BAFF controls B cell metabolic fitness through a PKCβ- and Akt-dependent mechanism

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B cell life depends critically on the cytokine B cell–activating factor of the tumor necrosis factor family (BAFF). Lack of BAFF signaling leads to B cell death and immunodeficiency. Excessive BAFF signaling promotes lupus-like autoimmunity. Despite the great importance of BAFF to B cell biology, its signaling mechanism is not well characterized. We show that BAFF initiates signaling and transcriptional programs, which support B cell survival, metabolic fitness, and readiness for antigen-induced proliferation. We further identify a BAFF-specific protein kinase C β–Akt signaling axis, which provides a connection between BAFF and generic growth factor–induced cellular responses.

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Abbreviations used: 4E-BP1, eIF4E-binding protein 1; BCR, B cell antigen receptor; BAFF, B cell–activating factor of the TNF family; BAFF-R, BAFF receptor; eIF4E, eukaryotic translation initiation factor 4E; GO, gene ontology; GSK-3, glycogen synthase kinase–3; PI3K, phosphoinositide 3–kinase; PKCβ and PKCδ, protein kinase C β and δ, respectively; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TMRE, tetramethylrhodamine ethyl ester; TSC2, tuberous sclerosis complex 2.

The regulation of B cell life span is essential for normal immunity. The short life of newly generated immature B cells assures their rapid turnover and permits selection of nonautoreactive and functionally fit mature B cells. The longevity of mature B cells is required to maintain a highly diverse immunoglobulin repertoire in preparation for antigenic challenge. The exceeded life span of antigen-experienced memory cells assures the most efficient immune response to recurring pathogens (1, 2).

The survival of B cells is sustained by a constant and apparently ligand-independent tonic signal mediated by the cell surface–expressed B cell antigen receptor (BCR) (3, 4). In addition, survival of B cells is supported by B cell–activating factor of the TNF family (BAFF; also known as BLyS) produced by stromal cells and various cell types of myeloid origin. BAFF promotes B cell survival by a triggering of the B cell surface–expressed BAFF receptor (BAFF-R) (5, 6).

Both BCR and BAFF-R signals are crucial for B cell survival. Genetic ablation of the BCR from mature B cells leads to B cell death (3, 4). Similarly, inactivating mutations in BAFF or BAFF-R or BAFF neutralization by a genetically engineered soluble BAFF-binding protein reduce B cell life span and lead to immunodeficiency (7–12). Conversely, increased levels of BAFF in the serum of mice that express both endogenous and transgenic BAFF extend B cell survival beyond physiological limits (13, 14), but the negative effect of this prolonged B cell life span causes BAFF-transgenic mice to develop lupus-like autoimmune disease.

The signaling mechanisms of BAFF-mediated B cell survival are not very well understood. It is known that binding of BAFF to BAFF-R causes processing of NF-κB2 precursor protein p100 to active p52, which has the potential to initiate transcription of antiapoptotic genes such as members of the Bcl-2 family (15, 16). BAFF also counteracts BCR-induced up-regulation of Bim in an Erk-dependent fashion (17). Furthermore, BAFF promotes the cytoplasmic retention of protein kinase C δ (PKCδ), thus containing the proapoptotic potential of PKCδ upon translocation to the nucleus (18). However, it is not clear whether this effect reflects a direct impact of BAFF on PKCδ or the overall physiological condition of BAFF-treated B cells.

It is well established that the bioenergetic state of cytokine-treated hematopoietic cells and T lymphocytes correlates directly with their survival (for review see reference 19). Prosurvival cytokines such as IL-3 and IL-7 promote nutrient uptake, increase glycolysis rates, and regulate mitochondrial energy homeostasis (20, 21). Therefore, it seems plausible that, similar to other prosurvival cytokines, BAFF also controls B cell survival by the regulation of B cell

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metabolic fitness. In this paper, we identify PKCβ and Akt as critical components of a BAFF-induced signaling chain that controls B cell survival and metabolic fitness.

RESULTS

BAFF promotes B cell metabolic fitness and prepares for antigen-induced proliferation

Incubation of mature B cells with BAFF drastically changes the state of B cell metabolism. Treatment with BAFF increases B cell size, cellular protein content, and mitochondrial membrane potential (Fig. 1, A–C). BAFF-treated cells show changes in gene transcription that direct these cells toward production of proteins required for glycolytic metabolism and cell cycle progression. Analysis of polyribosome-associated and, hence, actively translated mRNAs revealed two large mRNA clusters that were particularly prominent in BAFF-treated B cells (Fig. 2, A–C; and Table S1, available at http://www.jem.org/cgi/content/full/jem.20060990/DC1). The first cluster contains mRNAs collectively related to glycolysis. The second cluster overrepresented in BAFF-stimulated B cells contains mRNAs for proteins controlling cell cycle, chromosome condensation, and mitosis. The BAFF-induced up-regulation of cell cycle progression proteins such as cyclin D and cyclin E, Cdk4, Mcm2 and 3, the proliferation marker Ki67, and Survivin was independently confirmed by protein expression analysis (Fig. 3 A). Analysis of total RNA from BAFF-treated cells showed that the abundance of these transcripts reflects overall changes in the pattern of gene expression rather then BAFF-mediated recruitment of the selected mRNAs into polyribosomes (Table S2).

Stimulation with BAFF resulted not only in the transcription and translation of genes required for cell cycle progression but also in phosphorylation of the key cell cycle controlling Rb protein (Fig. 3 A, bottom), which is prerequisite for the release of E2F and cell cycle entry into S phase (22). Despite the substantial accumulation and modification of cell cycle–controlling proteins, BAFF does not induce B cell proliferation in vitro (Fig. 3 B). However, preincubation of B cells with BAFF accelerates proliferation in response to BCR stimulation compared with BCR-only triggered cells (Fig. 3 B). This result points to a role of BAFF in maintaining B cells in a state of immediate responsiveness to antigenic stimulation.

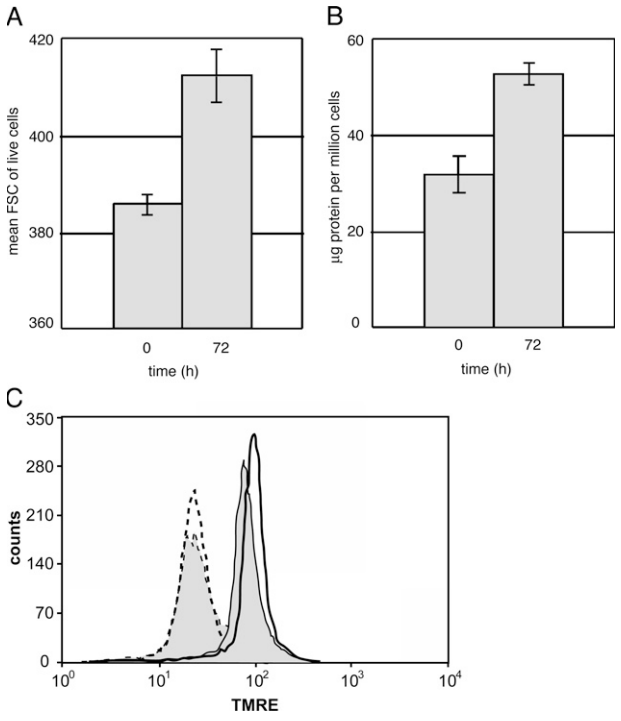

Figure 1. BAFF increases size, protein content, and mitochondrial membrane potential of B cells. Data represent three independent experiments. (A) Mature B cells were incubated in culture medium in the presence of BAFF, and the cell size was measured by FACS analysis of the forward scatter (FSC). (B) Protein amount per million B cells. Error bars in A and B represent SD. (C) Mitochondrial membrane potential was measured by TMRE labeling. Histograms show the TMRE fluorescence of B cells incubated in the absence (shaded area, continuous line) or presence (open area, bold line) of BAFF. In control samples (dashed lines), membrane potential was dissipated by addition of the uncoupling agent carbonyl cyanide m-chlorophenyl hydrazone.

BAFF activates Akt and induces phosphorylation of key regulatory Akt targets

Increased protein synthesis in response to BAFF suggests that BAFF controls activation of proteins required for translation. Indeed, treatment with BAFF caused phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) and its inhibitor, eIF4E-binding protein 1 (4E-BP1; Fig. 4 A). Both are required for active protein synthesis: phosphorylation of eIF4E increases its binding to capped mRNAs, whereas 4E-BP phosphorylation disrupts its binding to eIF4E. Treatment with BAFF also leads to phosphorylation of S6 ribosomal protein, a hallmark of active protein synthesis (23–25).

Phosphorylation of 4E-BP, which is known to be controlled by Akt and Pim-2 (19), suggested an involvement of these kinases in BAFF signaling. Activation of Akt requires its recruitment to the plasma membrane, where it binds to the lipid second messenger phosphatidyl inositol-3,4,5-triphosphate via its pleckstrin homology domain. This induces a conformational change, which allows for Akt phosphorylation at threonine 308 by PDK1 and at serine 473 by a second kinase, whose identity is still controversial (26, 27).

We found that treatment with BAFF led to a rapid phosphorylation of Akt at both S473 and T308, which, after a transitory decline after 1 h of stimulation, regained a stably high level by 24 h and beyond (Fig. 4 B and not depicted). Activation of Akt by BAFF was associated with the phosphorylation of several Akt targets known to control cell survival and metabolism (Fig. 4 C). First, treatment with BAFF lead to phosphorylation of the GTPase-activating protein tuberous sclerosis complex 2 (TSC2), which promotes protein synthesis through activation of mammalian target of

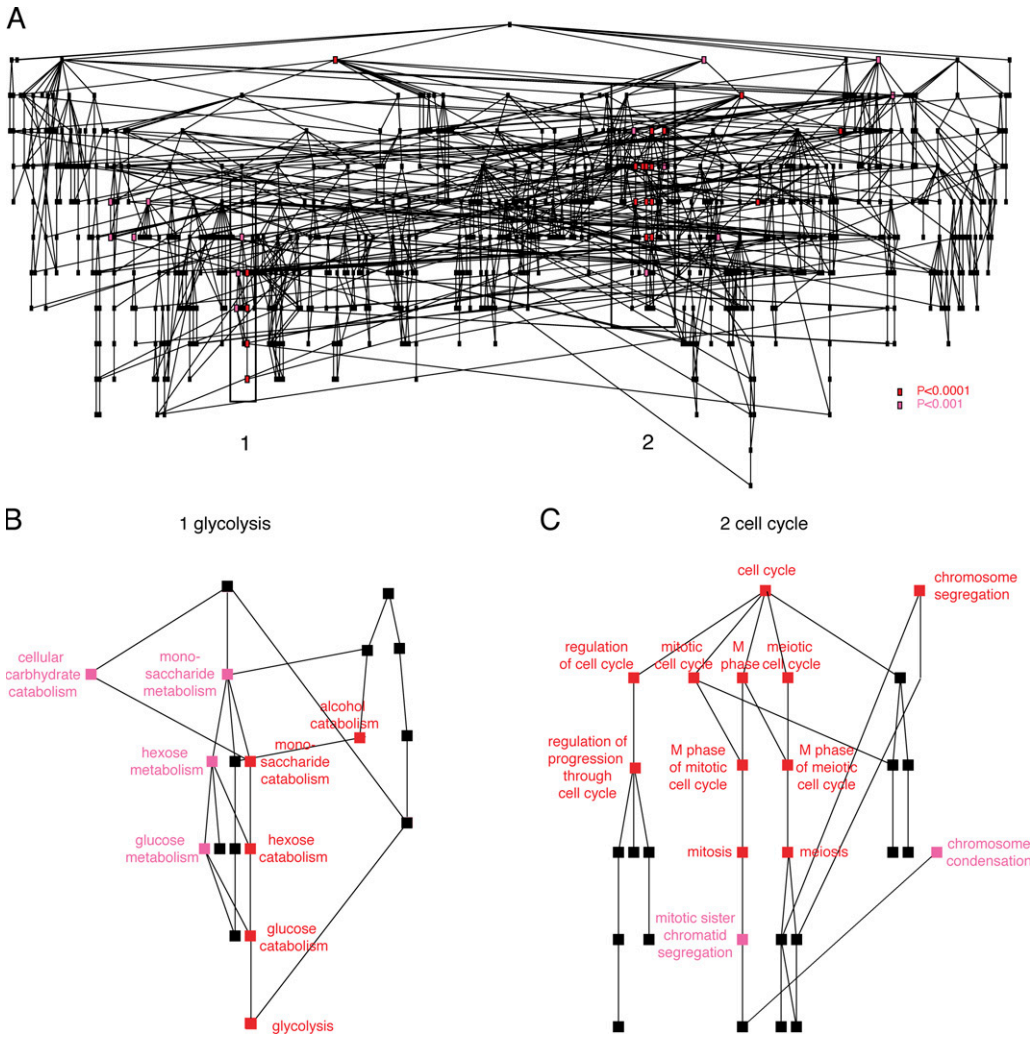

Figure 2. BAFF induces selective changes in gene transcription. The expression pattern and levels of polysome-associated mRNAs in unstimulated and BAFF-treated B cells were determined by Affymetrix microarray analysis (Table S2). The biological significance of BAFF-induced changes in gene expression was then analyzed using the GO tool GoMiner. GO categories representing biological processes are displayed as a directed acyclic graph, and those overrepresented in BAFF-stimulated cells are marked. (A) Colors indicate a statistical significance of $P < 0.001$ (pink) or $P < 0.0001$ (red). (B and C) Two prominent clusters of overrepresented GO categories are framed and displayed as 1 glycolysis and 2 cell cycle. Labeling represents GO "Biological Process" category names. Colors denote p-values as in A. Gene identities for the displayed GO categories are listed in Table S1.

rapamycin (28, 29). Second, BAFF induced the phosphorylation and, hence, inactivation of glycogen synthase kinase–3 (GSK-3). GSK-3 has been found to cause apoptosis by inducing MCL1 degradation and compromising mitochondrial membrane integrity (30). Finally, stimulation of B cells with BAFF led to phosphorylation of FoxO1 and its subsequent degradation. Members of the FoxO family of transcription factors are known to play a key role in the regulation of genes required for cell cycle progression, glycolytic metabolism, and survival (31, 32).

Growth factor stimulation of hematopoietic cells induces Pim-2 expression, which confers resistance to atrophy and cell death (33). Treatment with BAFF strongly induced all three isoforms of Pim-2 within 24 h of stimulation (Fig. 4 D).

BAFF-induced Akt activation depends on phosphoinositide 3-kinase (PI3K) activity

Akt-activation requires its binding to specific phosphoinositides, which are generated by PI3K at the plasma membrane (27). The predominant form of PI3K in B cells consists of the regulatory subunit p85α and the catalytic subunit p110δ (34). We found that treatment with BAFF led to tyrosine phosphorylation of a p85-associated protein with a molecular weight of approximately p110 (Fig. 5 A). Sequence analysis of a protein that co-migrated with phospho-p110 confirmed that this protein represents the catalytic subunit p110δ (unpublished data). It is therefore possible that BAFF activates PI3K by inducing the tyrosine phosphorylation of its catalytic subunit. A similar phenomenon has previously been reported in B cell lines stimulated through the BCR (35, 36).

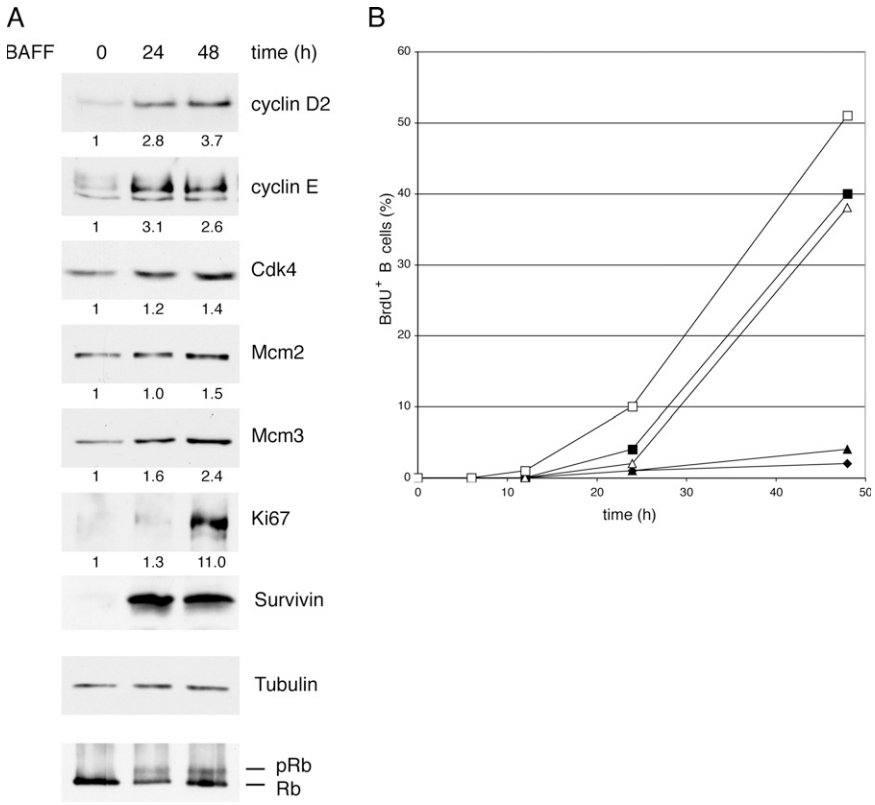

Figure 3. BAFF induces up-regulation of cell cycle–controlling proteins but not entry into S phase. (A) Purified B cells were incubated with BAFF for the indicated times, and the expression levels of cell cycle regulatory proteins cyclin D2, cyclin E, Cdk4, Mcm2, Mcm3, Ki67, Survivin, and Rb were measured by Western blotting. Protein loading was controlled by tubulin expression level analysis. Numbers represent the fold induction normalized to the Tubulin signal. Hyperphosphorylated Rb (pRb) is identified by a migratory shift. (B) B cell proliferation in vitro was measured by BrdU incorporation. Frequencies of $BrdU^+$ B cells incubated in medium alone (diamonds); in the presence of BAFF (closed triangles), anti-IgM (closed squares), or BAFF and anti-IgM (open triangles); or after preincubation with BAFF for 24h followed by anti-IgM treatment for the indicated times (open squares) are shown.

The activation of PI3K by BAFF is important for the regulation of Akt activation and B cell survival. Treatment of B cells with the PI3K-specific inhibitor LY294002 diminished BAFF-induced activation of Akt and phosphorylation of Akt target proteins (Fig. 5 B and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060990/DC1). Consistently, BAFF-mediated B cell survival was severely impaired by concentrations of LY294002, which abolish Akt activity (Fig. 5 C). The half-life of the phosphoinositide products of PI3K is limited by the lipid phosphatase and tensin homologue deleted on chromosome 10 (PTEN), but BAFF did not appear to affect PTEN activity (Fig. S2), as judged by phosphorylation of the inhibitory PTEN phosphorylation site serine 380 (37).

PKCβ controls BAFF-mediated activation of Akt

Akt activation requires two sequential events. Initially, Akt binding to the phosphoinositide products of PI3K induces a conformational change in the protein. This allows for its phosphorylation at two essential sites: T308 in the activation loop is phosphorylated by PDK1, whereas the identity of the kinase that phosphorylates the C-terminal S473 residue remains to be determined (26, 27). In search of the kinase, which is responsible for Akt S473 phosphorylation and B cell survival in response to BAFF, we tested the role of PKCβ in BAFF-mediated signaling. This choice was based on two major reasons. First, coincubation with recombinant PKCβ leads to Akt phosphorylation at S473 but not T308 (38). Second, PKCβ deficiency in mice causes a decline in B cell survival in vitro and in vivo (39, 40).

We initially assessed the ability of BAFF to activate PKCβ by analysis of BAFF-induced PKCβ translocation to the plasma membrane. This translocation is an activation hallmark for phospholipid-dependent classic PKCs, including PKCβ (41–43), and triggering of B cells through the BCR leads to translocation of PKCβ into specialized membrane microdomains called lipid rafts (44). We found that treatment of B cells with BAFF increased the amount of membrane-associated PKCβ (Fig. 6 A). A similar level of BAFF-induced membrane translocation was observed for Akt (Fig. 6 A). Notably, membrane translocation of PKCβ in response to BAFF occurred outside lipid rafts (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20060990/DC1). Because lipid rafts of BAFF-triggered B cells did also not contain Akt or PDK1, we conclude that BAFF-induced Akt activation is a raft-independent event.

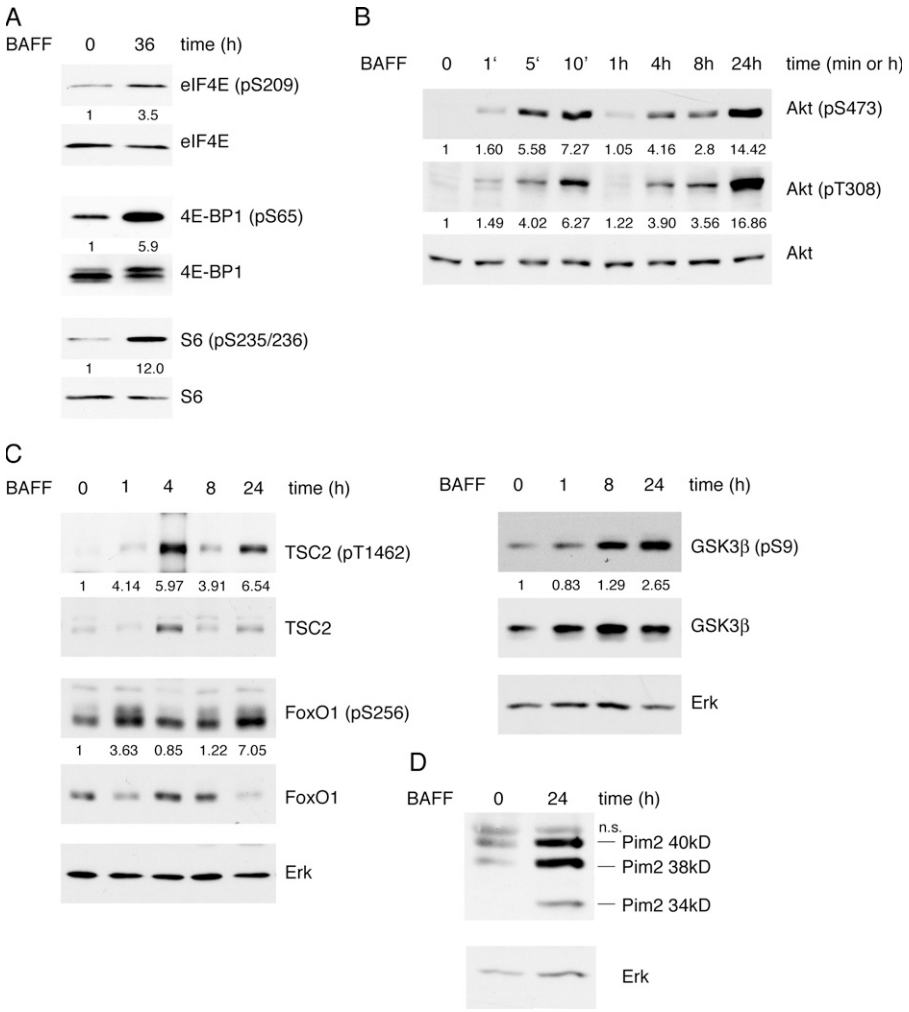

Figure 4. BAFF activates proteins required for protein translation and cell survival. (A) Purified B cells were incubated with BAFF for the indicated times, and phosphorylation of proteins that control translation was measured by Western blot analysis of eIF4E (pS209), 4E-BP1 (pS65), and S6 (pS235/236). Membranes were stripped and reprobed with nonphosphospecific antibodies against the respective proteins. Numbers represent the fold change of the phosphospecific signal normalized to the nonphosphospecific signal. (B) BAFF-induced Akt phosphorylation on S473 and T308 was measured by immunoblot analysis using the respective phosphospecific antibody (top). Akt expression was controlled by reprobing with a nonphosphospecific Akt antibody. Quantification was done as in A. (C) BAFF-induced phosphorylation of TSC2 at T1462 and of FoxO1 at S256 were measured by sequential immunoblot analysis (left). Erk is provided as a loading control to illustrate changes in FoxO1 total protein content. BAFF-induced phosphorylation of GSK-3β at S9 was measured in a similar fashion on a separate gel (right). In all cases, the amount of tested proteins was measured by reprobing the immunoblots with nonphosphospecific antibodies against the respective proteins. Quantification was done as in A. (D) BAFF induces expression of Pim-2. The positions of three different Pim-2 isoforms are indicated. Erk expression is provided as a protein loading control in C and D. n.s., nonspecific.

In addition to PKCβ translocation to the plasma membrane, BAFF promotes the association of PKCβ with Akt (Fig. 6 B). This association does not depend on PI3K activity (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20060990/DC1), but it is functionally important, as BAFF-induced Akt phosphorylation at S473 was greatly reduced in PKCβ-deficient B cells (Fig. 6 C). In contrast, Akt phosphorylation at the PDK1 target site T308 was similar between wild-type and mutant cells. The functional link between PKCβ and Akt is specific to BAFF signaling, as stimulation through the BCR induced Akt phosphorylation in PKCβ-deficient B cells at wild-type levels (Fig. 6 D).

Loss of PKCβ has an impact on BAFF-mediated cellular responses

PKCβ-deficient B cells exhibit poor survival in vitro in the absence of stimuli (Fig. 7 A) (40). Treatment with BAFF promotes the viability of mutant B cells, although they fail to reach wild-type survival levels regardless of the BAFF dose and the duration of treatment. Although PKCβ-deficient cells were partially responsive to the survival action of BAFF, its ability to support B cell growth in vitro was severed by lack of PKCβ (Fig. 7, A and B). These results suggest that the PKCβ-mediated Akt signaling element contributes mostly to BAFF-mediated B cell fitness rather than mere survival. Altered

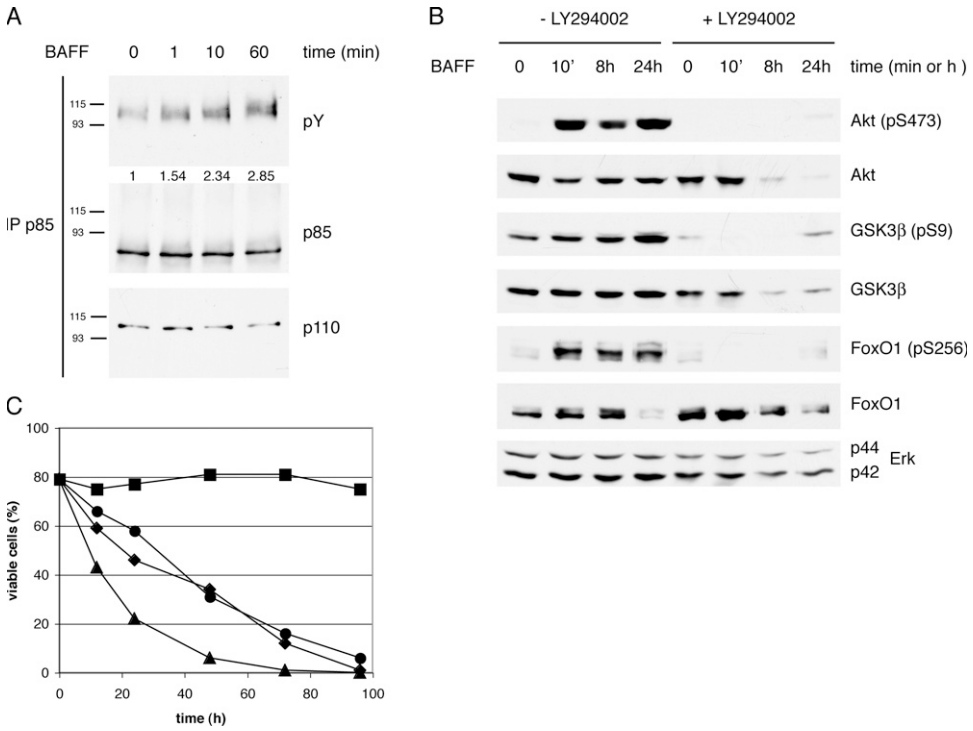

Figure 5. PI3K controls BAFF-induced Akt activation and B cell survival. (A) BAFF induces phosphorylation of p110. The PI3K subunit p85 was immunoprecipitated from extracts of BAFF-treated cells, and tyrosine-phosphorylated proteins in immunoprecipitates were analyzed by Western blotting using antiphosphotyrosine antibody. The amounts of p85 and p110 in the immunoprecipitates were measured by Western blot analysis. (B) PI3K inhibition suppresses BAFF-induced Akt, GSK-3β, and FoxO1 phsophorylation. B cells were stimulated with BAFF in the absence or presence of LY294002, and protein phosphorylation was measured as described in Fig. 4. (C) Inhibition of PI3K reduces survival of BAFF-treated cells. B cells were cultured in medium alone (circles) or in the presence of BAFF (squares), LY294002 (triangles), or BAFF together with LY294002 (diamonds). The frequencies of viable B cells were determined by FACS analysis.

cellular responses of PKCβ-deficient B cells were not caused by defective surface expression of BAFF-R (Fig. 7 C), arguing for a cell-intrinsic BAFF signaling defect in the absence of PKCβ.

The involvement of PKCβ in BAFF-mediated signaling is indirectly supported by the pattern of changes in the PKCβ-deficient, peripheral B cell compartment. In the absence of BAFF signaling, B cell maturation arrests between the T1 ($CD21^{lo}$) and the T2 ($CD21^{hi}$) stages (7, 10–12). PKCβ deficiency impairs B cell maturation in a fashion similar to failed BAFF signaling, albeit at a lower magnitude. In addition, the size of the peripheral PKCβ-deficient B cell compartment falls to ~70% of wild-type levels (39), and immature B cells prevail over mature B cells in the spleen of mutant mice, as judged by surface IgM and IgD expression (Fig. 7 D). Collectively, these observations suggest a partial defect in BAFF-mediated signaling and peripheral B cell maturation in the absence of PKCβ.

PKCβ does not control BAFF-induced regulation of NF-κB and PKCδ

BAFF's prosurvival function has been linked to its ability to activate the processing of the NF-κB2 protein p100 into the transcriptionally active form p52 (15, 16). We find that BAFF-induced p100-p52 processing was not affected by the absence of PKCβ (Fig. 8 A). We have previously shown that BAFF controls B cell survival through cytoplasmic retention of the proapoptotic kinase PKCδ (18). However, the ratio of cytoplasmic versus nuclear PKCδ was not altered by PKCβ deficiency (Fig. 8 B). We conclude that the BAFF-mediated regulation of NF-κB and PKCδ occurs independent of PKCβ.

DISCUSSION

Our findings show that like cytokines such as IL-3 and IL- 7, which promote survival of hematopoietic cells, BAFF supports B cell survival by increasing metabolic fitness. Treatment of B cells with BAFF induces transcription of mRNAs that encode components of carbohydrate metabolism. The BAFF-induced metabolic bias toward glycolysis might be especially important for B cell survival in lymphoid organs and at inflammatory sites where oxygen tension is low compared with arterial blood (45, 46). In this environment, activation of glycolysis will provide energy to sustain the active protein synthesis and cell growth caused by BAFF. Indeed, prolonged BAFF treatment led to expression of the hypoxia-inducible factor α (unpublished data).

Normally, accumulation of proteins, particular those controlling cell cycle, and increase in cell volume precedes cell

division. However, the lack of BrdU incorporation in BAFF-treated cells shows that BAFF does not induce DNA-replication, thus precluding cell division. Previous findings show persistent expression of Cdk inhibitor proteins p18 and p27 in BAFF-treated cells (47). Therefore, it is likely that high expression of these and possibly other cell cycle inhibitors prevents the proliferation of BAFF-treated B cells in the absence of an antigenic signal.

BAFF-stimulated cells enter BCR-induced proliferation more readily than untreated cells. This result suggests that the accumulation of cell cycle–controlling proteins in response to BAFF prepares resting B cells for an immediate immune response upon antigenic challenge. It is also attractive to speculate that, in response to BAFF, B cells might establish a storage pool of certain cell cycle–controlling mRNAs or proteins, which could be used for several rounds of cell division. Such a mechanism would help explain the astonishingly short replication time (~7 h) of B cells at the height of the germinal center response (48, 49).

Akt activation requires two potentially independent pathways. The PI3K-dependent pathway is shared both by BAFF-R and BCR. This feature of the pathway makes it particularly important in the regulation of B cell immunity and explains the poor survival of B cells in the absence of the p85 regulatory subunit of PI3K (50, 51). The pathway that involves PKCβ appears to be BAFF specific. Thus, PKCβ deficiency impairs phosphorylation of Akt on activating serine 473 in response to BAFF but not BCR stimulation. The residual BAFF-induced Akt S473 phosphorylation, which we observe in the absence of PKCβ, indicates an ability of other kinases to partially adopt this role. This would be expected, as a plethora of kinases has previously been implicated in mediating Akt S473 phosphorylation (52).

Increased translocation of PKCβ to the plasma membrane and PKCβ association with Akt in response to BAFF suggest a direct involvement of PKCβ in BAFF signaling. Furthermore, PKCβ dependency of Akt phosphorylation at S473 but not T308 agrees with in vitro data on Akt phosphorylation by PKCβ (38) and suggests a direct action of PKCβ on S473 of Akt upon BAFF stimulation.

Involvement of PKCβ in BAFF and BCR signaling provides a mechanistic explanation for the poor survival and

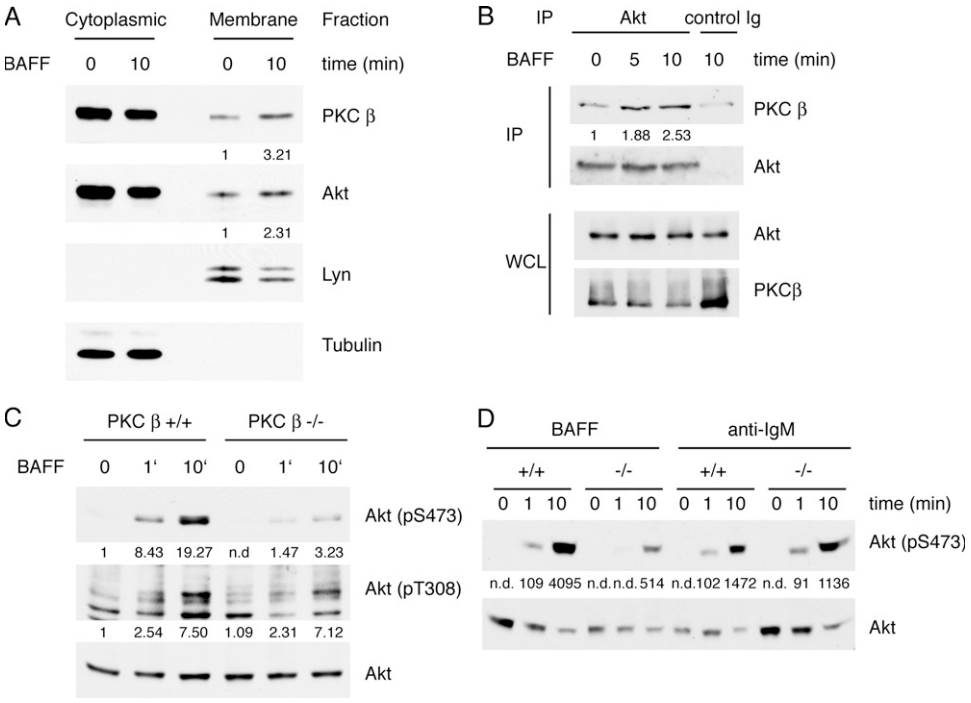

Figure 6. PKCβ controls BAFF-induced Akt phosphorylation. (A) BAFF induces membrane translocation of PKCβ. B cells were stimulated with BAFF followed by preparation of cytoplasmic and membrane protein extracts. PKCβ and Akt content in the extracts was measured by Western blotting. Fraction purity and protein loading was controlled by Western blotting using antibodies against Tubulin and Lyn. Numbers represent the fold change of PKCβ or Akt content in the membrane fractions normalized to the amount of Lyn. (B) BAFF induces PKCβ-Akt association. B cells were stimulated with BAFF, and protein extracts were treated with Akt antibody or control serum. The amounts of PKCβ or Akt in immunoprecipitates (IP) or whole cell lysates (WCL) were measured by Western blot analysis. Amounts of PKCβ in Akt-IPs were quantified as fold change over the corresponding Akt signal. (C) Akt phosphorylation levels in B cell lysates derived from control and BAFF-treated PKCβ$^{+/+}$ and PKCβ-deficient mice were measured and quantified as described in Fig. 4. n.d., no band detected by the quantification software (see Materials and methods). (D) B cells from PKCβ$^{+/+}$ and PKCβ-deficient B cells were stimulated with BAFF or anti-IgM antibody as indicated. Akt phosphorylation in cell lysates was measured and quantified as described in C. Because there was no band detected by the quantification software at time 0 (n.d.), numbers in D represent the total quantified area of the phosphospecific signal normalized to the total Akt content.

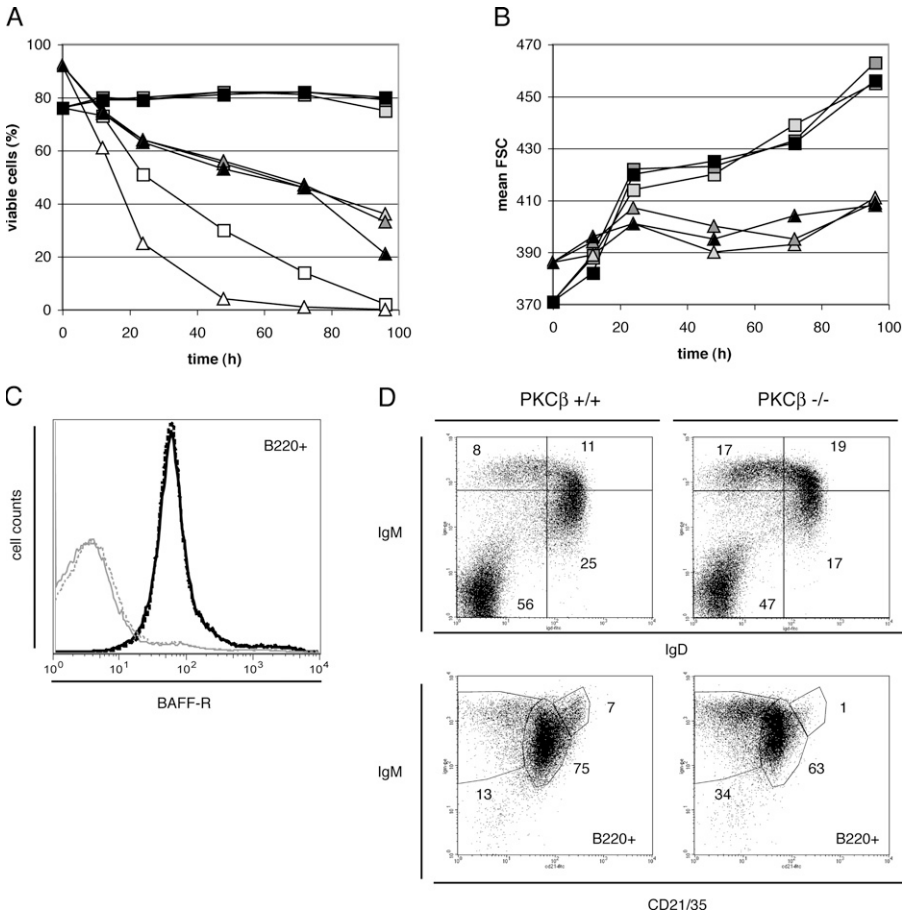

Figure 7. BAFF-mediated cellular responses of PKCβ-deficient B cells are altered in vitro and in vivo. (A) B cells from PKCβ$^{+/+}$ (squares) and PKCβ-deficient (triangles) B cells were cultured in medium alone (open symbols) or in the presence of 25 ng/ml (light gray symbols), 100 ng/ml (dark gray symbols), or 250 ng/ml (closed symbols) of BAFF, and the frequencies of viable B cells were measured by FACS analysis. (B) The cell size of live BAFF-treated PKCβ$^{+/+}$ (squares) and PKCβ-deficient (triangles) B cells was measured by FACS analysis of the forward scatter (FSC). Symbol shadings represent the same BAFF concentrations as in A. (C) BAFF-R expression on splenic B220-positive cells from PKCβ$^{+/+}$ (continuous black line) and PKCβ-deficient (dashed black line) was measured by FACS. Gray lines (continuous and dashed) represent staining with an isotype control antibody. (D) Maturity of splenic B cells from PKCβ$^{+/+}$ (left) and PKCβ-deficient (right) mice was assessed by expression of the surface markers IgD versus IgM (top) and CD21/35 versus IgM (bottom). Only live lymphocytes are shown, and the bottom panel is gated on B220-positve cells. Numbers in the top panels represent frequencies of cells in the respective quadrants. Gates in the bottom panels denote B cell developmental stages T1 (IgM^{hi} CD21^{lo}), T2 (IgM^{hi} CD21^{hi}), and mature (IgM^{int} CD21^{int}).

altered peripheral maturation of PKCβ-deficient B cells in vitro and in vivo. However, the relatively mild reduction of B cell numbers in the absence of PKCβ argues in favor of additional PKCβ-independent signaling pathways initiated by BAFF-R and/or BCR. Signaling from both receptors induces activation of NF-κB, albeit through distinct mechanisms. Although PKCβ has an important function in canonical NF-κB activation upon BCR-triggering, BAFF-induced NF-κB2 processing is independent of PKCβ. Another PKC family member, PKCδ, also plays an important role in the regulation of B cell survival, but it promotes cell death rather than survival. The proapoptotic potential of PKCδ is contained by BAFF, which prevents its accumulation in the nucleus. This BAFF-dependent survival mechanism can also function in the absence of PKCβ.

BAFF-mediated B cell survival likely represents the collective outcome of several BAFF-induced signaling features, including NF-κB activation, cytoplasmic retention of PKCδ, and Akt activation. It is thus conceivable that the loss of a single signaling branch can to some degree be compensated for. In this case, the loss of PKCβ would be expected to cause some damage to B cell survival, but it would be less severe than the complete abrogation of BAFF signaling. This could explain the more dramatic changes to the B cell compartment in BAFF- or BAFF-R–deficient mice than in PKCβ knockouts. Although PKCβ-deficient B cells were partially responsive to the survival action of BAFF, they appeared to be largely refractive to BAFF-mediated cell growth. This cellular process is closely associated with Akt- and mammalian target of rapamycin–dependent

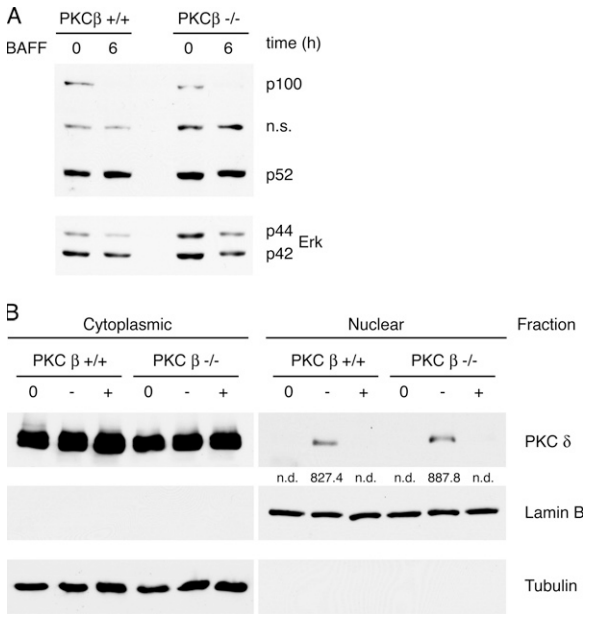

Figure 8. (A) Unaltered BAFF-induced NF-B2/p100 processing in the absence of PKC. The presence of p100 and p52 was determined by Western blot analysis using an antibody against NF-κB2. Protein loading was controlled by Erk (p44/p42) expression. (B) Unaltered BAFF-induced cytoplasmic retention of PKCδ in the absence of PKCβ. Cytoplasmic and nuclear extracts were prepared from PKCβ$^{+/+}$ and PKCβ-deficient B cells at time 0 and after 24 h of incubation in the absence (−) or presence (+) of BAFF. PKCδ expression was assessed by Western blotting. Fraction purity and protein loading were controlled by Western blotting using antibodies against Tublin and Lamin B. n.d., no band detected by the quantification software (see Materials and methods).

signaling pathways and represents an important distinction between growth factor–mediated cellular fitness on the one hand and mere cell survival on the other (19, 53). The latter can also be achieved through an altered ratio in the expression of pro- and antiapoptotic proteins, but, in this context, cells become progressively smaller (54). It appears that, perhaps in contrast to BAFF-induced NF-κB activation and cytoplasmic retention of PKCδ, which mediate B cell survival (55), PKCβ-dependent Akt activation preferentially regulates the fitness facet of BAFF-mediated cellular responses.

Our findings may have practical implications. There is increasing evidence that BAFF plays an important role in the control of autoreactive B cells (8, 9, 13, 14, 56, 57). Therefore, identification of Akt and PKCβ as components of BAFF signaling may suggest novel ways of pharmacological intervention in B cell–mediated autoimmune disorders.

MATERIALS AND METHODS

Mice. Mice were housed in the Laboratory Animal Research Center at the Rockefeller University under specific pathogen-free conditions. C57BL/6 mice were used as a source of wild-type B cells. PKCβ-deficient mice on a C57BL/6 genetic background were used for analysis (39, 40). Mice were used at ages of 8–12 wk. Protocols were approved by the Institutional Animal Care and Use Committee at the Rockefeller University.

B cell purification and in vitro culture. Resting mature CD43^{-}, CD62L^{+} B cells were isolated from spleen and lymph nodes by MACS (Miltenyi Biotec) according to the manufacturer's instructions. The purity of mature B cells was routinely >95%. Cells were cultured for 12–16 h in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-mercapto-ethanol, and 100 U/ml penicillin/streptomycin at 37°C in a CO_2 incubator to reduce the background BAFF signaling caused by endogenous BAFF in vivo. This preincubation did not alter B cell size. After preincubation, live cells were separated over a Ficoll gradient (Cedar Lanes), and their purity was assessed by trypan blue exclusion. Cells were stimulated in culture medium using 25 ng/ml BAFF (R&D Systems) or 1.3 μg/ml F(ab′)$_2$ fragment goat anti–mouse IgM (Jackson ImmunoResearch Laboratories). LY294002 (Calbiochem) was used at a 10-μM concentration.

FACS analysis. Cells were analyzed on a flow cytometer (FACSCalibur; Becton Dickinson) using CellQuest software (BD Biosciences). For measurement of mitochondrial membrane potential, cells were used after 12–16 h of preincubation without BAFF (time point 0) or after 72 h of BAFF stimulation. Cells were incubated in culture medium with or without BAFF and containing the potentiometric dye tetramethylrhodamine ethyl ester (TMRE; Invitrogen) at a 100-nM concentration. Carbonyl cyanide m-chlorophenyl hydrazone (Sigma-Aldrich) was added to control samples at a concentration of 50 μM as an uncoupler of the mitochondrial respiratory chain. After 30 min of incubation at 37°C in a CO_2 incubator, cells were immediately analyzed by FACS. For BrdU incorporation, B cells were cultured in the presence of 10 μM BrdU, and fixed and stained using the FITC BrdU Flow Kit (BD Biosciences). For cell death analysis, an aliquot of ∼10^5 cells was removed from the B cell culture at various time points ranging from 0 to 96 h. TO-PRO-3 (Invitrogen) was added at a concentration of 10 nM to distinguish live (TO-PRO-3^{-}) and dead (TO-PRO-3^{+}) cells in the culture samples. Upon TO-PRO-3 addition, cells were immediately analyzed by FACS. B cell surface marker expression was analyzed using antibodies against IgD, CD21/35, B220 (BD Biosciences), BAFF-R (R&D Systems), or a F(ab′)$_2$ fragment of goat anti–mouse IgM (Jackson ImmunoResearch Laboratories), as previously described (58).

Immunoblot analysis and immunoprecipitation. Cells were lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 1% NP-40, 10 μM NaF, 2 mM Na_3VO_4, and protease inhibitor cocktail (Sigma-Aldrich). Lysates were resolved by SDS-PAGE and transferred onto Immobilon-P membrane (Millipore) using standard procedures. Membranes were developed with the following antibodies against: pS6 (S235/236), S6, peIF4E (S209), eIF4E, p4E-BP1 (S65), 4E-BP1, pAkt (S473), pAkt (T308), pGSK-3β (S9), GSK-3β, pFoxO1 (S256), FoxO1, pTSC2 (T1462), pPTEN (S380), PTEN, and Erk (all obtained from Cell Signaling Technology); Pim-2, Akt, TSC2, and PKCβ (all obtained from Santa Cruz Biotechnology, Inc.); p85, p110δ, and pY (4G10; all obtained from Upstate Biotechnology); and tubulin (Sigma-Aldrich). Where applicable, membranes were first developed with phosphospecific antibodies, and stripped and reprobed with the respective control antibody. For Akt immunoprecipitation, lysates were precleared with goat-serum and protein G–Sepharose (GE Healthcare), followed by incubation with anti-Akt antibody overnight. Anti–ferritin heavy chain antibody (Santa Cruz Biotechnology, Inc.) was used as a goat immunoglobulin control. Immune complexes were precipitated with protein G–Sepharose, washed five times in lysis buffer, and eluted in SDS sample buffer for SDS-PAGE. For p85 immunoprecipitation, lysates were precleared with rabbit immunoglobulin and protein A–Sepharose (GE Healthcare), followed by incubation with p85 antibody–agarose conjugate overnight. Immune complexes were washed and eluted as described earlier in this section. For analysis of NF-κB2 p100 processing and for the expression of cell cycle proteins, cells were lysed in a buffer containing 350 mM NaCl, 20 mM Hepes/NaOH, pH 7.9, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 1% NP-40, 20% glycerol, 10 μM NaF, 2 mM Na_3VO_4, and protease inhibitor cocktail (Sigma-Aldrich). Lysates were analyzed by SDS-PAGE, and membranes were incubated with antibodies against NF-κB2 p52 (Upstate

Biotechnology) or cyclin D2, cyclin E, Cdk4, and Mcm3 (all obtained from Santa Cruz Biotechnology, Inc.); Mcm2 and Rb (obtained from BD Biosciences); Ki67 (DakoCytomation); and Survivin (Chemicon). Signal quantification was done using NIH Image 1.63 software.

Lipid raft isolation. Lipid rafts were prepared as described previously (59). The purity of the preparation was assessed by the presence of Lyn and the lipid raft marker ganglioside GM1, which was detected using horseradish peroxidase–coupled cholera toxin B subunit (Sigma-Aldrich). Proteins were detected using antibodies against PKCβ, Akt, and PDK1 (all obtained from Santa Cruz Biotechnology, Inc.) and Lyn (a gift from C. Lowell, University of California, San Francisco, San Francisco, CA).

Subcellular fractionation. Fractionation of primary mouse B cells into cytoplasmic and nuclear extracts was performed as described previously (18). Lysates were analyzed for expression of PKCδ using an antibody from Santa Cruz Biotechnology, Inc. Fraction purity was assessed by Western blot analysis using tubulin as cytoplasmic and lamin B (Santa Cruz Biotechnology, Inc.) as nuclear markers, respectively. For preparation of cytoplasmic and membrane fractions, cells were disrupted by hypotonic lysis for 15 min on ice in a buffer containing 10 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 10 μM NaF, 2 mM Na_3VO_4, and protease inhibitor cocktail (Sigma-Aldrich). Nuclei were removed by centrifugation at 400 *g* for 10 min. The supernatant was then centrifuged at 95,000 *g* for 1 h in a SW55Ti rotor (Beckman Coulter). The supernatant was removed and saved as the cytoplasmic fraction. The pellet containing the membrane fraction was resolved in SDS sample buffer. Fraction purity was assessed by Western blot analysis using tubulin as cytoplasmic and Lyn as membrane markers, respectively.

Polyribosome purification and RNA isolation. Polyribosomes were purified essentially as described previously (60) from unstimulated cells and those that had been cultured in the presence of BAFF for 36 h. In brief, cells were incubated in culture medium containing 100 μg/ml cycloheximide (CHX; Sigma-Aldrich) for 15 min, washed three times in PBS containing CHX, and lysed in 10 mM Hepes-KOH, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 1% NP-40, 0.5 mM DTT, 100 μg/ml CHX, 200 U/ml RNAsin (Promega), 100 U/ml SUPERase (Ambion), and EDTA-free protease inhibitor cocktail (Roche). All chemicals were of molecular biology–grade purity and nuclease free. Nuclei were removed from the extracts by centrifugation at 2,000 *g* for 10 min. An aliquot of the lysate was saved for preparation of total RNA. The remaining lysate was loaded onto a 20–50% wt/wt linear density sucrose gradient in 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, and 5 mM $MgCl_2$. Gradients were centrifuged for 2 h at 40,000 *g* at 4°C in a SW41 rotor (Beckman Coulter). Fractions of 0.7-ml volume were collected with continuous monitoring at 254 nm using an ISCO UA-6 UV detector. To identify polyribosome-containing fractions, the content of ribosomal S6 protein in the fractions after trichloroacetic acid precipitation was determined by Western blot. For RNA isolation, polysome-containing fractions were pooled, and RNA was isolated from total and polysomal samples using TRIzol LS (Invitrogen) according to the manufacturer's protocol.

Gene expression analysis. Quality of RNA was confirmed before labeling by analyzing 20–50 ng of each sample using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent Technologies). All samples had a 28S/18S ribosomal peak ratio of 1.8–2 and were considered suitable for labeling. 2 μg of total RNA was used for cDNA synthesis using an oligo(dT)-T7 primer and the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Synthesis, linear amplification, and labeling of cRNA were accomplished by transcription in vitro using the MessageAmp aRNA Kit (Ambion) and biotinylated nucleotides (Enzo Diagnostics). 10 μg of labeled and fragmented cRNA were hybridized to the mouse genome MOE430 2.0 array (Affymetrix), which interrogates ~39,000 transcripts at 45°C for 16 h. Automated washing and staining were performed using the Fluidics Station 400 (Affymetrix), according to the manufacturer's protocols. Finally, chips were scanned with a high numerical aperture and flying objective lens in the scanner (GS3000; Affymetrix). Raw expression data were analyzed using Microarray Analysis software (version 5.1; Affymetrix). The complete datasets can be accessed at http://www.ncbi.nlm.nih.gov/geo under accession no. GSE5985. Data were normalized to a target intensity of 500 to account for differences in global chip intensity. Genes, which were differentially expressed between unstimulated and BAFF-treated cells, were initially identified using GCOS software. 377 transcripts showed a change of twofold or more (Table S2) and were selected for further analysis. Approximately three quarters of these genes were up-regulated upon BAFF stimulation, whereas one quarter was down-regulated. For a biological interpretation of the differentially regulated genes, the GoMiner gene ontology (GO) tool was used. GO is a genome project that describes gene products in terms of their associated biological processes, cellular components, and molecular functions (61). GO categories are organized in directed acyclic graphs, a kind of hierarchy in which one category can have more than one "parent." GoMiner identifies GO categories that are over- or underrepresented in lists of genes of interest, such as differentially expressed genes from a microarray experiment, and calculates statistical significance as the one-sided nominal unadjusted p-value from Fisher's exact test (62). We report only those BAFF-induced biological processes in which the p-value was <0.001.

Protein identification. Gel-resolved proteins were digested with trypsin, batch purified on a reversed-phase microtip, and resulting peptide pools were individually analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS; ultraflex TOF/TOF; Bruker Daltronics Inc.) for peptide mass fingerprinting (PMF), as previously described (63). Selected peptide ions (m/z) were taken to search a nonredundant (NR) protein database (3,245,378 entries on 28 January 2006; National Center for Biotechnology Information) using the Peptide-Search algorithm (developed by Matthias Mann, Max-Planck-Institute for Biochemistry, Martinsried, Germany; an updated version of this program is currently available as PepSea from Applied Biosystems/MDS SCIEX). A molecular mass range up to twice the apparent molecular weight (as estimated from electrophoretic relative mobility) was covered, with a mass accuracy restriction of <35 ppm and a maximum of one missed cleavage site allowed per peptide. To confirm PMF results with scores ≤40, mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples using the ultraflex instrument in "LIFT" mode. Fragment ion spectra were taken to search the NR protein database using the Mascot MS/MS ion search program (version 2.0.04 for Microsoft Windows; Matrix Science Ltd.) (64). Any tentative confirmation (Mascot score ≥30) of a PMF result thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

Online supplemental materials. Fig. S1 shows BAFF-induced Akt phosphorylation in the absence or presence of LY294002. Fig. S2 shows PTEN phosphorylation at serine 380, as well as total PTEN content in cell extracts prepared at various time points of BAFF stimulation. In Fig. S3, the distribution of PKCβ, Akt, and PDK1 into lipid rafts was analyzed before and after BAFF stimulation. Lipid raft–containing fractions are marked by the presence of Lyn and GM1. Fig. S4 shows BAFF-induced PKCβ-Akt association, which is not affected by the presence of LY294002. Table S1 lists the gene IDs for the GO categories presented in Fig. 2 (B and C). Table S2 contains the complete list of Affymetrix probe sets that showed a fold change of two or more in BAFF-stimulated versus unstimulated cells. Results are given both for RNA samples isolated from polysomes and analyzed in Fig. 2 (sheet 1 = polysome), as well as RNA samples derived from total cell lysates (sheet 2 = total). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20060990/DC1.

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REFERENCES

1. Rajewsky, K. 1996. Clonal selection and learning in the antibody system. *Nature*. 381:751–758.
2. Fulcher, D.A., and A. Basten. 1997. B cell life span: a review. *Immunol. Cell Biol.* 75:446–455.
3. Lam, K.P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell*. 90:1073–1083.
4. Kraus, M., M.B. Alimzhanov, N. Rajewsky, and K. Rajewsky. 2004. Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. *Cell*. 117:787–800.
5. Mackay, F., P. Schneider, P. Rennert, and J. Browning. 2003. BAFF and APRIL: a tutorial on B cell survival. *Annu. Rev. Immunol.* 21:231–264.
6. Schneider, P. 2005. The role of APRIL and BAFF in lymphocyte activation. *Curr. Opin. Immunol.* 17:282–289.
7. Schiemann, B., J.L. Gommerman, K. Vora, T.G. Cachero, S. Shulga-Morskaya, M. Dobles, E. Frew, and M.L. Scott. 2001. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science*. 293:2111–2114.
8. Gross, J.A., S.R. Dillon, S. Mudri, J. Johnston, A. Littau, R. Roque, M. Rixon, O. Schou, K.P. Foley, H. Haugen, et al. 2001. TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease: impaired B cell maturation in mice lacking BLyS. *Immunity*. 15:289–302.
9. Gross, J.A., J. Johnston, S. Mudri, R. Enselman, S.R. Dillon, K. Madden, W. Xu, J. Parrish-Novak, D. Foster, C. Lofton-Day, et al. 2000. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. *Nature*. 404:995–999.
10. Yan, M., J.R. Brady, B. Chan, W.P. Lee, B. Hsu, S. Harless, M. Cancro, I.S. Grewal, and V.M. Dixit. 2001. Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency. *Curr. Biol.* 11:1547–1552.
11. Thompson, J.S., S.A. Bixler, F. Qian, K. Vora, M.L. Scott, T.G. Cachero, C. Hession, P. Schneider, I.D. Sizing, C. Mullen, et al. 2001. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science*. 293:2108–2111.
12. Sasaki, Y., S. Casola, J.L. Kutok, K. Rajewsky, and M. Schmidt-Supprian. 2004. TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. *J. Immunol.* 173:2245–2252.
13. Mackay, F., S.A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, J. Tschopp, and J.L. Browning. 1999. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* 190:1697–1710.
14. Khare, S.D., I. Sarosi, X.Z. Xia, S. McCabe, K. Miner, I. Solovyev, N. Hawkins, M. Kelley, D. Chang, G. Van, et al. 2000. Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice. *Proc. Natl. Acad. Sci. USA*. 97:3370–3375.
15. Claudio, E., K. Brown, S. Park, H. Wang, and U. Siebenlist. 2002. BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nat. Immunol.* 3:958–965.
16. Kayagaki, N., M. Yan, D. Seshasayee, H. Wang, W. Lee, D.M. French, I.S. Grewal, A.G. Cochran, N.C. Gordon, J. Yin, et al. 2002. BAFF/BLyS receptor 3 binds the B cell survival factor BAFF ligand through a discrete surface loop and promotes processing of NF-kappaB2. *Immunity*. 17:515–524.
17. Craxton, A., K.E. Draves, A. Gruppi, and E.A. Clark. 2005. BAFF regulates B cell survival by downregulating the BH3-only family member Bim via the ERK pathway. *J. Exp. Med.* 202:1363–1374.
18. Mecklenbräuker, I., S.L. Kalled, M. Leitges, F. Mackay, and A. Tarakhovsky. 2004. Regulation of B-cell survival by BAFF-dependent PKCdelta-mediated nuclear signaling. *Nature*. 431:456–461.
19. Hammerman, P.S., C.J. Fox, and C.B. Thompson. 2004. Beginnings of a signal-transduction pathway for bioenergetic control of cell survival. *Trends Biochem. Sci.* 29:586–592.
20. Vander Heiden, M.G., D.R. Plas, J.C. Rathmell, C.J. Fox, M.H. Harris, and C.B. Thompson. 2001. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol. Cell. Biol.* 21:5899–5912.
21. Barata, J.T., A. Silva, J.G. Brandao, L.M. Nadler, A.A. Cardoso, and V.A. Boussiotis. 2004. Activation of PI3K is indispensable for interleukin 7–mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J. Exp. Med.* 200:659–669.
22. Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell*. 81:323–330.
23. Minich, W.B., M.L. Balasta, D.J. Goss, and R.E. Rhoads. 1994. Chromatographic resolution of in vivo phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: increased cap affinity of the phosphorylated form. *Proc. Natl. Acad. Sci. USA*. 91:7668–7672.
24. Ruggero, D., and N. Sonenberg. 2005. The Akt of translational control. *Oncogene*. 24:7426–7434.
25. Hay, N., and N. Sonenberg. 2004. Upstream and downstream of mTOR. *Genes Dev.* 18:1926–1945.
26. Chan, T.O., and P.N. Tsichlis. 2001. PDK2: a complex tail in one Akt. *Sci. STKE*. 2001:PE1.
27. Vanhaesebroeck, B., and D.R. Alessi. 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* 346:561–576.
28. Manning, B.D., and L.C. Cantley. 2003. Rheb fills a GAP between TSC and TOR. *Trends Biochem. Sci.* 28:573–576.
29. Manning, B.D., and L.C. Cantley. 2003. United at last: the tuberous sclerosis complex gene products connect the phosphoinositide 3-kinase/Akt pathway to mammalian target of rapamycin (mTOR) signaling. *Biochem. Soc. Trans.* 31:573–578.
30. Maurer, U., C. Charvet, A.S. Wagman, E. Dejardin, and D.R. Green. 2006. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol. Cell.* 21:749–760.
31. Burgering, B.M., and G.J. Kops. 2002. Cell cycle and death control: long live Forkheads. *Trends Biochem. Sci.* 27:352–360.
32. Tran, H., A. Brunet, E.C. Griffith, and M.E. Greenberg. 2003. The many forks in FOXO's road. *Sci. STKE*. 2003:RE5.
33. Fox, C.J., P.S. Hammerman, R.M. Cinalli, S.R. Master, L.A. Chodosh, and C.B. Thompson. 2003. The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor. *Genes Dev.* 17:1841–1854.
34. Okkenhaug, K., and B. Vanhaesebroeck. 2003. PI3K in lymphocyte development, differentiation and activation. *Nat. Rev. Immunol.* 3:317–330.
35. Kuwahara, K., T. Kawai, S. Mitsuyoshi, Y. Matsuo, H. Kikuchi, S. Imajoh-Ohmi, E. Hashimoto, S. Inui, M.D. Cooper, and N. Sakaguchi. 1996. Cross-linking of B cell antigen receptor-related structure of pre-B cell lines induces tyrosine phosphorylation of p85 and p110 subunits and activation of phosphatidylinositol 3-kinase. *Int. Immunol.* 8:1273–1285.
36. Craxton, A., A. Jiang, T. Kurosaki, and E.A. Clark. 1999. Syk and Bruton's tyrosine kinase are required for B cell antigen receptor-mediated activation of the kinase Akt. *J. Biol. Chem.* 274:30644–30650.
37. Birle, D., N. Bottini, S. Williams, H. Huynh, I. deBelle, E. Adamson, and T. Mustelin. 2002. Negative feedback regulation of the tumor suppressor PTEN by phosphoinositide-induced serine phosphorylation. *J. Immunol.* 169:286–291.
38. Kawakami, Y., H. Nishimoto, J. Kitaura, M. Maeda-Yamamoto, R.M. Kato, D.R. Littman, M. Leitges, D.J. Rawlings, and T. Kawakami. 2004. Protein kinase C betaII regulates Akt phosphorylation on Ser-473 in a cell type- and stimulus-specific fashion. *J. Biol. Chem.* 279:47720–47725.

39. Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, and A. Tarakhovsky. 1996. Immunodeficiency in protein kinase cbeta-deficient mice. *Science*. 273:788–791.
40. Saijo, K., I. Mecklenbräuker, A. Santana, M. Leitges, C. Schmedt, and A. Tarakhovsky. 2002. Protein kinase C β controls nuclear factor κB activation in B cells through selective regulation of the IκB kinase α. *J. Exp. Med*. 195:1647–1652.
41. Newton, A.C. 2001. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem. Rev*. 101:2353–2364.
42. Babwah, A.V., L.B. Dale, and S.S. Ferguson. 2003. Protein kinase C isoform-specific differences in the spatial-temporal regulation and decoding of metabotropic glutamate receptor1a-stimulated second messenger responses. *J. Biol. Chem*. 278:5419–5426.
43. Collazos, A., B. Diouf, N.C. Guerineau, C. Quittau-Prevostel, M. Peter, F. Coudane, F. Hollande, and D. Joubert. 2006. A spatiotemporally coordinated cascade of protein kinase C activation controls isoform-selective translocation. *Mol. Cell. Biol*. 26:2247–2261.
44. Su, T.T., B. Guo, Y. Kawakami, K. Sommer, K. Chae, L.A. Humphries, R.M. Kato, S. Kang, L. Patrone, R. Wall, et al. 2002. PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling. *Nat. Immunol*. 3:780–786.
45. Makino, Y., H. Nakamura, E. Ikeda, K. Ohnuma, K. Yamauchi, Y. Yabe, L. Poellinger, Y. Okada, C. Morimoto, and H. Tanaka. 2003. Hypoxia-inducible factor regulates survival of antigen receptor-driven T cells. *J. Immunol*. 171:6534–6540.
46. Caldwell, C.C., H. Kojima, D. Lukashev, J. Armstrong, M. Farber, S.G. Apasov, and M.V. Sitkovsky. 2001. Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. *J. Immunol*. 167:6140–6149.
47. Huang, X., M. Di Liberto, A.F. Cunningham, L. Kang, S. Cheng, S. Ely, H.C. Liou, I.C. Maclennan, and S. Chen-Kiang. 2004. Homeostatic cell-cycle control by BLyS: induction of cell-cycle entry but not G1/S transition in opposition to p18INK4c and p27Kip1. *Proc. Natl. Acad. Sci. USA*. 101:17789–17794.
48. Hanna, M.G., Jr. 1964. An autoradiographic study of the germinal center in spleen white pulp during early intervals of the immune response. *Lab. Invest*. 13:95–104.
49. MacLennan, I.C. 1994. Germinal centers. *Annu. Rev. Immunol*. 12:117–139.
50. Fruman, D.A., S.B. Snapper, C.M. Yballe, L. Davidson, J.Y. Yu, F.W. Alt, and L.C. Cantley. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science*. 283:393–397.
51. Suzuki, H., Y. Terauchi, M. Fujiwara, S. Aizawa, Y. Yazaki, T. Kadowaki, and S. Koyasu. 1999. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. *Science*. 283:390–392.
52. Dong, L.Q., and F. Liu. 2005. PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. *Am. J. Physiol. Endocrinol. Metab*. 289:E187–E196.
53. Massague, J. 2004. G1 cell-cycle control and cancer. *Nature*. 432:298–306.
54. Rathmell, J.C., M.G. Vander Heiden, M.H. Harris, K.A. Frauwirth, and C.B. Thompson. 2000. In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. *Mol. Cell*. 6:683–692.
55. Sasaki, Y., E. Derudder, E. Hobeika, R. Pelanda, M. Reth, K. Rajewsky, and M. Schmidt-Supprian. 2006. Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. *Immunity*. 24:729–739.
56. Zhang, J., V. Roschke, K.P. Baker, Z. Wang, G.S. Alarcon, B.J. Fessler, H. Bastian, R.P. Kimberly, and T. Zhou. 2001. Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J. Immunol*. 166:6–10.
57. Pers, J.O., C. Daridon, V. Devauchelle, S. Jousse, A. Saraux, C. Jamin, and P. Youinou. 2005. BAFF overexpression is associated with autoantibody production in autoimmune diseases. *Ann. N. Y. Acad. Sci*. 1050:34–39.
58. Mecklenbräuker, I., K. Saijo, N.Y. Zheng, M. Leitges, and A. Tarakhovsky. 2002. Protein kinase Cdelta controls self-antigen-induced B-cell tolerance. *Nature*. 416:860–865.
59. Dobenecker, M.W., C. Schmedt, M. Okada, and A. Tarakhovsky. 2005. The ubiquitously expressed Csk adaptor protein Cbp is dispensable for embryogenesis and T-cell development and function. *Mol. Cell. Biol*. 25:10533–10542.
60. Stefani, G., C.E. Fraser, J.C. Darnell, and R.B. Darnell. 2004. Fragile X mental retardation protein is associated with translating polyribosomes in neuronal cells. *J. Neurosci*. 24:7272–7276.
61. The Gene Ontology Consortium. 2000. Gene ontology: tool for the unification of biology. *Nat. Genet*. 25:25–29.
62. Zeeberg, B.R., W. Feng, G. Wang, M.D. Wang, A.T. Fojo, M. Sunshine, S. Narasimhan, D.W. Kane, W.C. Reinhold, S. Lababidi, et al. 2003. GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol*. 4:R28.
63. Winkler, G.S., L. Lacomis, J. Philip, H. Erdjument-Bromage, J.Q. Svejstrup, and P. Tempst. 2002. Isolation and mass spectrometry of transcription factor complexes. *Methods*. 26:260–269.
64. Perkins, D.N., D.J. Pappin, D.M. Creasy, and J.S. Cottrell. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 20:3551–3567.