B Cell Antigen Receptor-Induced Activation of Akt Promotes B Cell Survival and Is Dependent on Syk Kinase

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Signaling through the B cell Ag receptor (BCR) is a key determinant in the regulation of B cell physiology. Depending on additional factors, such as microenvironment and developmental stage, ligation of the BCR can trigger B lymphocyte activation, proliferation, or apoptosis. The regulatory mechanisms determining B cell apoptosis and survival are not known. Using the chicken B lymphoma cell line DT40 as a model system, we investigated the role of the serine/threonine kinase Akt in B cell activation. While parental DT40 cells undergo apoptosis in response to BCR cross-linking, cells overexpressing Akt show a greatly diminished apoptotic response. By contrast, limiting the activation of Akt, either by inhibiting phosphatidylinositol 3-kinase or by ectopic expression of the phospholipid phosphatase MMAC1, results in a significant increase in the percentage of apoptotic cells after BCR cross-linking. Using various DT40 knockout cell lines, we further demonstrate that the tyrosine kinase Syk is required for Akt activation and that Lyn tyrosine kinase inhibits Akt activation. Taken together, the data demonstrate that Akt plays an important role in B cell survival and that Akt is activated in a Syk-dependent pathway.

The response of B lymphocytes to foreign Ag is mediated by the multisubunit B cell receptor (BCR) (/ 2). Engagement of the BCR by Ag or anti-receptor Abs can lead to multiple cellular responses depending upon microenvironment, context of Ag, isotype of Ag receptor, expression levels of coreceptors, and, most significantly, stage of development. For example, stimulation of immature B cells through the Ag receptor typically leads to apoptosis or anergy, whereas mature B cells respond to Ag by proliferating and differentiating into memory or Ab-secreting cells (1, 3). In recent years, great progress has been made in elucidating the signaling pathways activated by BCR. The regulatory mechanisms underlying the decision between B cell survival and apoptosis, however, are not well understood.

Among the first steps in BCR signaling is the recruitment of the protein tyrosine kinases Lyn and Syk to the receptor complex in a phosphorylation-dependent manner (2, 3). B cells from Syk–/– mice accumulate in the late pro-B stage, suggesting an important function for Syk in B cell development (4, 5). Phosphorylation targets identified for Syk include the adapter molecule BLNK/SLP-65 (6, 7) and phospholipase C-γ (PLC-γ) (8). Phosphorylation of BLNK/SLP-65 is required for membrane localization of PLC-γ, NCK, and Vav. BLNK/SLP-65 also binds the GRB2-SOS complex, and thus contributes to the activation of the Ras/mitogen-activated protein-kinase cascade (6, 7). In contrast to Syk, Lyn serves both positive and negative functions during BCR signaling. Lyn phosphorylates and activates the pleckstrin homology (PH) domain-containing tyrosine kinase Btk. Btk and Syk cooperate to activate PLC-γ, which is required for the BCR-stimulated Ca2+ response (9–11). Lyn mediates its negative regulatory function by phosphorylating the BCR coreceptor CD22. Once phosphorylated, CD22 recruits and activates the protein tyrosine phosphatase SHP-1, which leads to down-regulation of BCR signaling (12–15). Another important component of the BCR signaling cascade is the coreceptor CD19. CD19 is tyrosine phosphorylated in response to BCR ligation and recruits Vav and phosphatidylinositol 3-kinase (PI 3-kinase) (13, 16, 17).

While effects of mitogen-activated protein kinase activation and calcium mobilization have been well studied in B cells (reviewed in Ref. 3), the consequences of PI 3-kinase activation have only recently been addressed. Mice that lack the regulatory p85 subunit of PI 3-kinase exhibit profound defects in B cell function, including decreased proliferative responses and impaired B cell survival (18, 19). PI 3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3) (reviewed in Ref. 20). The PH domains of several signaling molecules have been demonstrated to specifically bind PIP3, resulting in the recruitment of the respective proteins to the plasma membrane. In B cells, the PH domain-containing kinases Btk and Akt (also termed protein kinase B) are activated in a PI 3-kinase-dependent manner (2, 21, 22). While Btk is found to play a role in the sustained calcium response, a function for the serine/threonine kinase Akt in B cells has not yet been established in BCR signaling. Akt is the cellular homologue of the product of v-Akt, the oncogene of the acutely transforming rodent retrovirus Akt-8 that causes T cell leukemias and lymphomas in mice (23, 24). To date, three closely related isoforms of Akt (Akt1, Akt2, and Akt3) have been identified in mammals (4, 25–29). All three isoforms are widely expressed and are regulated by the levels of PIP3 in the plasma membrane. Upon activation in response to various growth factors, Akt phosphorylates its target proteins, thereby altering their activities.
factors, such as platelet-derived growth factor, epidermal growth factor, insulin, and insulin-like growth factor-1, Akt generates a survival signal in nonhemopoietic cells (29–31). Consistent with its survival function, overexpression of Akt can lead to cell transformation (30, 32). Recently, the tumor suppressor MMAC1 (also termed PTEN and TEP-1) has been identified as an important negative regulator of Akt (33). MMAC1 is a phospholipid phosphatase and catalyzes the dephosphorylation of the D3 position inPIP3, thereby antagonizing PI 3-kinase. Interestingly, heterozygous MMAC1 knockout mice develop spontaneous tumors with a high frequency of lymphomas (19, 34, 35), suggesting an important role for MMAC1 in lymphocyte biology. While it is clear that PI3 kinase-containing signaling molecules such as Btk and PLC-γ are regulated by PIP3 levels and important for B cell function, a role for Akt in lymphocyte activation has not been addressed.

In this study, we asked whether Akt plays a role in the regulation of B cell survival and how the activity of Akt is regulated. Using the B cell lymphoma line DT40 as a model system, we found that overexpression of Akt in these cells blocked receptor-induced apoptosis, indicating that Akt does provide a survival signal. Consistent with a requirement for PI 3-kinase in Akt activation, inhibition of PI 3-kinase abolished BCR-induced Akt activation and resulted in enhanced apoptosis. Similarly, exogenous expression of MMAC1 led to enhanced apoptosis and inhibition of Akt. Furthermore, our data suggest antagonistic roles for Syk and Lyn kinases in regulating Akt activation.

Materials and Methods

Cells, Abs, and reagents

DT40 cell lines carrying targeted deletions in btk, lyn, syk, or plc-γ have been described previously (8, 9). All DT40 cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 1% chicken serum (Sigma, St. Louis, MO), penicillin, streptomycin, glutamine, and 50 μM mercaptopethanol. The chicken cell lines SL29 and GdT were obtained from American Type Culture Collection (Manassas, VA) and cultured as recommended. U373 cells and U373 cells ectopically expressing MMAC1 (36) were grown in DMEM, supplemented with 10% FCS, penicillin, and streptomycin. Anti-chicken mAb M4 was described previously (9). Anti-Akt and anti-phosphoserine Akt were purchased from New England Biolabs (Beverly, MA). Anti-phosphotyrosine Ab 4G10 was from Upstate Biotechnology (Lake Placid, NY). Anti-MMAC1 Ab BL72 was described previously (36). PI 3-kinase inhibitors wortmannin and LY294002 were from Calbiochem (La Jolla, CA). Histone H2B and insulin were from Sigma.

Cloning and transfections

Avian Akt was cloned from a DT40 cDNA library (prepared by T. McClanahan, DNAX, Palo Alto, CA) by PCR using primers based on the chicken Akt1 cDNA sequence (GenBank accession AF039943). The human MMAC1 cDNA and the catalytically inactive MMAC1 mutant MMAC1(55) were described previously (36, 37). Both avian Akt and human MMAC1 were subcloned into the pAPuroFL vector, and cells were stably transfected by electroporation (9).

Biochemical analysis

Cells were washed twice in room temperature PBS, resuspended in serum-free RPMI medium, and then incubated at 37°C for 2 h. For inhibition of PI 3-kinase, cells were pretreated for 15 min with inhibitor before stimulation with M4 or insulin. A total of 2–4 × 10^7 cells was lysed in 1% Nonidet P-40, 50 mM MOPS, pH 7, 150 mM NaCl, 2 mM EDTA, 5 mM sodium orthovanadate, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium fluoride, and 2 mM PMSE (lysis buffer) for 15 min on ice. Cell lysates were clarified by centrifugation at 12,000 × g for 10 min. Abs were added to clarified lysates, incubated on ice for 45 min, followed by addition of protein A-agarose beads. Lysates were mixed at 4°C for 1–2 h and washed in lysis buffer. Precipitated proteins were separated by SDS-PAGE, followed by Western blot analysis. For in vitro Akt kinase assays, the precipitates were washed three times in washing buffer (25 mM HEPES, pH 7, 1 mM NaCl, 0.1% BSA, 10% glycerol, 1% Triton X-100) and once in kinase buffer (20 mM HEPES [pH 7], 10 mM MgCl2, 10 mM MnCl2, 0.2 mM EGTA). After the final wash, beads were resuspended in kinase buffer containing 1 mM DTT, 5 mM ATP, 10 mM [γ-32P]ATP (Amersham), and 500 ng histone H2B (Roche Molecular Biochemicals, Burlington, Boehringer Mannheim, Indianapolis, IN). Reactions were incubated for 30 min at 30°C and terminated by the addition of SDS sample buffer.

Apoptosis assay

Cells (5 × 10^5/ml) were incubated with mAb M4 at 2 μg/ml. Flow cytometry analyses of apoptotic cells were conducted after 20 h using the TUNEL in situ cell death detection kit (Roche Molecular Biochemicals). Flow cytometry was performed using a FACScan (Becton Dickinson, San Diego, CA) and analyzed using CellQuest software (Becton Dickinson).

Results

Activation of Akt following BCR ligation

The ability of lymphoid cells to respond to extracellular stimuli with the activation of cell death or survival pathways is an important regulatory mechanism during an immune response. The identification of Akt as the cellular homologue of v-Akt, which induces lymphomas in rats (24), suggests that this kinase might play an important role in lymphoid cell survival. To investigate a possible function of Akt in B cell survival, we chose the chicken DT40 cell system, an established model for the analysis of BCR-mediated signal transduction.

Comparison of the primary structure of Akt kinases from various species reveals a high degree of sequence conservation. Chicken Akt, for example, shares 96% sequence identity with human Akt1, thus allowing use of anti-human Akt reagents for detection. DT40 B cells express easily detectable levels of Akt when either immunoprecipitated and blotted with an anti-Akt Ab (Fig. 1, left panel) or by blotting whole cell lysate (not shown). Using a variety of different Akt antibodies, we could only detect expression of one Akt isoform in DT40 cells (data not shown). Stimulation of DT40 cells through the B cell Ag receptor using an anti-chicken IgM Ab, M4, led to increased Akt kinase activity after 5 min of stimulation, as measured by phosphorylation of histone H2B in an in vitro kinase assay (Fig. 1, right panel).

While activation of Akt has been shown to provide a survival signal to many cell types, stimulation of DT40 cells through the Ag receptor leads to apoptosis (8). To determine whether quantitative or temporal differences of Akt activation may explain the lack of survival in DT40 cells, a time course of Akt activation stimulated with either anti-Akt Ab and either immunoablotted with anti-Akt Ab (left panel) or assayed in vitro for kinase activity using H2B as a substrate (right panel).
apoptosis (Fig. 2B). In addition, costimulation of the insulin receptor and BCR did not augment Akt activation nor enhance survival (Fig. 2B). Similarly, a 30-min pretreatment with insulin followed by M4 did not reduce the apoptotic response (not shown). This suggests that other signaling pathways besides Akt contribute to the outcome of B cell survival and that these pathways exist in a balance, which together produce the appropriate B cell response.

Activation of Akt by Ag receptor stimulation provides a survival signal
To investigate the hypothesis that the balance of death and survival pathways determines cell survival, we artificially altered this balance to favor either Akt activation or inhibition. To shift the balance of signals to favor Akt activation, stable clones overexpressing chicken Akt were compared with parental cells for their ability to rescue BCR-induced apoptosis. Cells were stimulated with M4 and cultured for 18 h. Apoptotic cells were enumerated using the TUNEL assay method. As shown in Fig. 3A, high levels of Akt expression (clones 7 and 9) led to increased cell survival (Fig. 3A). In the lower expressing clone (clone 3), while still expressing more Akt than parental cells, no effect on apoptosis was observed, suggesting that activation of Akt is tightly controlled by factors other than expression levels, and only when levels of Akt are above a certain threshold can this regulation be overcome. The inhibition of apoptosis observed with clone 9 was not sensitive to the PI 3-kinase inhibitor LY294002 (data not shown), suggesting that high levels of Akt are active independent of PI 3-kinase.

To rule out the possibility that the antiapoptotic effect of Akt overexpression, as seen with clones 7 and 9, is merely a peculiarity of two isolated clones, we repeated the experiment and established another series of independent clonal lines (Fig. 3B). DT40 cells were transfected with the Akt expression vector (clones 6', 8', and 13') or with empty vector as control (C), and stable lines were selected as before. Akt clone 6' and the vector control (C) show Akt expression levels comparable with parental DT40 cells (P). In contrast, Akt clones 8' and 13' exhibit highly elevated Akt protein levels.
levels. In this experiment, we also analyzed the constitutive phosphorylation of Akt on S473 as a relative readout for activity. As shown in Fig. 3B (bottom panel), only in clone 13, which shows the highest level of Akt expression, could we detect constitutive S473 phosphorylation. Consistent with the previous experiment (Fig. 3A), BCR-induced apoptosis is significantly reduced (24% of the rate seen with parental DT40 cells) in clone 13, but not in clone 8, which shows high levels of Akt, but no constitutive activity (Fig. 3B, bar graph).

Inhibition of Akt activation by LY294002 or exogenous expression of MMAC1 enhances apoptosis

Since artificially shifting the balance of BCR-induced signals to favor Akt activation-promoted cell survival, we explored the effect of inhibiting Akt signals. Our initial approach was to inactivate Akt by homologous recombination. While we were successful in inactivating one allele, we were not able to obtain viable clones in which both alleles are inactivated. Given our previous observations that inactivation of both alleles is lethal in these cells. Therefore, for this purpose, we used inhibitors of PI 3-kinase to study the function of Akt in DT40 cells.

Others have shown that chemical inhibition of PI 3-kinase activity effectively inhibits activation of Akt in DT40 cells (38). To investigate the effect of Akt inhibition on cell survival, DT40 cells were pretreated with LY294002 compound or vehicle, DMSO, stimulated with M4, and assayed for Akt activation (Fig. 4A) and cell survival (Fig. 4B). In agreement with previous reports, activation of Akt is efficiently blocked by LY294002 in DT40 cells and, consistent with Akt playing a survival role, LY294002 significantly enhanced BCR-induced apoptosis from 37.4% (M4 alone) to 74.8% (M4 plus LY294002) (Fig. 4B). Similar results were observed with wortmannin (data not shown).

A recently described lipid phosphatase, MMAC1 or PTEN, has been shown to regulate Akt activity in a number of cell types by catalyzing the reverse reaction of PI 3-kinase, effectively diminishing levels of PIP3 in the plasma membrane following growth factor stimulation with M4, and assayed for Akt activation (Fig. 4). We next investigated the signaling mechanisms required for activation of Akt. Early signaling events in BCR activation have been well characterized both biochemically and genetically through the use of cell lines and genetically altered mice. Specific requirements for the activation of PI 3-kinase and Akt in B cells, however, have only recently been addressed. Two recent studies that analyzed the requirements for Akt activation in DT40
cells reported contradicting results (21, 39). The study by Li et al. (21) provided evidence that Syk kinase is a requirement for Akt activation and, further, that Lyn kinase acts as an endogenous inhibitor of Akt activation. In contrast, Gold et al. reported that activation of Akt was completely dependent on Lyn kinase and that Syk kinase was required for sustained phosphorylation of Akt. Thus, to definitively identify upstream molecules required for Akt activation in B cells, we utilized mutant DT40 cell lines previously established by Kurosaki and coworkers (reviewed in Ref. 3). DT40 lines, which lack expression of Lyn, Syk, PLC-\(\gamma\), and Btk, were analyzed for their ability to activate Akt upon BCR ligation. Activation of Akt in Btk\(^{-/-}\) and PLC-\(\gamma\)\(^{-/-}\) cells was not significantly altered compared with parental cells, indicating their functions are not critical for Akt activation (Fig. 7, panels 1, 2, and 3). Surprisingly, DT40 cells, which lack Lyn tyrosine kinase, consistently gave an earlier and more robust response compared with parental cells (Fig. 7, panel 4), suggesting a negative regulatory role for Lyn in Akt activation. By contrast, cells lacking Syk tyrosine kinase showed no detectable activation of Akt, arguing that Syk is an essential molecule in this activation pathway (Fig. 7, lower panels). In the double knockout cell line, Lyn\(^{-/-}\)Syk\(^{-/-}\), a slight yet reproducible increase in Akt activation was observed, possibly due to basal PI 3-kinase activity from other receptors. Our findings are in agreement with Li et al. (21), and support that Akt activation in response to BCR cross-linking occurs via activation of PI 3-kinase in a pathway initiated by Syk activation.

### Discussion

Signals from the B cell Ag receptor and coreceptors such as CD19, CD22, and FcγRIIB become integrated inside the cell, leading to a response, which is largely determined by antigenic history, stage of development, and microenvironment of the B cell. While multiple pathways, which become activated following BCR ligation, have been identified, the contribution of each path to cell fate is unclear. This study addresses two important questions relating to the role of Akt in B cell function. First, does activation of Akt following BCR ligation influence B cell fate? And second, how is activation of Akt regulated in B cells?
As reported by others, Akt is activated following BCR ligation in DT40 cells, an immature B cell line, in A20 cells, a memory B cell line, and in primary B cells (21, 22, 38, 39). The functional consequence of Akt activation in B cells, however, has not been explored. We addressed this question in the DT40 cells. It is quite intriguing that Akt, a well-known survival factor, is activated following BCR ligation in DT40 cells, yet the cell’s response to this stimulus is programmed cell death. Thus, we suggest that both death and survival pathways are activated by BCR cross-linking, and that these pathways are in a fine balance with each other, and, depending on internal and external factors, the balance may be shifted. To test this, we artificially shifted the balance of pathways away from Akt activation either chemically with PI 3-kinase inhibitors, LY294002, or wortmannin (not shown), or by exogenous expression of MMAC1, a lipid phosphatase. BCR-induced apoptosis was significantly enhanced, suggesting that the PI 3-kinase/Akt pathway provides a survival signal to the cell. Furthermore, shifting the balance of pathways to favor Akt activation by increasing the expression levels of Akt led to an almost complete block of receptor-induced apoptosis, further supporting the idea that Akt activation sends a survival signal to B cells following Ag receptor ligation. Interestingly, at this high Akt expression level, the activity of Akt was PI 3-kinase independent, suggesting that localization to the plasma membrane is not necessary for activation in this case. High levels of Akt protein were also necessary to observe constitutive Akt phosphorylation/activity in DT40 transfectants, and it is possible that Akt levels need to exceed a certain threshold level for this constitutive phosphorylation to occur. Only in DT40 cells that showed constitutive Akt phosphorylation was BCR-mediated apoptosis significantly inhibited, suggesting that Akt is directly mediating the antiapoptotic effect in these cells. Consistent with this interpretation are findings of Li et al. (40), who studied the apoptotic effect of MMAC in human tumor cell lines. Interestingly, a constitutively active form of Akt, but not active PI 3-kinase, was able to suppress MMAC-mediated apoptosis in their experiments. Although the mechanism of Akt activation in our overexpressing DT40 clones is at present unclear, it is consistent with the transforming potential of Akt and the fact that it is overexpressed in a number of human tumors.

Interestingly, stimulation of DT40 cells with insulin or M4 resulted in Akt activation with very similar kinetics. Further co-stimulation with insulin plus M4 or pretreatment of cells with insulin had no significant effect on the apoptotic response or the overall activation of Akt kinase. This could suggest that in DT40 cells the majority of available Akt protein is activated in response to BCR stimulation. Alternatively, activation of an upstream component in the Akt signaling pathway, such as PI 3-kinase, could be a limiting factor in these cells. The data further underscore the notion that BCR ligation results in the activation of a proapoptotic pathway, which overrides the antiapoptotic signal generated by Akt.

Our findings that quantitative differences in Akt activation influence B cell fate may advance our understanding of why stimulation through the B cell Ag receptor can lead to multiple outcomes: anergy, apoptosis, proliferation, or differentiation. The integration of numerous signaling pathways influenced by both extrinsic factors such as microenvironment and context of Ag, and intrinsic factors such as levels of accessory molecules and coreceptors, leads to the appropriate response from the cell. We would predict that activation of the PI 3-kinase/Akt pathway would play a major role in determining the outcome of BCR ligation in B cells. Immature B cells generally respond to Ag receptor ligation with apoptosis, while mature B cells typically respond with proliferation and differentiation. A number of factors could potentially contribute to the signal balance. For instance, while immature B cells express only IgM isotype Ag receptors and lower levels of CD19, mature B cells express both IgM and IgD Ag receptors and elevated levels of CD19 (41–44). While differences in signal transduction through IgM and IgD Ag receptors remain to be identified, it is conceivable that components of the respective receptor complexes may differ, leading to qualitatively distinct outcomes. In addition, memory B cell lines typically express IgG, IgE, or IgA Ag receptors. A possible shift in balance of signals may occur via the YXXM motif found in the cytoplasmic tail of IgG and IgE Ag receptors. This motif is characterized as a PI 3-kinase-binding region and may function in memory cells to enhance Akt activation either through bypassing the requirement for CD19 phosphorylation and its negative regulation by CD22 or by additive enhancement of PI 3-kinase activation. These questions remain to be addressed.

The second objective of this study was to identify mechanisms for regulation of Akt activity in B cells. While regulation of PI 3-kinase activity in B cells has not been well characterized, two conflicting reports indicate that the tyrosine kinases Syk and Lyn play significant roles in Akt activation in B cells (21, 39). Our findings are in agreement with Li et al., who demonstrate that Syk kinase is absolutely required for BCR-induced Akt activation and that Lyn kinase plays an antagonistic role in Akt activation. Similar findings have recently been reported by Craxton et al. (45). Craxton et al. (45) also reported that Btk was required for full Akt activation. In our experiments, however, we could not detect a significant effect on Akt activation in Btk−/− cells.

CD22, which is tyrosine phosphorylated by Lyn upon BCR ligation (13), and CD19 have been shown to have counterregulatory effects on BCR-mediated signaling pathways, including activation of extracellular signal–related kinase 2, c-Jun N-terminal kinase, and p38 (46). Negative regulation by CD22 occurs via recruitment of SHP-1 to the phosphorylated tyrosine in the immunoreceptor tyrosine-based inhibitory motif. As one substrate for SHP-1 is CD19 (47), and phosphorylation of CD19 is the major pathway for PI 3-kinase activation in B cells (16), we postulate that activation of Akt following BCR ligation is regulated by Syk phosphorylation of CD19 and that phosphorylation of CD22 mediated by Lyn kinase may inhibit the activation of Akt by inhibiting activation of PI 3-kinase. Experiments are in progress to further analyze the regulation of Akt in B cells.

At many stages during B cell development, the decision to live or die is mediated through Ag receptor signals. This study points to the PI 3-kinase/Akt pathway as a major factor in determining cell fate. While Akt has been shown to play an important role in maintenance of cell survival in fibroblasts and epithelial cells (48–50), its effects on cell survival have not been investigated in lymphocytes. Our data clearly demonstrate for the first time that the PI 3-kinase/Akt is critical in determining B cell fate by preventing apoptosis. While a number of potential substrates for Akt have been identified in many cell types, further studies will be necessary to identify the specific targets Akt utilizes to block apoptosis in lymphocytes and especially in tumor cells.

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References


