Negative Feedback Regulation of Antigen Receptors through Calmodulin Inhibition of E2A

Jiyoti Verma-Gaur, Jannek Hauser and Thomas Grundström

J Immunol 2012; 188:6175-6183; Prepublished online 11 May 2012;
doi: 10.4049/jimmunol.1103105
http://www.jimmunol.org/content/188/12/6175

Supplementary Material  http://www.jimmunol.org/content/suppl/2012/05/11/jimmunol.1103105.DC1

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References  This article cites 27 articles, 8 of which you can access for free at:
http://www.jimmunol.org/content/188/12/6175.full#ref-list-1

Subscription  Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Negative Feedback Regulation of Antigen Receptors through Calmodulin Inhibition of E2A

Jiyoti Verma-Gaur,¹ Jannek Hauser,¹ and Thomas Grundström

Signaling from the BCR is used to judge Ag-binding strengths of the Abs of B cells. BCR signaling enables the selection for successive improvements in the Ag affinity over an extremely broad range of affinities during somatic hypermutation. We show that the mouse BCR is subject to general negative feedback regulation of the receptor proteins, as well as many coreceptors and proteins in signal pathways from the receptor. Thus, the BCR can downregulate itself, which can enable sensitive detection of successive improvements in the Ag affinity over a very large span of affinities. Furthermore, the feedback inhibition of the BCR signals for most of its proteins, as well as most other regulations of genes by BCR stimulation, is to a large extent through inhibition of the transcription factor E2A by Ca²⁺/calmodulin.

Materials and Methods

Activation and infection of B lymphocytes from mouse spleens

Primary B lymphocytes were purified from mouse spleens, maintained as previously described (5), and stimulated with 5 ng/ml IL-4 (PeproTech) and either 200 ng/ml CD40L (R&D Systems) or 10 μg/ml LPS (Calbiochem) for 48 h. The BCR of stimulated cells at 10⁶ cells/ml, where indicated, was activated by incubation with goat F(ab)² anti-mouse IgM at 2.5 μg/ml (5 μg/ml for Fig. 3A and 3B, and various concentrations, as indicated, for Fig. 3C) for the indicated times. The MSCV-IRESV-GFP–based retroviruses encoding wild-type and CaM-resistant m8N47 mutant of the E12 splice form of mouse E2A were described previously (5). Retrovirus concentrated by centrifugation was added with 5 μg/ml polybrene, 0.5×10⁶ purified B cells after activation with CD40L plus IL-4 for 14 h (Figs. 4, 6B) or 24 h (Fig. 7). After 12 h of incubation, the infection was repeated for 12 h, followed by incubation for an additional 22 h postinfection in fresh complete medium with the stimulants to allow for expression of GFP and E12. In addition to CD40L plus IL-4, the medium was supplemented with LPS (200 ng/ml) during retroviral infection incubations to enable efficient infection. Where indicated, anti-mouse IgM (2.5 μg/ml) was added for the indicated times. For DNA microarray and Western blot analyses of infected B lymphocytes, the B cells were purified from spleens of mice heterozygous for knockout of the E2A gene (6). Infected cells were purified for DNA microarray and real-time RT-PCR analyses with a FACSAria cell sorter (BD Biosciences), using their green fluorescence from expression of the GFP.

DNA microarray analysis

Gene expression changes upon Ag receptor activation with anti-mouse IgM were analyzed using the Illumina BeadChip system (Illumina, San Diego, CA). For in vitro transcription amplification, 200 ng RNA from the time course and 100 ng RNA from the comparison of gene expression in B cells expressing wild-type and CaM-resistant E12 were used with the Illumina RNA Amplification Kit (Ambion). Amplified RNA (1.5 μg) was hybridized to the Sentrix MouseRef-6 Expression BeadChip containing 47,667 probes. The primary data were collected from the BeadChip using the manufacturer’s BeadArray Reader and analyzed using the supplied scanner software. Data normalization was performed by cubic spline normalization using Illumina’s BeadStudio v3 software; fold changes ≥ 1.5 with p < 0.05 were considered significant. Clustering of certain selected genes was

department of Molecular Biology, Umeå University, SE-901 87 Umeå, Sweden. E-mail address: Thomas.Grundstrom@molbiol.umu.se

Abbreviations used in this article: CaM, calmodulin; GC, germinal center; PLA, proximity-ligation assay; SH, somatic hypermutation.

© 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00

Address correspondence and reprint requests to Prof. Thomas Grundström, Department of Molecular Biology, Umeå University, SE-901 87 Umeå, Sweden. E-mail address: Thomas.Grundstrom@molbiol.umu.se

Received for publication November 1, 2011. Accepted for publication April 12, 2012.

This work was supported by grants from the Swedish Research Council and the Swedish Cancer Society.

The datasets presented in this article have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession numbers GSE35747 and GSE35748.

HARVESTED lymphocytes were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% FCS in PBS. 

Proximity-ligation assay

Harvested lymphocytes were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% FCS in PBS. 

Proximity ligation assay (PLA) (10–13) was performed with the Duolink system using in situ PLA kits from Olink Bioscience. In brief, cells were stained for intracellular proteins using one primary mouse or goat Ab that was biotinylated using an Ab biotinylation kit (Miltenyi Biotec) together with a secondary rabbit or mouse Ab. The Abs used were the same as for the determination of protein levels by FACS, except that the goat anti–CD19 Ab C-20, the mouse anti-SYK Ab SYK-01, and the mouse anti-CD79B (anti-Igβ) Ab B29/123 (all from Santa Cruz Biotechnology) were used. Antibiot IgG and anti-rabbit IgG secondary Abs conjugated with oligonucleotides (PLA probes) were subsequently used, according to the manufacturer’s protocol, to generate fluorescence signals only when the two biotinylated Abs were in close proximity to each other. 

We recently performed DNA microarray analysis of the genome-wide transcriptional changes in 3 h when stimulating B cells to SH by CD40L plus IL-4 (14). The expression of a large set of genes (≥4600) differed significantly between the BCR-stimulated and nonstimulated B cells, and the numbers of upregulated and downregulated genes were approximately equal, at 7% of the total number of genes (14). Interestingly, the DNA microarray data for receptor components, coreceptors, and downstream signaling factors of the BCR showed a reduction in the expression of almost all these genes after BCR stimulation of the cells (Fig. 1). This reduction indicates very broad negative feedback regulation of almost the entire receptor–signaling network of the BCR at the mRNA level. This general downregulation was specific to the BCR and not a general property of all the receptors and signaling proteins, because analysis of other signaling networks (Jak/Stat signaling, TLR signaling, receptor tyrosine kinases, TGF-β signaling, ERK signaling, and BAFF receptor signaling) showed no significant effect of BCR stimulation on the expression of those other signaling networks. 

To examine how fast the Ag receptor-driven transcriptional changes of genes for BCR-signaling proteins occurred, we performed a time course analysis of activated primary splenic B cells after BCR activation. The mRNA expression levels of almost all analyzed BCR proteins, coreceptors, and downstream-signaling factors decreased progressively (Fig. 2). The mRNA levels for IgH fragments were from Jackson ImmunoResearch]. When using primary Ab biotinylated using an Ab biotinylation kit (Miltenyi Biotec), the secondary Ab was streptavidin-PerCP (BD Pharmingen) or streptavidin-allophycocyanin (eBioscience). Stainings were done for ≥30 min at room temperature in the dark. Flow cytometry was performed with a FACSCalibur, and analysis was done using CellQuest software (Becton Dickinson Biosciences). 

Protein levels by FACS, except that the goat anti–CD19 Ab C-20, the mouse anti-SYK Ab SYK-01, and the mouse anti-CD79B (anti-Igβ) Ab B29/123 (all from Santa Cruz Biotechnology) were used. Antibiot IgG and anti-rabbit IgG secondary Abs conjugated with oligonucleotides (PLA probes) were subsequently used, according to the manufacturer’s protocol, to generate fluorescence signals only when the two biotinylated Abs were in close proximity to each other. 

RESULTS

Receptor activation downregulates BCR signaling components

We recently performed DNA microarray analysis of the genome-wide transcriptional changes in 3 h when stimulating B cells to SH by CD40L plus IL-4 (14). The expression of a large set of genes (≥4600) differed significantly between the BCR-stimulated and nonstimulated B cells, and the numbers of upregulated and downregulated genes were approximately equal, at 7% of the total number of genes (14). Interestingly, the DNA microarray data for receptor components, coreceptors, and downstream signaling factors of the BCR showed a reduction in the expression of almost all these genes after BCR stimulation of the cells (Fig. 1). This reduction indicates very broad negative feedback regulation of almost the entire receptor–signaling network of the BCR at the mRNA level. This general downregulation was specific to the BCR and not a general property of all the receptors and signaling proteins, because analysis of other signaling networks (Jak/Stat signaling, TLR signaling, receptor tyrosine kinases, TGF-β signaling, ERK signaling, and BAFF receptor signaling) showed no significant effect of BCR stimulation on the expression of the vast majority of those genes. Furthermore, and also in sharp contrast with the BCR-signaling network, upregulations and downregulations were approximately equally common among the genes that did change expression in those other signaling networks.

To examine how fast the Ag receptor-driven transcriptional changes of genes for BCR-signaling proteins occurred, we performed a time course analysis of activated primary splenic B cells after BCR activation. The mRNA expression levels of almost all analyzed BCR proteins, coreceptors, and downstream-signaling factors decreased progressively (Fig. 2). The mRNA levels for IgH
and the signaling proteins BLK and JNK2 decreased <2-fold. However, in most cases, the reductions were 2–10-fold after 3 h: a minority of the reductions occurred within 1 h, but a majority of the reductions occurred within 2 h (Fig. 2). The signaling protein Shp-1 and the coreceptors CD21 and CD72 were exceptions whose mRNA level was unchanged or even showed an increase (Figs. 1, 2). Thus, activation of BCR signaling leads to negative feedback regulation of almost all receptor-signaling components (Figs. 1, 2). However, in most cases, the reductions were 2–10-fold after 3 h: a minority of the reductions occurred within 1 h, but a majority of the reductions occurred within 2 h (Fig. 2). The signaling protein Shp-1 and the coreceptors CD21 and CD72 were exceptions whose mRNA level was unchanged or even showed an increase (Figs. 1, 2). Thus, activation of BCR signaling leads to negative feedback regulation of almost all receptor-signaling components (Figs. 1, 2). We performed flow cytometry analyses to determine whether the rapid reductions in the mRNA levels of analyzed genes after BCR activation resulted in corresponding changes in the protein levels. Most mRNA reductions led, with a delay, to reductions in the protein (Fig. 3A). At 3 h, when the mRNA levels for most genes were already reduced 50–90% (Figs. 1, 2), the protein levels were rarely reduced >30%; several proteins showed very small reductions or no reductions at all (Fig. 3A). At 5 h after BCR activation, the protein levels were reduced 30–60%, with few exceptions. CD81 was an exception for which the reduction in mRNA was not followed by a reduction in protein. Surprisingly, despite the increase in CD21 mRNA when activating the BCR (Fig. 2), the CD21 protein level was reduced ∼2-fold. Thus, the mechanism of CD21 downregulation after triggering the BCR appears to be posttranscriptional. With regard to BLK, for which the reduction in mRNA was small or absent (Figs. 1, 2), no significant reduction was found in the protein level (Fig. 3A).

BCR signaling reduces the sensitivity of the BCR
To analyze whether there is indeed a negative feedback regulation of Ag receptor function as indicated by the rapid reductions in many mRNA and protein levels, we compared the activating phosphorylation of JNK, ERK, and p38 proteins downstream of the BCR after an initial BCR stimulation with anti-IgM and a second stimulation 6 h later. These proteins were chosen because, in contrast to most other BCR signalosome proteins, none or very little reduction was seen in Jnk2 and Erk mRNA, and the different p38 mRNA showed opposite responses to BCR stimulation (Fig. 1). Thus, for these proteins, phosphorylation upon a second BCR stimulation would show the ability of the whole receptor to function the second time, rather than reflecting only the level of the individual protein, provided that the protein level reflects the mRNA level. The activating phosphorylation was strongly induced 15 and 30 min after the initial BCR stimulation for all three proteins (Fig. 3B, quantifications in Supplemental Fig. 2). After 4 h with anti-IgM (5 μg/ml), followed by 2 additional hours of incubation without anti-IgM, the phosphorylation level of all three was close to background (Fig. 3B, quantifications in Supplemental Fig. 2). Importantly, after these 6 h, a second BCR stimulation was unable to induce any of the activating phosphorylations. This was not due to a corresponding reduction in the individual protein, because JNK and ERK protein levels did not change, in contrast to the BTK control (Fig. 3B, quantifications in Supplemental Fig. 2). With regard to p38, for which only the p38α mRNA was reduced (Figs. 1, 2), there was a reduction in p38α protein; however, it was too small to account for the complete block of induction of phospho-p38 upon the second BCR stimulation (Fig. 3B, quantifications in Supplemental Fig. 2). Thus, activation of the BCR leads to a strong reduction in signaling through the receptor (see also below).

Next, we analyzed whether BCR signaling reduces the sensitivity of the BCR. A weaker stimulation of the BCR (0.75 μg/ml anti-IgM) inhibited its ability to respond to a second stimulation of the same strength (Fig. 3C). Importantly, however, BCR signaling was also induced the second time when the strength of the stimulation was increased (cf. BCR restimulations at concentrations ≥0.75 μg/ml of anti-IgM in Fig. 3C). Thus, BCR signaling reduces the sensitivity of the BCR.

Genome-wide role of inhibition of E2A by Ca2+/CaM in B cells after BCR activation
BCR activation leads to a signaling complex below the receptor that rapidly produces a combination of Ca2+ signaling and a cascade of phosphorylations (15). To investigate whether Ca2+ sig-
naling and/or serine protein kinases were essential for negative feedback after BCR stimulation, we first analyzed the activation in the presence of various inhibitors of the signaling pathways for one selected downregulated gene, CD19. The Ca\(^{2+}\) chelator BAPTA-AM completely blocked the reduction in CD19 mRNA after BCR stimulation, and inhibitors of L-type or IP\(_3\) receptor Ca\(^{2+}\) channels partially or completely blocked the effect, especially when combined (Supplemental Fig. 1C). Thus, Ca\(^{2+}\) signaling is essential, even though inhibitors of Ca\(^{2+}\)/CaM-dependent protein kinase (KN93) or the Ca\(^{2+}\)/CaM-dependent phosphatase calcineurin (cyclosporin A) did not block this negative feedback (Supplemental Fig. 1C). The MAPK inhibitor PD98059, but not the protein kinase C inhibitor bisindolylmaleimide, blocked the reduction in CD19 mRNA (Supplemental Fig. 1C), suggesting that, as with Ca\(^{2+}\) signaling, MAPK signaling is important. We decided to analyze the Ca\(^{2+}\)-signaling dependence for the entire group of transcriptionally downregulated proteins.

Previous studies showed that the main Ca\(^{2+}\)-sensor protein CaM, when Ca\(^{2+}\) loaded, can bind to and inhibit transcriptional activities of E2A transcription factors (5, 7, 9, 14, 16, 17). The E2A proteins have important roles in the transcriptional regulatory network that promotes commitment to and differentiation of B cells (18, 19). We reported a series of mutants of the E2A isoform E12 that gains resistance to CaM to different extents (9). To elucidate the genome-wide role of Ca\(^{2+}\)/CaM inhibition of E2A in the regulatory effects of BCR activation, we used the CaM-resistant E12 mutant m8N47, which has three amino acid substitutions in the CaM binding site and retains the sensitivity of E2A to Id protein inhibitors (20). Purified primary splenic B cells from mice heterozygous for the E2A gene were infected with retroviruses expressing either wild-type E12 or CaM-resistant E12, together with GFP. Infected B cells were purified by cell sorting based on their GFP expression, and the BCR was activated for half of these cells by anti-IgM treatment for 3 h. Comparison of the changes in the DNA microarray profile upon BCR-stimulation of wild-type E2A-infected B cells with those of noninfected B cells (Figs. 1, 2) revealed a very strong overlap (data not shown), which validated our experimental conditions. Comparison of the expression levels of BCR-regulated genes in wild-type E2A-infected B cells with those of CaM-resistant E12-infected B cells showed that 83% of the genes that were downregulated and 87% of the genes that were upregulated when infected to express wild-type E12 lost >50% of the BCR stimulation effect on their expression when infected to express CaM-resistant E12 (Fig. 4A). Thus Ca\(^{2+}\)/CaM inhibition of E2A is needed for a majority of the regulatory effect for most of the genes that are rapidly up- or downregulated by activation of the BCR of the splenic B cells. In summary, Ca\(^{2+}\)/CaM inhibition of E2A is very broadly used to shift the transcriptional program upon activation of the BCR of these cells.

**Mechanism of feedback regulation of BCR-signaling components**

BCR activation downregulated the mRNA levels of most of the BCR-signaling components in the cells expressing wild-type E12 as in the case of uninfected B cells (Figs. 2, 4B), although the extent of this effect was reduced in some cases, presumably due to the infection, the overexpression of wild-type E12, or both. In contrast, the mRNA expression levels of Igl, Iga, Cd20, Cd22, Cd72, Cd81, Lyn, Blk, Btk, Pcr2, Prkcb, Gab2, and p38a showed no reduction after BCR activation in the infected cells expressing CaM-resistant E12 (Fig. 4B). Thus, the downregulation of all of these genes by BCR activation was dependent on CaM inhibition of E2A. For all other genes analyzed, with the exception of BLNK, most of the reducing effect of anti-IgM on the expression in infected cells expressing wild-type E12 had disappeared in the mutant (Fig. 4B), indicating that the expression of these genes is regulated, to a large extent, by CaM inhibition of E12. Taken together, these results suggest that CaM sensitivity of E2A regulates the inhibitions of expression of most BCR-signaling components after BCR activation.

**Reduction of in vivo proximity of BCR-signaling components after BCR activation depends on CaM sensitivity of E2A**

BCR activation leads to formation of a BCR-associated protein complex composed of tyrosine kinases, adaptors proteins, lipid-metabolizing enzymes, and serine/threonine kinases that is often referred to as a “signalsome” (15, 21, 22). We followed the interactions between proteins within the BCR signalosome after BCR activation using in situ PLA with the Duolink system, which enables visualization and quantification of protein interactions in...
microscopy. Primary splenic mouse B cells were stained using Abs against different combinations of two BCR-signaling proteins and PLA probes (secondary Abs conjugated with oligonucleotides). This generates fluorescence signals, detected as distinct individual red dots, only when the two PLA probe-marked proteins are in close proximity (≤40 nm) (10–13). We obtained specific signals of proximity for many protein pairs, and the levels of background signals were minimal when one or both of the primary Abs were omitted (Fig. 5, Supplemental Fig. 3). As expected for the formation of a signalosome (15, 21, 22), BCR activation for 15 min increased the number of signal dots for many protein pairs, including CD19-LYN, CD19-SYK, CD19-BLNK, CD22-SYK, CD22-BLNK, CD22-PLCγ2, SYK-LYN, Igβ-BTK, and Igβ-PLCγ2 (Fig. 5, Supplemental Fig. 3). For each of these protein pairs, the increase in proximity was followed by a reduction (within 5 h) in prolonged anti-IgM treatment (Fig. 5, Supplemental Fig. 3). The increases in the number of dots at 15 min after BCR activation ranged from 2–5-fold; after 5 h of anti-IgM treatment, the numbers of dots were reduced 2–5-fold (Fig. 6A). All of the p values for the reductions that occurred between 15 min and 5 h after BCR activation were p < 0.015, with the exception of CD22-SYK (p = 0.07) and SYK-LYN (p = 0.08). The reductions in the CD22-SYK and SYK-LYN pairs were greater and were associated with lower p values in the analyses of infected cells below, a finding that supports that these pairs are also reduced. In summary, the results show that BCR activation after the initial formation of the BCR signalosome rapidly reduces the number of proteins interacting in the signalosome, which further supports the notion of negative-feedback regulation of the BCR signalosome.

To further study the mechanism of the negative feedback of the BCR signalosome, we performed PLA for purified primary splenic B cells infected with retrovirus expressing either wild-type or CaM-resistant E12 protein, together with GFP, which enabled selective analysis of the infected cells. The protein pairs of CD19-LYN, CD19-SYK, CD19-BLNK, CD22-SYK, CD22-BLNK, CD22-PLCγ2, SYK-LYN, Igβ-BTK, and Igβ-PLCγ2 showed 2–5-fold decreases in the numbers of dots/cell between 15 min and 5 h after BCR activation for the primary B cells infected with virus expressing wild-type E12 (Fig. 6B). The reductions were not sig-

![Image](https://via.placeholder.com/150)
significantly different between noninfected and wild-type–infected cells for any of the nine protein pairs (Fig. 6). Also, the largest difference, from 50 ± 24% to 79 ± 13% for CD22-SYK, was not statistically significant (p = 0.26). Importantly, and in sharp contrast to wild-type E12, the reductions that occurred between 15 min and 5 h after BCR activation disappeared almost completely postinfection when using virus expressing CaM-resistant E12 (Fig. 6B). All of the differences in the reductions between wild-type and CaM-resistant E12 were significant (p ≤ 0.03). In summary, the results show that CaM inhibition of E12 is essential for negative-feedback regulation of most of the BCR-signaling components.

**FIGURE 4.** Losses of effect of BCR stimulation in B cells with CaM-resistant E12. Purified primary splenic B cells from mice heterozygous for deletion of the E2A gene that encodes E12 were infected with a retrovirus expressing either wild-type or CaM-resistant E12, together with GFP, followed by BCR activation of half of the cells by anti-IgM treatment for 3 h. Infected B cells (30–50% of the cells) were purified by cell sorting based on their GFP expression, and microarray analysis of the B cells infected with virus expressing wild-type and CaM-resistant E12, with or without BCR stimulation, was performed with three independent mice using the Illumina Mouse BeadChip system. (A) Overview of genome-wide loss of effect of BCR stimulation in B cells with CaM-resistant E12. The bar graph shows the percentage of the genes regulated by BCR stimulation (≥1.33-fold change and p < 0.05) in B cells infected with wild-type E12 that lost their BCR regulation in B cells infected with CaM-resistant E12 mutant to the extent indicated. The analysis included 1598 upregulated genes and 1684 downregulated genes. (B) Loss of inhibition of expression of mRNA for BCR components, coreceptors, and signaling proteins upon BCR stimulation when E12 is CaM resistant. The changes in expression levels of key genes in BCR signaling were analyzed as described in (A). The mRNA levels before the addition of anti-IgM were set as 100% in both wild-type and CaM-resistant E12-infected B cells (not represented in the bar graphs). The mRNA levels of Iga, Cd72, Cd81, Lyn, Btk, Blnk, Pkcγ2, and Gab2 were determined by real-time RT-PCR as for some genes in Fig. 2, because the signals were not clearly above background for Lyn and Gab2, and the spread of the data was too great for the other six genes in the DNA microarray analysis. Data are representative of three different mice and are expressed as mean ± SD.

**FIGURE 5.** Transitory in vivo proximity of CD19 and LYN upon BCR stimulation. B cells from mouse spleen were activated with CD40L plus IL-4 activators for 48 h, followed by stimulation of the BCR by addition of anti-IgM for the indicated times. The detection of the proximal location of CD19 and LYN (shown as red dots) by in situ PLA was as described in Materials and Methods. The B cells used in controls with only the anti-CD19 or the anti-LYN primary Ab or with no primary Ab (−Ab) were stimulated with anti-IgM for 0.5 h. All samples were counterstained with Hoechst 33342 dye (blue) to visualize nuclei. Original magnification ×100.

**Feedback inhibition of BCR signaling after BCR activation depends on CaM sensitivity of E2A**

To analyze whether negative-feedback regulation of BCR function after BCR stimulation depends on CaM sensitivity of E2A, we compared the activating phosphorylation of JNK, ERK, and p38 downstream of the BCR after an initial BCR stimulation with anti-IgM and after a second BCR stimulation 6 h later. Purified and activated primary splenic B cells from mice heterozygous for deletion of the E2A gene that encodes E12 were infected with a retrovirus expressing either wild-type or CaM-resistant E12, together with GFP. The activating phosphorylations were all strongly induced 15 and 30 min after the initial BCR stimulation when infecting the B cells with virus expressing wild-type E12 or CaM-resistant E12 (Fig. 7A), as shown above for noninfected B cells (Fig. 3B). In contrast, the result of the second BCR stimulation differed remarkably between B cells expressing wild-type or CaM-resistant E12 (Fig. 7A). As for noninfected B cells (Fig. 3B), the second BCR stimulation was totally unable to induce any of the activating phosphorylations postinfection of the B cells with wild-type E12, whereas all three phosphorylation levels were high 6 h after the initial BCR stimulation, and especially upon 15 or 30 min of restimulation in the B cells in which infection was with CaM-resistant E12 (Fig. 7A, quantifications in Supplemental Fig. 4). This dramatic difference was especially remarkable, because the majority of the cells in these Western blot analyses were noninfected. Based on each quantified measurement of the level of the activating phosphorylation in the total cell population postinfection, as well as in cells analyzed in parallel that were not infected, and knowing the percentage of infected cells in the individual infection (∼40%), the levels of the induced phosphorylations in the infected cell populations were calculated. Importantly, and in contrast to B cells infected with wild-type E12 that had downregulated all three phosphorylations and lacked response to the second BCR stimulation, this analysis showed that the B cells infected with CaM-resistant E12 had lost the downregulation and were nevertheless, at least for phosphorylation of p38 and ERK, responding to the second BCR stimulation by in-
increased phosphorylation (Fig. 7B). The p values for the differences between wild-type and CaM-resistant E12 in the phosphorylations were all ≤0.04, ≤0.02, and ≤0.01 after 0, 15, and 30 min, respectively, of restimulation of the BCR. Thus, feedback inhibition of BCR signaling after BCR activation depends on inhibition of E2A by CaM.

**Discussion**

Regulation of Ag receptor signaling is essential for the development of specific immunity while retaining tolerance to self. Although a great deal of progress has been made in the identification of various signaling factors and their role in BCR signaling, the precise molecular mechanisms of how this activation of B cells regulates their differentiation remains poorly understood. In this study, we show that activation of signaling by the BCR leads to negative feedback regulation of most BCR-signaling components, including the BCR proteins, coreceptors, and downstream-signaling factors. We also show that signaling through the BCR is strongly reduced after activation of the receptor. Within 5 h after BCR activation, the individual proteins were reduced 30–60%, with a few exceptions. For each protein component, this can be considered a relatively modest downregulation. However, together, >15 such downregulations (Fig. 3A) could explain the very large downregulation of BCR signaling. Our PLA analysis of interacting protein pairs showed 2–5-fold reductions for a large number of pairs (Fig. 6A). The reductions in the protein pairs were close to the products of the levels of the reductions of the individual proteins. The four pairs, CD19-BLNK, CD22-PLCγ2, SYK-LYN, and Igβ-BTK, which have no protein in common, all showed 2–5-fold reductions in PLA between 15 min and 5 h after BCR stimulation. This finding suggests that the reduction in the complete BCR signalosomes with all proteins in appropriate amounts is much greater and could exceed the product of these four reductions, because at least as many other proteins of the BCR signalosome were also downregulated. Furthermore, all signaling proteins of the BCR signalosome were not analyzed at the protein level, and there is no reason why the downregulations should be limited to the studied ones. In addition, at least one analyzed protein was downregulated posttranscriptionally. Therefore, it is possible that other signalosome proteins are also downregulated posttranscriptionally. Hence, the total downregulation of the BCR signalosome can be at least several orders of magnitude and can explain that activation of the BCR leads to a strong feedback reduction in signaling through the receptor (Fig. 3B).

What is the physiological role of the strong negative feedback regulation of the BCR signalosome in the immune system? The reason for this feedback is most likely because the BCR has to detect improvements in Ag affinity over an extremely broad range of affinities. BCR signaling should detect differences in affinity, even when relatively small, for Ag-binding strengths all the way from 10^(-6) M(-1) or less to 10^(-10) M(-1) and greater. The BCR is used to select the B cells with highest Ag affinity for the extrafollicular plasmablast responses to optimize the initial wave of Ab
defender against infections, whereas B cells with less strong Ag binding are selected for the GC, where they undergo SH and selection for improved Ag affinity. Finally, the GC B cells with highest affinity are selected for plasma cell differentiation (1–3). High-affinity GC B cell clones have increased their Ag-binding strength by many orders of magnitude (from \(10^{-8} \text{M}^{-1}\) to at least \(10^{10} \text{M}^{-1}\) and typically have 5–10 mutations in the variable regions of their Ig genes. They typically have gone through several rounds of SH. Each round leads to one or a few mutations, followed by selection for increased affinity; after a number of such cycles, the B cells with highest Ag affinity are selected for plasma cell differentiation and, thereby, mass production of their high-affinity Ab (1–3). The threshold for BCR signaling has to increase successively during the GC reaction, because, during this process, the responder B cells with successively higher and higher affinities will compete successively during the GC reaction, because, during this process, the B cells with highest Ag affinity are selected for plasma cell differentiation (1–3). However, the experimental data supporting this remain weak (3), and mathematical modeling suggests that competition for Ag binding is not the most efficient selection mechanism, even when including limiting Ag binding sites on the follicular dendritic cells in the simulations (2). In this study, we examined whether the increasingly stringent requirement for BCR affinity during the GC reaction could be due, at least in part, to an effect of activation of BCR signaling on the BCR signalosome itself. We show that the BCR can downregulate itself. Furthermore, BCR restimulations of different strengths after a weak initial stimulation directly showed that BCR signaling reduces the sensitivity of the BCR to the next stimulation. This could enable sensitive detection of successive improvements in Ag affinity of the receptor over a very large span of affinities during SH. This discovered property of the B cell-selection system does not exclude the previously suggested selection mechanisms. Instead, it is likely that the impressive efficiency of affinity maturation in the GC is due to a combination of selection mechanisms (2). One such mechanism is affinity-dependent T cell help (2, 3, 23). It is notable that in our analysis of the genome-wide transcriptional changes upon stimulation of the BCR of mouse splenic B cells activated to SH, three of the most strongly upregulated genes are those for the chemokines XCL1/lymphotactin-\(\alpha\), CCL3 (MIP-1-\(\alpha\)), and CCL4 (MIP-1-\(\beta\)), which are all chemotactic for T cells. Their mRNA levels increased 50-, 32-, and 24-fold, respectively, in 3 h. For CCL3 and CCL4, BCR stimulation was also shown to induce secretion of the proteins from tonsillar B cells (24). Thus, BCR stimulation leads to a concerted downregulation of the BCR signalosome and upregulation of chemokines recruiting T cell help, which could provide an efficient system for selection of additional successive improvements in Ag affinity.

Antigen-reactive B cells are eliminated by apoptosis, receptor editing, and anergy. In the last case, autoreactive cells persist but have become unresponsive to Ag. Desensitized BCR of anergic B cells exhibit a defect at the earliest events in BCR signaling, consistent with failed transmission of signals through the receptor complex (25, 26). Importantly, a consistent feature of anergic T and B lymphocytes is increased basal intracellular free Ca\(^{2+}\) (26), which indicates the possibility of a long-term reduction in E2A activity by Ca\(^{2+}\)-loaded CaM in such cells. Therefore, our findings could indicate that the mechanism reported in this article is also important in desensitization of the BCR in anergy. This mechanism could function together with other mechanisms of BCR desensitization in anergy, as discussed by Yarkoni et al. (26).

We show that inhibition of the transcription factor E2A by Ca\(^{2+}/\text{CaM}\) is important for the feedback inhibition of BCR signaling and most of the proteins of the BCR signalosome. How much of the downregulation is through inhibition of this transcription factor? Most of the negative feedback of the BCR appears to be through inhibition of E2A, because mutation of this protein to CaM resistance leads to loss of the downregulation of BCR signaling, as well as loss of most of the negative feedback effect of BCR stimulation on expression of BCR signalosome components. We previously reported that AID protein in SH and repressors of initiation of plasma cell development were regulated through inhibition of the transcription factor E2A by Ca\(^{2+}/\text{CaM}\) (5, 14). Our global analysis of the effects of mutation of E2A to CaM resistance on mRNA levels after BCR stimulation showed that the previously analyzed genes were no exceptions. In fact, >80% of all significantly downregulated genes lost >50% of their downregulation in cells expressing a CaM-resistant E2A.

There were approximately as many genes with upregulation that is dependent on CaM sensitivity of E2A as there were genes with
downregulation that is dependent on this sensitivity. How can this occur? It could be due to CaM-dependent relief of repression by E2A, because E2A represses some of its target genes (27). Inductions could also be the result of a shift from CaM-sensitive E-protein homodimers to a CaM-resistant E-protein heterodimer with another bHLH transcription factor, analogous to the Ca\textsuperscript{2+}/CaM dependent shift to E-protein heterodimers with MyoD, which is important in muscle development (20). In summary, we report that inhibition of E2A by Ca\textsuperscript{2+}/CaM plays a major role in the responses of B cells to BCR stimulation and, in particular, in a broad negative feedback regulation of the BCR signalosome. The role of defects in Ca\textsuperscript{2+}/CaM inhibition of E2A, leading to dysregulated BCR signaling responses in development of humoral immune disorders and B cell lymphomas, will be important to explore.

Acknowledgments

We thank Drs. J. Forssell and P. Sideras for the kind gift of the Ab against BTK.

Disclosures

The authors have no financial conflicts of interest.

References