess a PH domain, but is a well known activator of NFAT signaling [45–47]. As expected, the expression of exogenous Vav1-uGFP augmented the BCR-stimulated activation of NFAT following BCR crosslinking (Fig. 2B). These results indicate that an elevation in the level of Akt2 in DT40 cells is inhibitory to the BCR-stimulated activation of NFAT.

3.2. Akt2, but not Akt1, inhibits NFAT activation

In breast cancer cells, Akt1, but not Akt2, is a negative inhibitor of NFAT [21]. To test for a possible isoform specificity to NFAT inhibition in DT40 cells, we co-transfected Syk-deficient DT40 cells with plasmids coding for Syk-EGFP plus a plasmid for either Akt2-flag or flag-HA-Akt1, along with the NFAT reporter construct. Interestingly, the expression of flag-HA-Akt1 with Syk-EGFP had no significant effect on NFAT activity (Fig. 2C). Western blotting analyses verified the expression of Syk-EGFP and the two Akt isoforms in the transfected cells. The inability of Akt1 to inhibit NFAT signaling did not arise from a lack of activity as a constitutively active form of Akt1 (HA-Akt1-DD) in which the activating phosphorylation sites at T308 and S473 were both mutated to the phosphomimic, Asp, had only a modest effect on BCR signaling to NFAT (Fig. 2C). Akt1 and Akt2 recognize the same consensus sequence for phosphorylation with isoform specificities being due, in part, to their differential localization within the cell with a larger proportion of Akt2 found concentrated at the plasma membrane or on mitochondria [22, 48]. To investigate if the localization of Akt1 might be related to its lack of inhibitory activity, we overexpressed Myr-Akt1, a form of Akt1 that contains an N-terminal myristoylation site for constitutive membrane localization. Interestingly, Myr-Akt1 inhibited NFAT activation to an extent similar to Akt2 (Fig. 2C). These results suggest that the localization of active Akt may be a key factor in its ability to negatively regulate the activation of NFAT.

3.3. Endogenous Akt is a negative regulator of NFAT activation

To explore further an inhibitory role for Akt2 in BCR-signaling, we examined the consequences of reducing the activity of the endogenous kinase. Since the localization of Akt to the membrane, which is a function of its PH domain, appeared to be important for its inhibitory activity, we examined the effects of triciribine, an Akt inhibitor that binds to the PH domain to prevent membrane recruitment [49, 50]. The phosphorylation and activation of endogenous Akt in response to BCR cross-linking was reduced in the presence of triciribine (Fig. 3A). Triciribine at 10 μ M reduced anti-IgM activated phosphorylation of

Akt by 1.8 $\pm$ 0.1 fold. The treatment with triciribine of Syk-expressing DT40 cells reversed the inhibitory effects of overexpressed Akt2 on NFAT activity (Fig. 3B). In cells containing only endogenous Akt, triciribine significantly enhanced NFAT activity induced by BCR ligation (Fig. 3B). Similarly, the treatment of Jurkat T cells with triciribine enhanced, while the overexpression of Akt2 inhibited, the activation of NFAT triggered by the aggregation of the TCR with anti-CD3 antibodies (Fig. 4A). These data suggest that active Akt negatively regulates the activation of NFAT in both DT40 B cells and Jurkat T cells.

In DT40 cells, both the ectopic expression of Vav1 and the elimination of the inhibitory phosphorylation site at Y317 on Syk via expression of Syk(Y317F) greatly amplify signaling through the PLC- γ pathway, leading to the enhanced activation of NFAT following BCR crosslinking [45, 46, 51–53]. Y317, when phosphorylated, is the binding site for c-Cbl, an E3 ubiquitin ligase and inhibitor of Syk-dependent signaling [53–55]. Since the inhibition of Akt with triciribine also enhanced NFAT activity in these cells, we asked if these mechanisms were redundant. However, in cells that expressed either exogenous Syk-EGFP plus Vav1-uGFP, or Syk(Y317F)-EGFP, the enhanced BCR-induced NFAT activation was even further increased upon inhibition of Akt (Fig. 4B). In fact, the elimination of the Y317 phosphorylation site on Syk or the ectopic expression of Vav1, when coupled with the inhibition of Akt, enhanced the BCR-induced activation of NFAT 15- to 20-fold over that seen in cells expressing wild-type Syk alone. Thus, even under conditions where the activation of PLC- γ and the mobilization of calcium are greatly enhanced by the expression of Vav1-uGFP or Syk(Y317F)-EGFP, Akt still functions to restrain the extent to which NFAT is activated by receptor engagement.

3.4. Selective inhibition of NFAT-dependent transcription by Akt2

We asked if the inhibitory effects of Akt2 were specific to NFAT or reflected a more general mechanism of regulating transcription factor activity. We examined the effect of expression of Akt1 or Akt2, along with Syk, on a luciferase reporter gene under the regulation of the promoter for thymidine kinase (TK). Interestingly, ectopic expression of Akt2-flag was stimulatory while flag-HA-Akt1 was inhibitory (Fig. 5A). Since the TK promoter contains three binding sites for SP1, we examined the effects of Akt1 and Akt2 expression on transcription from an SP1 driven promoter. Again, expression of flag-HA-Akt1 inhibited while Akt2-flag stimulated transcription (Fig. 5B). Thus, Akt2 is not a general inhibitor of gene transcription or protein synthesis. These data further indicate that Akt1 and Akt2 have non-redundant functions and may have opposing effects on the activity of certain transcription factors. These observations are consistent with previous indications that Akt positively modulates SP1 activity [55, 56] and suggestions that this is a property specific to Akt2 versus Akt1 [57].

The function of NFAT as a transcription factor often requires its cooperation with the transcription factor AP-1, which binds to an adjacent site on the promoter [58]. This is the case for the NFAT-driven luciferase construct used in this study. To determine if Akt2 affects NFAT transcriptional activity through effects on AP-1, we employed a reporter plasmid to monitor AP-1 activity alone. However, while Syk-EGFP expression enhanced the BCR-mediated activation of AP-1, neither the expression of exogenous Akt2-flag nor treatment with triciribine caused a significant change in AP-1 activity (Fig. 5C). Thus, Akt2 exerts its effects selectively on NFAT.

Another important transcription factor in immune cell function that is regulated by BCR engagement and calcium mobilization is NF- κ B [59]. We therefore asked if ectopic expression of Akt2 also had an inhibitory effect on its activation. We expressed Akt2-flag both with and without Syk-EGFP in Syk-deficient cells along with an NF- κ B-driven

luciferase construct. However, Akt2-flag had no significant effect on basal NF- κ B activity and was slightly stimulatory for the BCR-induced, Syk-dependent activation of NF- κ B (Fig. 5D).

3.5. Akt2 enhances the tyrosine-phosphorylation of Syk

An early event in BCR signaling is the increased phosphorylation of proteins on tyrosine. To determine if Akt2 expression inhibited receptor stimulated protein phosphorylation, we activated cells expressing either Syk-EGFP alone or Syk-EGFP plus Akt2-flag by crosslinking the BCR with anti-IgM. Lysates were analyzed by Western blot for changes in the level of phosphotyrosine-containing proteins. The expression of Akt2-flag did not inhibit, but rather enhanced receptor-mediated increases in tyrosine phosphorylation (Fig. 6A). Interestingly, a phosphotyrosine-containing protein corresponding to the migration position of Syk-EGFP appeared upon BCR ligation, but was also present in the unstimulated cells in which Akt2-flag was expressed (Fig. 6B). To verify that Syk was tyrosine phosphorylated in the absence of stimulation upon exogenous expression of Akt2-flag, cells expressing Syk-EGFP alone or with Akt2-flag were activated with anti-IgM or left unactivated. Tyrosine phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine (4G10) and probed by Western blot with an antibody for Syk. As shown in Fig. 6C, Syk was immunoprecipitated with 4G10 antibodies from unstimulated cells only when Akt2-flag was expressed. Thus, the exogenous expression of Akt2 enhances Syk tyrosine phosphorylation even in the absence of BCR aggregation.

To determine if the enhanced basal phosphorylation of Syk on tyrosine accounted for the ability of Akt2 to inhibit BCR signaling, we tested a series of selective phosphotyrosine phosphatase (PTP) inhibitors for their abilities to modulate the extent of Syk phosphorylation in the absence of receptor engagement. An inhibitor of Lyp/PTPN22 caused a large increase in the basal phosphorylation of Syk on tyrosine (Fig. 6D). To determine if this enhanced phosphorylation inhibited the BCR-stimulated activation of NFAT, we compared NFAT activation in cells treated with or without the Lyp inhibitor. However, the inhibition of Lyp failed to decrease receptor mediated signaling (Fig. 6E).

An inhibition of protein tyrosine phosphatase activity could, in theory, result in the inhibition of the Src-family kinase, Lyn, through increased phosphorylation of its C-terminal inhibitory tyrosine. Lyn also is a kinase that phosphorylates Syk on the inhibitory site at Y317 [51]. Therefore, the BCR-induced activation of NFAT in the presence or absence of exogenously expressed Akt2 was measured in DT40 cells lacking both Syk and Lyn [51], reconstituted with Syk-EGFP. The expression of Akt2-flag reduced NFAT activation to a similar extent in the cells lacking Lyn as in cells containing the endogenous kinase (Fig 6F).

3.6. Akt2 expression alters calcium signaling

A principal target of the receptor-proximal signaling complex that forms upon BCR aggregation is PLC- γ , whose phosphorylation is a critical event for signaling to NFAT. To determine if Akt2 overexpression inhibited PLC- γ activity, we assessed BCR ligation-induced IP3 production in cells expressing Syk-EGFP alone compared to cells expressing Syk-EGFP and Akt2-flag. As an indication of IP3 levels, the downstream metabolite IP1 was measured using an ELISA assay. IP1 levels increased significantly following BCR aggregation, but no change was detected between cells expressing or not expressing Akt2-flag (Fig. 7A). This indicated that PLC- γ function and activation-induced IP3 levels were not affected by Akt2.

The activation of NFAT occurs as a consequence of increases in intracellular calcium. NFAT in lymphocytes can be stimulated and the need for receptor engagement bypassed by

treatment with ionomycin, a potent calcium ionophore. Therefore, we considered the effect of Akt2-flag expression on ionomycin-induced NFAT activation. Cells expressing Syk-EGFP alone or Syk-EGFP plus Akt2-flag were treated with increasing concentrations of ionomycin and analyzed for NFAT activity. As shown in Fig. 7B, no significant difference in BCR-stimulated NFAT activation was found between cells expressing or lacking exogenously expressed Akt2-flag at multiple concentrations of ionomycin. Thus, signaling downstream from calcium mobilization appears intact in cells overexpressing Akt2.

IP3 produced by PLC- γ binds to IP3 receptors located on the ER membrane, resulting in the efflux of calcium from intracellular stores and subsequent opening of plasma membrane CRAC channels for influx of extracellular calcium. Since ionomycin-induced calcium influx does not require functional CRAC channels, we asked if Akt2 affected CRAC channel function. Thapsigargin, a sarco/endoplasmic reticulum calcium ATPase (SERCA) inhibitor, depletes ER stores and elevates intracellular calcium leading to subsequent CRAC channel opening. However, thapsigargin-induced NFAT activation was unchanged in cells expressing Syk-EGFP and Akt2-flag as compared with those expressing Syk-EGFP alone (Fig. 7C). Thus, CRAC channel function was not affected by the expression of Akt2.

Because signaling both upstream and downstream of calcium efflux from the ER remained intact, we focused on calcium release from intracellular stores. To detect intracellular calcium levels in DT40 cells, we transfected cells with a plasmid coding for GCaMP3, a modified form of GFP that functions as a fluorescent calcium indicator [36]. This allowed us to detect calcium flux only in transfected cells within a mixed population. Syk-deficient DT40 cells were transiently transfected with plasmids for GCaMP3 and myc-Syk, with or without Akt2-flag. Fluorescence was measured as a function of time after IgM stimulation. As shown in Fig. 7D, the initial increase in intracellular calcium was not reduced in Akt2-flag-expressing cells. Interestingly, however, calcium returned to basal levels more rapidly in cells expressing Akt2-flag. Multiple trials indicated a significant decrease in sustained calcium release in cells expressing Akt2 (Supplemental Fig. 1A and B).

3.7. Akt and regulation of the IP3 receptor

The IP3 receptor (IP3R) has been reported to exist in a complex with activated Akt and to become phosphorylated on a serine located near the C-terminus [60, 61]. To confirm an interaction between IP3R and Akt, we co-expressed an EYFP tagged form of IP3R1 along with flag-HA-Akt1 and Akt2-flag in DT40 cells. EYFP-IP3R1 was immunoprecipitated using an immobilized GFP-binding protein (GFP-Trap) [62]. Western blotting analyses confirmed the presence of both flag-HA-Akt1 and Akt2-flag in the EYFP-IP3R1 immune complexes (Fig. 8A). To explore a role for IP3R1 phosphorylation in modulating the effects of Akt2 on calcium signaling, we generated two forms of EGFP-tagged IP3R1, one wild-type and one in which the serine phosphorylated by Akt was replaced by an alanine. We then examined the ability of exogenously expressed EGFP-IP3R1 or EGFP-IP3R1(S2681A) to reverse the Akt2-induced inhibition of NFAT activity. However, the ectopic expression of neither form of EGFP-IP3R1 had any detectable effect on the ability of Akt2-flag to inhibit the activation of NFAT in DT40 cells (Fig. 8B). A similar lack of effect was seen when EGFP-IP3R1(S2681A) was expressed with and without Akt2-flag in Jurkat T cells and cells were activated with anti-CD3 (data not shown).

The release of calcium from the ER through IP3 receptors is also modulated by direct interactions with members of the Bcl-2 family of anti-apoptotic proteins. For example, Bcl-2 interacts with IP3R through its BH4 domain, which binds to the regulatory and coupling domain of the calcium channel [62]. This interaction can be blocked by introduction into cells of a peptide, TAT-IDP, that is modeled on the Bcl-2 binding site on IP3R [38]. We asked whether or not blocking the interaction of Bcl-2 with IP3R might reverse the

inhibitory effects of Akt2 on calcium mobilization. We transiently transfected Syk-deficient DT40 cells with Syk-EGFP and GCaMP3 expression plasmids in the presence or absence of a plasmid directing the expression of Akt2-flag and treated the cells with TAT-IDP_{DD/AA}, which is a protease resistant form of the inhibitory peptide [38]. Cells were activated with anti-IgM and changes in intracellular calcium were monitored as a function of time. Detection of receptor aggregation-induced calcium flux demonstrated that TAT-IDP_{DD/AA} enhanced prolonged calcium flux in Syk-expressing cells (Fig. 8C). Multiple trials indicated a significant increase in sustained calcium release in cells treated with TAT-IDP_{DD/AA} (Supplemental Fig. 1C). Interestingly, the peptide completely rescued calcium levels from the inhibition caused by the expression of Akt2-flag (Fig. 8D).

4. Discussion

The clustering of BCR complexes leads to the activation of Syk, the phosphorylation of the adaptor protein BLNK and the formation of a “signalosome”, a complex of proteins that regulates calcium mobilization and includes Syk, BLNK, Btk, Vav, and PLC- γ [2, 6, 64]. The assembly and proper distribution of the signalosome to the membrane is promoted by the presence of PIP3 generated by activated PI3K [64]. DT40 B cells deficient in p110 δ (the major B cell isoform of the catalytic subunit of PI3K), B cells from mice deficient in p110 δ , or B cells from mice expressing an inactive form of p110 δ all exhibit a diminished, but not abrogated mobilization of calcium [11–14]. Likewise, loss of PIP3 phosphatases from cells increases intracellular levels of PIP3 and results in lymphocytes that are hyperresponsive to antigen receptor engagement [41, 65, 66]. Consequently, inhibitors of PI3K generally suppress signaling through the PLC- γ pathway and diminish calcium fluxes in B cells [67]. This is also true in DT40 B cells to which the addition of wortmannin to inhibit PI3K depresses the Syk-dependent activation of NFAT. Of proteins with PH domains that operate downstream of the BCR, Btk and Vav-family proteins are clearly required for the effective activation of PLC- γ [5, 7, 68–71]. The overexpression of Vav1 further enhances BCR signaling [45, 47]. Thus, it is likely that the requirement for the participation of these signaling molecules in the regulation of calcium mobilization accounts for some of the repressive effect of PI3K inhibitors on the activation of NFAT.

In contrast, the effect of PI3K inhibitors on receptor-stimulated, NFAT-dependent gene transcription in T cells is variable depending on the cell system examined and the nature of the activating signal, but in many cases is stimulatory [15–17]. Thus, the consequences of inhibiting the PI3K pathway on the antigen receptor-induced activation of NFAT are not universally inhibitory. Even in B cells, the activation of PI3K following BCR engagement simultaneously generates signals that are both stimulatory and inhibitory to NFAT-mediated gene transcription. Our data indicate that Akt2, which has a PH domain and whose activation is dependent on PI3K, is a net inhibitor of both the BCR- and TCR-mediated activation of NFAT. When the activity of Akt2 is increased by overexpressing the kinase, the activation of NFAT is reduced in both DT40 B cells and Jurkat T cells. Similarly, if the activity of endogenous Akt is reduced by the addition of the small molecule inhibitor triciribine, the activation of NFAT is enhanced in both cell types. Thus, Akt2 is likely the protein responsible for much of the inhibitory effect of PI3K on NFAT activity.

We found no measureable effect of Akt2 on signaling either prior to or subsequent to the release of intracellular calcium other than an increase in the basal level of tyrosine phosphorylation of Syk. The mechanism for this is not completely clear; however, Akt is known to phosphorylate and inhibit the activity of PTP1B/PTPN1 and could have as substrates other tyrosine phosphatases [72]. The phosphatase whose inhibition most greatly affected the tyrosine-phosphorylation of Syk was Lyp/PTPN22, which is consistent with the reduced level of phosphorylated Syk observed in B cells from patients expressing a

hyperactive Lyp mutant [72]. Regardless, the inhibition of Lyp failed to decrease BCR signaling to the activation of NFAT, making it an unlikely participant in the Akt2 inhibitory pathway. Furthermore, lack of phosphorylation of Syk on the only site known to limit its activity (Y317) did not alter the responsiveness of cells to Akt2 expression or inhibition as measured in cells either lacking Lyn or expressing Syk(Y317F).

We found instead that the inhibition of NFAT by Akt2 is a consequence of its ability to limit calcium mobilization, most likely by modulating calcium release from the ER. The calcium release resulting from BCR cross-linking, although initially unaffected by Akt2 expression, is more transient in the presence of excess Akt2. This effect on calcium mobilization likely explains the ability of Akt2 to negatively affect the activation of NFAT while having no inhibitory effect on NF- κ B. The degree to which BCR engagement leads to the differential activation of NF- κ B versus NFAT is a result of the magnitude and duration of calcium mobilization [74]. NF- κ B is preferentially activated by large, transient increases in intracellular calcium, which in our study are not inhibited by Akt2 expression. In contrast, NFAT activation requires low and more sustained increases in intracellular free calcium, which are attenuated in the presence of Akt2. This mechanism for the Akt-dependent inhibition of NFAT is distinct from that reported previously in breast cancer cells where it is Akt1 rather than Akt2 that inhibits NFAT [21]. The lack of effect of Akt2 expression on the ionomycin or thapsigargin-stimulated activation of NFAT makes it unlikely that Akt2 has any significant effect on the level of NFAT protein in DT40 cells.

The differential abilities of Akt1 and Akt2 to regulate calcium release from the ER are an interesting example of isoform specificity. Studies on the localization of each indicate that, while both are cytoplasmic, more of Akt2 than Akt1 localizes to the plasma membrane and mitochondria [22, 48]. This localization is interesting in light of the close interactions that take place between mitochondria and the ER to regulate calcium homeostasis [75, 76] and the fact that Bcl-2 is found associated with both the outer mitochondrial membrane and the ER [77]. Thus, the specific ability of Akt2 as compared to Akt1 to modulate calcium release may be a consequence of its differential localization. The addition of the myristoyl moiety to Akt1 to form Myr-Akt1 may be sufficient to target this isoform to the same locations as activated Akt2, explaining why Myr-Akt1 and not Akt1-DD is an effective inhibitor of NFAT activity. This is reasonable as myristoylation targets some proteins such as BID, CBR and DES1 to mitochondria and a myristoylated form of Akt1 modulates mitochondrial hexokinase activity [78–80].

Several mechanisms by which Akt localized at or near IP3R might modulate calcium release are possible. A direct interaction between Akt and IP3R has been demonstrated previously [60, 61] and we also can detect an association using a co-immunoprecipitation assay. However, it appeared that IP3R1 could interact with either Akt2 or Akt1 in this assay. Akt phosphorylates IP3R on S2681. The consequence of this phosphorylation was reported in one study to restrict calcium release from the ER [60], but in another not to significantly affect channel function [61]. In both studies, phosphorylation promoted cell survival in response to apoptotic stimuli. We were unable in our studies to detect an effect of the overexpression of a nonphosphorylatable mutant of IP3R1 on the ability of Akt2 to dampen calcium responses stimulated by BCR crosslinking, at least those leading to the activation of NFAT.

The properties of IP3 receptors are modulated by direct interactions with members of the Bcl-2 family of pro-apoptotic proteins including Bcl-2 and Bcl-xl [75]; each binds to separate regions of the IP3R channel to inhibit calcium release. By using a synthetic peptide to disrupt specifically the Bcl-2-IP3R interaction, we were able to reverse the inhibitory effect of Akt2 on calcium release from the ER. Although we were unable to sustain high

enough levels of the inhibitory IDP-TAT peptide in cells long enough to measure NFAT activity without inducing considerable cytotoxicity, an increase in the prolonged release of calcium from the ER is a well known prerequisite for NFAT activation [74]. These studies help pinpoint the place within the signal transduction pathway where Akt2 exerts its negative effects on NFAT signaling. However, the exact mechanism by which Akt2 influences the channel properties of IP3R is not completely understood. It is known that cells lacking the Bcl-2 binding partners Bax and Bak have a higher level of free Bcl-2, which increases its interaction with IP3R1 and inhibits calcium-induced apoptosis [81]. It is possible that BAD, which like Bax and Bak regulates apoptosis by sequestering Bcl-2 and is phosphorylated by Akt2 resulting in the release of Bcl-2 [82], is the Akt2 target for NFAT inhibition (Fig. 9). This mechanism suggests a new role for Bcl-2 outside of regulating apoptosis; one in which it regulates transcription factor activation for determining B cell responses to receptor ligation.

5. Conclusion

1. Activation of the PI3K signaling pathway by engagement of the B cell antigen receptor has both positive and negative effects on the downstream signaling pathways that lead to the mobilization of calcium and activation of the NFAT transcription factor.
2. Inhibitory effects of the PI3K pathway on NFAT signaling are mediated by the serine/threonine kinase Akt2, whose activation attenuates prolonged release of calcium from the ER.
3. Calcium levels can be restored by the treatment of cells with a peptide that blocks the interaction between Bcl-2 and the IP3 receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCR	B cell receptor for antigen
Bcl-2	B-cell lymphoma 2
CRAC	calcium release activated channel
EGFP	enhanced green fluorescent protein
EYFP	enhanced yellow fluorescent protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK3β	glycogen synthase kinase 3 beta
IP3	inositol 1,4,5-trisphosphate
IP3R	inositol 1,4,5-trisphosphate receptor
NFAT	nuclear factor of activated T cells
PH	pleckstrin homology

PI3K	phosphoinositide 3-kinase
PLC-γ	phospholipase C gamma
PMA	phorbol myristate acetate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Highlights

The activation of Akt through the PI3K signaling pathway negatively regulates coupling of the B cell antigen receptor to the NFAT transcription factor.

The overexpression of Akt2, but not Akt1, inhibits antigen receptor-mediated activation of NFAT.

Akt2 fails to block receptor-mediated signaling either upstream or downstream of calcium release from the endoplasmic reticulum.

Akt2 inhibits sustained calcium release from the endoplasmic reticulum.

The inhibitor effect of Akt2 can be reversed by a peptide inhibitor of the Bcl-2 interaction with the IP3 receptor.

Fig. 1.

The activation of NFAT in DT40 cells is inhibited by wortmannin. (A) NFAT activity measured in anti-IgM-activated DT40 B cells lacking Syk (Syk⁻) or expressing Syk-EGFP (Syk) and treated without or with (+wort) wortmannin. Luciferase activity was normalized to a value of 1.0 for cells expressing Syk-EGFP. Histograms represent the mean $\pm$ SEM of three replicate experiments, $*p < 0.01$ when compared to no wortmannin control. (B) DT40 cells expressing Syk-EGFP (Syk) or EGFP (Syk⁻) were activated with anti-IgM (+) or left unactivated (-). Lysates were analyzed by Western blot for expression of Syk-EGFP, Akt phosphorylated on S473 (pAkt), total Akt, or GAPDH as a loading control. (C) DT40 cells expressing Syk-EGFP (Syk) were activated with anti-IgM (IgM), pervanadate (PV), or were left unactivated (-). Immune isolated with phosphotyrosine antibodies were assayed for PI3K activity. The arrow indicates the migration position of PI3P. Stimulation with anti-IgM and with pervanadate led to a 3.9 $\pm$ 0.6 and 14.3 $\pm$ 1.3 fold increase in PI3K activity, respectively, based on three trials.

Fig. 2.

The activation of NFAT is inhibited by the overexpression of Akt2. Syk-deficient DT40 cells expressing Syk-EGFP (Syk), the NFAT-luciferase reporter plasmid, and either Akt2-flag (Akt2) (A) or Vav1-uGFP (Vav1) (B) were activated with anti-IgM. Cell lysates were analyzed by Western blot with antibodies against Syk, Akt, phosphorylated Akt (pAkt) or Vav1. Luciferase activity was normalized to a value of 1.0 for cells expressing Syk-EGFP. Data represent the mean $\pm$ SEM of triplicate experiments; * $p < 0.01$, ** $p < 0.001$ when compared to cells lacking Akt2. (C) NFAT activity was measured in anti-IgM-activated DT40 cells expressing Syk-EGFP without (Syk) or with flag-HA-Akt1 (Akt1), Akt2-flag (Akt2), HA-Akt1-DD (Akt1-DD) or Myr-Akt1 (Myr-Akt1). Activity was normalized to 1.0 for cells expressing Syk-EGFP. Data represent the mean $\pm$ SEM of triplicate experiments, ** $p < 0.001$. Cell lysates were analyzed by Western blotting for Syk-EGFP (Syk), Akt1 or Akt2 (Akt) and GAPDH.

Fig. 3.

The activation of NFAT is enhanced by triciribine, an Akt1/2 inhibitor. (A) DT40 cells expressing Syk-EGFP were pretreated with increasing concentrations of triciribine or DMSO for 1 h prior to activation with anti-IgM for 5 min. Lysates were analyzed by Western blotting for phosphorylation of Akt at S473 (pAkt) and for total Akt (Akt). The relative extent of phosphorylation of Akt as compared to unstimulated cells is indicated. (B) NFAT activity was measured in DT40 cells lacking (-) or expressing Syk-EGFP (+) and Akt2-flag as indicated. Cells were pretreated for 1 h with 10 μ M triciribine (Tri) or DMSO prior to activation with anti-IgM. Luciferase activity was normalized to 1.0 for cells expressing Syk-EGFP. Data represent the mean $\pm$ SEM of triplicate experiments, * $p < 0.001$ when compared to cells not treated with triciribine. Lysates were analyzed by Western blotting for expression of Syk-EGFP (Syk), Akt or GAPDH.

Fig. 4.

Triciribine, enhances NFAT activation in T and B cells. (A) NFAT activity was measured in Jurkat T cells with (+) or without (-) Akt2-flag expression in the presence or absence of 10 μ M triciribine (Tri) prior to activation with anti-CD3 and PMA. Luciferase activity was normalized to a value of 1.0 for cells activated by anti-CD3, but lacking triciribine or exogenous Akt2. Data represent the mean $\pm$ SEM of triplicate experiments, * $p < 0.001$. Lysates were analyzed by Western blotting with antibodies against Akt and GAPDH. (B) NFAT activity was measured in DT40 cells expressing Syk-EGFP (Syk), Syk(Y317F)-EGFP (Y317F) or Syk-EGFP with or without Vav1-uGFP (Vav1) in the presence or absence of 10 μ M triciribine (Tri). Cells were activated with anti-IgM. Luciferase activity was normalized to a value of 1.0 for cells expressing Syk-EGFP. Data represent the mean $\pm$ SEM of triplicate experiments, * $p < 0.001$.

SEM of triplicate experiments, * $p < 0.01$ when compared to cells not treated with triciribine. Cell lysates were analyzed by Western blotting for expression of Syk-EGFP (Syk), Vav-uGFP (Vav1) and GAPDH.

Fig. 5.

Akt2 is not a general inhibitor of gene transcription. Syk-EGFP-expressing DT40 cells were transiently transfected with pRL-TK (A), pGL2-Basic-SP-1 (B), Akt1, Akt2 or empty vector plasmids (Ctrl) and activated using anti-IgM (A) or trichostatin A (B) for 6 h and assayed for luciferase activity. The expression level of Syk-EGFP (insert, upper panels) was used as a measure of transfection efficiency. Relative luciferase values were normalized to a value of 1.0 for control cells. Data represent the mean $\pm$ SEM of triplicate experiments; * p < 0.05, ** p < 0.01 when compared to control cells. The expression of Syk-EGFP (insert, upper panels) and Akt isoforms (insert, lower panels) was confirmed by Western blotting. (C) Syk-deficient DT40 cells were transfected with pGL2-AP-1 reporter construct and plasmids to express EGFP (Syk⁻), Syk-EGFP (Syk) or both Syk-EGFP and Akt2-flag (Akt2). Cells expressing Syk-EGFP were pretreated for 1 h with triciribine (Tri). Cells were then activated with anti-IgM for 6 h and luciferase activity measured. Data represent the mean $\pm$ SEM of triplicate experiments. (D) NF- κ B activity was measured in Syk-deficient DT40 cells (Syk⁻) expressing Syk-EGFP (Syk) and/or Akt2-flag (Akt2). Luciferase activity from anti-IgM-activated cells activity was normalized to values obtained when cells were treated with a mixture of PMA and ionomycin. Data represent the mean $\pm$ SEM of triplicate experiments, * p < 0.05 when compared to cells expressing Syk, but lacking Akt2. The expression of Syk-EGFP (inset, upper panel) and Akt2 (inset, lower panel) were confirmed by Western blotting.

Fig. 6.

The expression of Akt2 enhances the phosphorylation of Syk on tyrosine. (A) DT40 cells expressing Syk-EGFP and increasing amounts of Akt2-flag (Akt) were activated with anti-IgM for 5 min, lysed and analyzed by Western blot with antibodies against phosphotyrosine (pTyr) or GAPDH. (B) DT40 cells lacking (–) or expressing (+) Syk-EGFP (Syk) and/or Akt2-flag (Akt2) were treated with or without anti-IgM for 5 min and analyzed by Western blot with antibodies against phosphotyrosine (pTyr), Syk and GAPDH. The region corresponding to the migration position of Syk-EGFP is illustrated in the top panel. (C) DT40 cells lacking (–) or expressing (+) Syk-EGFP (Syk) and/or Flag-Ha-Akt1 (1) or Akt2-flag (2) were treated with or without anti-IgM for 5 min. Phosphotyrosine containing proteins were immunoprecipitated and analyzed by Western blot with an antibody against Syk (upper panel). Cell lysates were probed with antibodies against Syk (middle panel) and GAPDH (bottom panel). (D) Lysates from DT40 cells expressing Syk-EGFP and treated for 1 h with a panel of inhibitors targeted against the indicated phosphatase or with DMSO alone (–) were analyzed by Western blotting with antibodies against phosphotyrosine (pTyr) and Syk. (E) NFAT activity was measured in anti-IgM-activated DT40 cells lacking (Syk[–]) or expressing Syk-EGFP (Syk) with or without Akt2-flag (Akt2). Cells were pretreated for 1 h with the Lyp inhibitor (Lyp) where indicated. Luciferase activity was normalized to a value of 1.0 for cells expressing Syk-EGFP. Data represent the mean ± SEM of triplicate experiments, **p* < 0.01 when compared to cells expressing Syk, but lacking Akt2. The expression of Syk-EGFP and Akt were confirmed by Western blotting in Syk-deficient cells (lane 1), cells expressing Syk-EGFP (lane 2) and cells expressing both Syk-EGFP and

exogenous Akt2 (lane 3). (F) NFAT activity was measured in anti-IgM-activated DT40 cells deficient in both Syk and Lyn (Syk⁻) expressing Syk-EGFP (Syk) with or without Akt2-flag (Akt2). Luciferase activity was normalized to a value of 1.0 for cells expressing Syk-EGFP and endogenous Lyn. Data represent the mean $\pm$ SEM of triplicate experiments, * p < 0.001 when compared to cells expressing Syk, but lacking Akt2. The expression of Syk-EGFP and Akt were confirmed by Western blotting (insert).

Fig. 7.

Akt modulates calcium release from intracellular stores. (A) IP1 levels were measured in Syk-deficient DT40 cells expressing EGFP (Syk^{-/-}), Syk-EGFP (Syk), or Syk-EGFP and Akt2-Flag (Syk+Akt2) and activated with anti-IgM for 1 h in the presence of LiCl. Bars represent the means and standard errors of three experiments. The expression of Syk-EGFP and Akt were confirmed by Western blotting. (B) NFAT activity was measured in DT40 cells expressing Syk-EGFP (Syk) with or without Akt2-flag (Akt2). Cells were treated with PMA (50 ng/ml) and increasing concentrations of ionomycin. Luciferase activity was measured in cell lysates. (C) NFAT activity was measured in DT40 cells expressing Syk-EGFP (Syk) with or without Akt2-flag (Akt2). Cells were activated by treatment with anti-IgM (α-IgM) or 10 μM thapsigargin (TG). Luciferase activity was normalized to a value of 1.0 for cells expressing Syk-EGFP and activated by anti-IgM. Data represent the mean ± SEM of triplicate experiments, **p* < 0.001 when compared to cells expressing Syk, but lacking Akt2. (D) Syk-deficient cells were transfected with plasmids encoding GCaMP3 and empty vector (Syk^{-/-}) or myc-Syk (Syk) with or without Akt2-flag (Akt2). Cells were activated with anti-IgM and fluorescence changes monitored. Measurements were

normalized to fluorescence measured at time 0. Cell lysates were analyzed by Western blot for expression of Syk-EGFP (Syk) and Akt2-flag (Akt2) for cells expressing Syk-EGFP alone (lane 1) or Syk-EGFP plus exogenous Akt2 (lane 2).

Fig. 8.

Inhibition of calcium release by Akt2 is reversed by TAT-IDP. (A) Syk-deficient DT40 cells expressing myc-Syk, YFP-IP3R1, and flag-HA-Akt1 (1) or Akt2-flag (2) were activated with anti-IgM and lysed. GFP-IP3R1 was isolated using GFP-Trap beads. Bound proteins were analyzed by Western blot for YFP-IP3R1 (upper panel) using anti-GFP antibodies (GFP) and for Akt isoforms (middle panel). Lysates were analyzed for Akt expression by Western blotting (bottom panel). (B) Anti-IgM-stimulated NFAT activity was measured in DT40 cells expressing Syk-EGFP with or without Akt2-flag (Akt2), GFP-IP3R1 (IP3R) or GFP-IP3R1(S2681A) (SA). Luciferase activity was normalized to cells expressing only Syk-EGFP. Data represent the mean $\pm$ SEM of triplicate experiments, * $p < 0.001$ when compared to cells expressing Syk, but lacking Akt2. (C) Syk-deficient DT40 cells expressing GCaMP3 and myc-Syk were treated without (Syk) or with 20 μ M TAT-

IDP_{DD/AA} (IDP) just prior to activation with anti-IgM. Fluorescence changes were monitored and readings normalized to fluorescence measured at time 0. (D) Syk-deficient DT40 cells expressing GCaMP3 and myc-Syk were transfected without (Syk) or with (Akt2) a plasmid for Akt2-flag. Cells were treated without (Syk, Akt2) or with (Akt2+IDP) 20 μ M TAT-IDP_{DD/AA} just prior to activation with anti-IgM. Fluorescence changes were monitored and readings normalized to fluorescence measured at time 0.

Fig. 9.
Proposed model for the negative regulation of NFAT by Akt2.