Induction of Activation-Induced Cytidine Deaminase–Targeting Adaptor 14-3-3γ Is Mediated by NF-κB–Dependent Recruitment of CFP1 to the 5′-CpG-3′–Rich 14-3-3γ Promoter and Is Sustained by E2A

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Induction of Activation-Induced Cytidine Deaminase–Targeting Adaptor 14-3-3γ Is Mediated by NF-κB–Dependent Recruitment of CFP1 to the 5′-CpG-3′–Rich 14-3-3γ Promoter and Is Sustained by E2A

Thach Mai, Egest J. Pone, Guideng Li, Tonika S. Lam, J’aime Moehlman, Zhenming Xu, and Paolo Casali

Class switch DNA recombination (CSR) crucially diversifies Ab biologic effector functions. 14-3-3γ specifically binds to the 5′-AGCT-3′ repeats in the IgH locus switch (S) regions. By interacting directly with the C-terminal region of activation-induced cytidine deaminase (AID), 14-3-3γ targets this enzyme to S regions to mediate CSR. In this study, we showed that 14-3-3γ was expressed in germinal center B cells in vivo and induced in B cells by T-dependent and T-independent primary CSR-inducing stimuli in vitro in humans and mice. Induction of 14-3-3γ was rapid, peaking within 3 h of stimulation by LPSs, and sustained over the course of AID and CSR induction. It was dependent on recruitment of NF-κB to the 14-3-3γ gene promoter. The NF-κB recruitment enhanced the occupancy of the CpG island within the 14-3-3γ promoter by CFP1, a component of the COMPASS histone methyltransferase complex, and promoter-specific enrichment of histone 3 lysine 4 trimethylation (H3K4me3), which is indicative of open chromatin state and marks transcription-competent promoters. NF-κB also potentiated the binding of B cell lineage-specific factor E2A to an E-box motif located immediately downstream of the two closely-spaced transcription start sites for sustained 14-3-3γ expression and CSR induction. Thus, 14-3-3γ induction in CSR is enabled by the CFP1-mediated H3K4me3 enrichment in the promoter, dependent on NF-κB and sustained by E2A. *The Journal of Immunology*, 2013, 191: 000–000.

Upon encounter with Ags, such as those on bacteria and viruses, B lymphocytes proliferate and differentiate into plasma cells and memory B cells (1–3). T-dependent Ab responses depend on engagement of B cell CD40 by trimeric CD154 expressed on the surface of Th cells (4, 5). T-independent Ab responses are elicited by Ags that trigger weak or no T cell responses and are boosted by (innate immune) TLRs, which are engaged by microbe-associated molecular patterns (6–8). TLR-signaling also plays an important role in T-dependent responses before the emergence of specific Th cells and can synergize with BCR-signaling to induce B cell proliferation and differentiation (9). The maturation of the Ab response depends on Ig somatic hypermutation (SHM) and class switch DNA recombination (CSR). SHM inserts mainly point mutations in DNA of the V(D)J regions, thereby providing the structural substrate for the positive selection by Ag of higher-affinity Ab mutants (10, 11). CSR replaces an IgH constant (Cγ) region, (e.g., Cμ) with a downstream Cγ region (Cγ, Cα, or Cε), thereby diversifying the biologic effector functions of an Ab without changing its specificity or affinity for Ag (12). Both SHM and CSR require activation-induced cytidine deaminase (AID), which is specifically expressed in activated B cells. AID deamminates deoxycytosines in V(D)J regions and switch (S) region DNA for SHM and CSR, respectively (13–15). High densities of deoxyuracils, as generated following AID-mediated DNA deamination (16), are processed by uracil DNA glycosylase (Ung), as recruited by Rev1 for the generation of dsDNA breaks in the upstream (donor) and downstream (acceptor) S regions (17, 18). Resolution of these dsDNA breaks results in looping out of the intervening DNA as an S circle, formation of S-S junction, leading to juxtaposition of the expressed VγDJHγ DNA to the downstream Cγ DNA (19, 20).

Induction of CSR requires primary and secondary stimuli (12). Primary stimuli comprise T-dependent and T-independent stimuli: 1) engagement of CD40 constitutively expressed on B cells by CD154 expressed on activated T cells; 2) dual engagement of TLRs and BCR, as effected by microbe-associated molecular patterns and repetitive microbial antigenic epitopes, respectively (9, 21); and 3) dual engagement of TLRs and TACI (TACI is engaged by BAFF or APRIL secreted from activated immune cells or epithelial cells) or BCR and TACI (7, 9, 22). The only known microbial component that can directly induce efficient CSR is bacterial LPS, because of the ability of LPS to engage TLR4 (on mouse B cells) through the lipid A moiety and to crosslink the BCR of a large proportion of B cells through the repetitive polysaccharidic moiety, O-antigen (8). Primary stimuli induce expression of AID and other important CSR factors through activation of NF-κB, HoxC4, and other transcription factors (14, 23). They also enable secondary stimuli—that is, cytokines that include IL-4 and TGF-β (and IFN-γ in the mouse) to induce germline IγR-S-Cγ transcription and histone...
 modifications in the donor and acceptor S regions (24–28)—thereby directing CSR to predetermined Ig isotypes. IL-4 and TGF-β can enhance induction of AID by primary stimuli (14).

14-3-3 adapter proteins (seven isoforms: 14-3-3β, 14-3-3ε, 14-3-3γ, 14-3-3ζ, 14-3-3r, 14-3-3r, and 14-3-3t) specifically bind the evolutionarily conserved 5′-AGC-3′ repeats in S region DNA and target the S regions that will undergo recombination through interaction with the combinatorial histone modifications H3K9acS10ph (29, 30, and G. Li, E.J. Pone, T.S. Lam, D.C. Tran, C.A. White, K.L. Hayama, H.Zan, Z. Xu, and P. Casali, submitted for publication). 14-3-3 adaptors recruit and stabilize AID to transcribed S regions through direct protein-protein interaction (29).

14-3-3 interaction with AID depends on the AID C-terminal region, which is dispensable for the AID DNA deamination activity but critical for AID to mediate CSR (31, 32). As shown previously, CSR is significantly reduced in 14-3-3γ−/− B cells, indicating that this isoform plays a critical role in the AID targeting in CSR (29).

As suggested by our previous findings, 14-3-3 proteins, like AID, are expressed in a B cell differentiation stage-specific fashion, such as in germinal center B cells, in which CSR occurs at a high level, and in B cells induced to undergo CSR by CD154 or LPS plus IL-4 (29). Significant advances have been made in the understanding of the regulation of the AID gene (AICDA/Aicda) expression, including the role of HoxC4 and NF-κB transcription factors and role of microRNAs (33), but it remains unclear how the expression of 14-3-3γ, as a critical AID-targeting CSR factor, is regulated. In this study, we have analyzed induction of 14-3-3γ by the same stimuli that induce AID and CSR and induction kinetics. Using comparative gene analysis and 5′-RACE, we have mapped the 14-3-3γ transcription start sites (TSSs) and identified unique and evolutionarily conserved features of the 14-3-3γ promoter. Using chromatin immunoprecipitation (ChIP), we have also addressed the molecular mechanisms underlying 14-3-3γ induction by analyzing 14-3-3γ locus-wide recruitment of transcription factors NF-κB and E2A and epigenetic changes. Finally, we have used multiple molecular approaches, including small-molecule antagonistic compounds, knockout (KO) mice, inhibitory proteins, and a luciferase reporter system involving the 14-3-3γ promoter, to define the modality of NF-κB and E2A-mediated 14-3-3γ induction.

Materials and Methods

Human B cells

Single-cell suspensions of IgD+CD38−CD19+ germinal center B cells and IgD+CD38+ CD19+ naive B cells were prepared from human tonsil surgical specimens, stained with fluorescein-conjugated mAbs to CD19, IgD, and CD38 (BD Biosciences) and sorted using a MoFlo (Beckman Coulter). Human PBMCs were prepared from buffy coat (obtained from the Blood Bank of the University of California–Irvine Medical Center) by a Ficoll–Hypaque Plus density gradient (GE Healthcare). Naïve IgD+ B cells were then purified using a MACS-based naïve B cell isolation kit (Miltenyi Biotec) following the manufacturer’s instructions (typically yielding >98% of IgD+ B cells and no IgG+ or IgA+ B cells) and cultured at 5 × 10^6 cells/ml in RPMI 1640 (Invitrogen) supplemented with FBS (10% v/v, Hyclone), penicillin-streptomycin (1% v/v; Invitrogen), amphotericin B (1% v/v; Invitrogen) and 50 μM β-mercaptoethanol (FBS-RPMI). IgD+ B cells were stimulated with an agonistic mAb to human CD40 (1 μg/ml, clone 2G8-5, ATCC) plus recombiant human IL-4 (30 ng/ml; Genzyme). All protocols were in accordance to the rules and regulations of the University of California–Irvine Institutional Regulatory Board.

Mouse B cells

Spleen and lymph node B cells were prepared as described (8). B cells were cultured (5 × 10^6 cells/ml in FBS-RPMI) in the presence of: CD154 (3 μg/ml, mouse CD154-containing membrane fragments of baculovirus-infected Sf21 insect cells) (23, 29), LPS (5 μg/ml, from Escherichia coli, serotype 055:B5 and deproteinized by chloroform extraction; Sigma-Aldrich), TR7L ligand R848 (30 ng/ml; Invivogen) or TRL9 ligand Cpg ODN1826 with a phosphorothioate backbone (Cpg ODN, 1 μM, sequence 5′-TCCATGACGTTCCTGACGTG-3′; Operon). IL-4 (3 ng/ml; R&D Systems) or anti-Ig-μ mAb (clone 11-26)-conjugated dextran (anti-Ig-μ mAb/dex, 10 ng/ml; Fina Biosolutions) were added as indicated. For inhibition of IKK-2 using [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide (TPCA-1, EMD Milipore), B cells were pretreated with 1 μM TPCA-1 for 1 h in FBS-RPMI before stimuli were added.

Retrotransional analysis

The S-003 and S003-Id3 retroviral vectors were provided by Dr. C. Murre (University of California–San Diego). For the generation of retrovirus, retroviral cDNA templates were cloned along with the pM-CCL–E2A retroviral packaging vector (Imgenex) into HEK293T cells using the ProFectin Mammalian Transfection System (Promega). Transfected cells were cultured in FBS-RPMI in the presence of chloroquine (25 μM) for 8 h. After the removal of chloroquine, retrovirus-containing culture supernatants were harvested every 12 h. For transduction and CSR analysis, mouse B cells were activated with LPS for 24 h and then centrifuged at 500 × g together with viral particles in the presence of 6 μg/ml polybrene (Sigma-Aldrich) for 90 min at 25°C. Transduced B cells were then cultured in virus-free FBS-RPMI in the presence of LPS plus IL-4 for 48 h for transcript analysis, or for 72 h for flow cytometry analysis of surface IgG1 and B220 expression in GFP+B cells (GFP translation is initiated by the IRES in the S-003 vector; GFP+ B cells indicating transduced B cells), as described previously (23, 29). Dead (7-AAD+) cells were excluded from analysis.

5′ Rapid amplification of cDNA ends (5′-RACE)

FirstChoice RLM-RACE kit (Ambion) was used according to the manufacturer’s specifications to identify the TSSs of the 14-3-3γ gene. Total RNA was extracted from mouse B cells cultured with LPS plus IL-4 for 24 h and then treated with calf alkaline intestinal phosophatase at 37°C for 1 h to remove the 5′-phosphate group from RNA and DNA molecules degraded at their 5′ ends. After reaming with 4 M of ammonium acetate and purification by phenol/chloroform extraction, RNA was treated at 37°C for 1 h with tobacco acid pyrophosphatase, which cleaves the 7′-methyl-guanosine cap (m7G cap) of mRNA, to expose the 5′-phosphate group in mRNA molecules originally with an intact m7G cap (mRNA molecules originally degraded at the 5′ end thus would not have a 5′-phosphate group). After a 5′-RACE RNA adapter was added to the newly exposed 5′-phosphate group, mRNA molecules were reverse transcribed into full-length cDNA with amplification of the 5′ end of 14-3-3γ cDNA by two rounds of PCR involving 5′-RACE adapter-specific outer and nested inner primers (both forward primers) and 14-3-3γ-specific outer primer (reverse primer, priming DNA ~ 230 nt DNA downstream of the putative promoter) and inner primer (nested reverse primer, priming DNA ~ 90 nt DNA downstream of the putative promoter; primers are listed in Supplemental Table I). Amplified 14-3-3γ-specific cDNA was analyzed by agarose gel electrophoresis and cloned into the pCR-Blunt II-TOPO vector (Invitrogen) for sequencing of individual cDNA molecules.

Transcript analysis by quantitative real-time PCR

RNA was extracted from B cells (5 × 10^6) using an RNasy Mini Kit (Qiagen) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 2 μg of RNA using the SuperScript III System with an oligo-dT primer (Invitrogen) and then analyzed by quantitative real-time PCR (QRT-PCR) using appropriate primers (Supplemental Table I) and SYBR Green (Dynamo HS Kit; New England Biolabs). PCR was performed in the MyQ Single-color Real-Time PCR Detection System (Bio-Rad Laboratories) according to the following protocol: 95°C for 30 s, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis was performed at 72–95°C. The ΔΔCT method was used to analyze levels of transcripts, and data were normalized to the level of Cd79b, which encodes the BCR Igβ-chain constitutively expressed in B cells.

Chromatin immunoprecipitation assays

ChIP assays were performed as described previously (35). B cells (1 × 10^7) were treated with 1% formaldehyde in PBS at 25°C for 10 min to crosslink chromatin. After quenching with 100 μM of glycine (pH 8.0) and washing with cold PBS containing a mixture of protease inhibitors (Sigma-Aldrich), B cells were resuspended in SDS-lysis buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA, 0.1% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, and protease inhibitor mixture). Chromatin was sonicated to yield 200–500-bp DNA fragments, precleared with agarose beads conjugated with protein A (Pierce) and then incubated with a polyclonal rabbit Ab to CFP1, monomethylation of histone 3 lysine 4 (H3K4me1), trimethylation of histone 3 lysine 4 (H3K4me3), p65, p52, or E2A at 4°C (Abs are listed in CTGACGTG-3′; Operon). IL-4 (3 ng/ml; R&D Systems) or anti-Ig-μ mAb (clone 11-26)-conjugated dextran (anti-Ig-μ mAb/dex, 10 ng/ml; Fina Biosolutions) were added as indicated. For inhibition of IKK-2 using [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide (TPCA-1, EMD Milipore), B cells were pretreated with 1 μM TPCA-1 for 1 h in FBS-RPMI before stimuli were added.
Supplemental Table II). After overnight incubation, immune complexes were isolated using agarose-beads conjugated with protein A, washed, and eluted in buffer containing 50 mM Tris-HCl, 0.5% SDS, 200 mM NaCl, and 100 μg/ml proteinase K (pH 8.0). Eluates were incubated at 65°C for 4 h to reverse formaldehyde crosslinks. DNA was purified using a QIAquick PCR purification column (Qiagen) and analyzed with QRT-PCR using specific primers. For ChIP assays involving anti-α-κ-b (p65 or p52) Abs, B cells were subjected to crosslinking with disuccinimidyl glutarate (1 mM; Pierce) for 30 min and then washed with cold PBS immediately before crosslinking with formaldehyde.

For two-step ChIP assays, crosslinked protein-DNA complexes were immunoprecipitated by the first Ab, as captured by protein A agarose beads. After washing and centrifugation, the pelleted beads were resuspended in 40 μl freshly made Re-ChIP buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 125 mM KCl, 10 mM Na2EDTA, 1.1% [v/v] Triton X-100, supplemented with 10 μg purified salmon sperm DNA, 100 μg purified yeast tRNA (Invitrogen), and 1 mg purified BSA (New England Biolabs) and precipitated by the second Ab. The procedures following the second immunoprecipitation, including binding to agarose beads bearing protein A, reverse crosslinking, and DNA purification, were the same as for regular ChIP assays.

Luciferase reporter assays
A 310-bp DNA sequence encompassing the two 14-3-3y TSSs (~270 to ~400 bp) was amplified by PCR from mouse genomic DNA using specific primers and cloned into the pGL3-basic luciferase (Pluttius pyrelicus) luciferase gene reporter vector (Promega). Mutant gene reporter constructs containing a truncated 14-3-3y promoter were generated by PCR-based mutagenesis and confirmed by DNA sequencing. Reporter constructs were cotransfected with the pR-L-TK vector (Promega), which drives constitutive expression of Renilla reniformis luciferase, into mouse CH12F3 B cells by electroporation (250 V and 900 V) in a Gene Pulser II (Bio-Rad), yielding typically more than 40% transfection efficiency. Transfected CH12F3 B cells were then cultured in FBS-RPMI in the presence of CD154, IL-4, typically more than 40% transfection efficiency. Transfected CH12F3 B cells (10^6) were fixed and permeabilized using the Cytofix/Cytoperm Fixation and Permeabilization solution (BD Biosciences) followed by flow cytometry.

Immunoblotting
B cells (1 × 10^6) were harvested by centrifugation at 500 × g for 5 min, resuspended in 0.5 ml lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% [v/v] NP-40) supplemented with phosphatase inhibitors, protease inhibitors (Sigma-Aldrich), cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes for immunoblotting involving specific Abs. Membranes were then stripped with 200 mM glycine (pH 2.5), equilibrated in PBS, and subjected to immunoblotting with an anti–p52-actin mAb (Abs are listed in Supplemental Table II).

Intracellular staining analysis of 14-3-3y expression in B cells
B cells (1 × 10^6) were fixed and permeabilized using the Cytofix/Cytoperm Fixation and Permeabilization solution (BD Biosciences) following the manufacturer’s instructions. After washing with PBS, cells were resuspended in 50 μl of the Ab diluent solution containing a polyclonal Ab specific for 14-3-3y (Supplemental Table II) and incubated at 4°C for 30 min. B cells were then washed, resuspended in PBS, and analyzed by flow cytometry.

Immunohistochemistry
Human tonsil specimens were fixed in formalin and embedded in paraffin. Tissue sections (6 μm) were mounted onto glass slides. After deparaffinization and rehydration, slides were processed following a typical staining procedure involving Abs specific for 14-3-3y, AID, CD20, or CD3, and then goat anti-mouse or goat anti-rabbit IgG secondary Abs conjugated with HRP-labeled polymers (Dako). After color development using the DAB+ chromogen (Dako), slides were dehydrated and counterstained with hematoxylin before being examined by microscopy.

Results
14-3-3y is upregulated in germinal center B cells and is induced by primary stimuli
We first analyzed the expression of the 14-3-3y isoform in germinal center B cells. 14-3-3y was highly expressed in human tonsils and upregulated in those CD20+ B cells that expressed AID at high levels (Fig. 1A). Like AID, 14-3-3y was expressed at higher levels in germinal center B cells (IgD+CD38+CD19+ cells in humans and PNA+B220+ cells in mice) than in nongermline center B cells by immunoblotting or QRT-PCR analyses (Fig. 1B, 1C). To determine the nature of stimuli that induce 14-3-3y, we stimulated purified naïve IgD+B cells, in which 14-3-3y was expressed at a basal low level, with primary CSR-inducing stimuli or primary plus secondary stimuli. 14-3-3y together with AID was induced in human and mouse B cells stimulated by CD40 engagement plus IL-4 (Fig. 1D). High 14-3-3y levels were also induced by TLR ligand LPS, R-848, or CpG ODN and were not further enhanced by IL-4 or anti–α mAb/dex, which crosslink the BCR in IgD+B cells (Fig. 1D, 1E).

We next analyzed kinetics of 14-3-3y induction in B cells stimulated by LPS. LPS triggers both TLR and BCR signaling and induce high levels of AID, germline Iα-S-Cα transcription, and enrichment of combinatorial histone modifications in the Sy3 region and, eventually, CSR to IgG3 (8, 30, and G. Li et al., submitted for publication). Induction of 14-3-3y by LPS was rapid, peaking (20–30-fold) within 3 h of stimulation, and then sustained beyond 48 h (Fig. 2A). At 48 h, the levels of Aicda expression and germline Iα-Sy3-Cy3 transcription peaked and CSR from Sμ to Sy3 (as quantified by levels of circle Iα-Cy3 transcripts and postrecombination Iμ-Cy3 transcripts) was readily detectable. In addition, within 3 h of stimulation by LPS, 14-3-3y was induced at levels higher than all nineteen genes we analyzed that were involved in B cell differentiation, such as Irf4, which upregulates AID and CSR (2, 36), and A20, which negatively regulates TLR signaling (37) (Fig. 2B).

Thus, 14-3-3y is expressed in germinal center B cells and is induced in B cells by primary CSR-inducing stimuli, including CD154 (in the presence of IL-4) and TLR ligands. 14-3-3y induction is rapid and is sustained over the period of induction of other molecular events that are critical for CSR, such as AID expression and germline Iα-S-Cα transcription.

14-3-3y promoter has two close TSSs and is highly rich in 5'- CpG-3' dinucleotide motifs
We next addressed molecular mechanisms underlying the rapid 14-3-3y induction, starting by identifying critical cis-regulatory elements in the 14-3-3y locus. By performing comparative gene analysis, we identified in the ~50-kb human and mouse 14-3-3y locus two evolutionarily conserved noncoding sequences, which can function as the promoter and 3' untranslated region (3' UTR), respectively (Fig. 3A). Using 5'-RACE, we next mapped two dominant TSSs within the putative promoter, with the upstream TSS responsible for 66% of mouse 14-3-3y transcripts and
FIGURE 1. 14-3-3-y is upregulated in germinal center B cells in vivo and by primary CSR-inducing stimuli in vitro. (A) Immunohistochemistry analysis of expression of 14-3-3-y (top), AID (middle), and CD20 (a B cell marker, pink signal) together with CD3 (a T cell marker, brown signal, bottom) in serial human tonsil sections. Yellow line–bounded ellipses indicate the germinal center (GC) outer zone, in which B cells express high levels of AID but not Bcl6 (29). Original magnification ×100. Data is representative of three independent experiments. (B) Immunoblotting analysis of 14-3-3-y and AID expression in human tonsil cells and sorted naive (IgD+CD38−) and GC (IgD+CD38+/CD19−) tonsil cells (left panels), as well as in mouse spleen cells and sorted non-GC (PNAhiB220+) and GC (PNAloB220+) B cells from C57BL/6 mice 14 d after immunization with NP16-CGG (right panels). (C) QRT-PCR analysis of expression of 14-3-3-y and Aicda in mouse spleen GC and non-GC B cells, as in (B). Data are expressed as ratios of values in GC B cells to those in non-GC B cells (set as 1.2 for graphic purposes). Depicted are mean and SEM of data from three independent experiments. (D) Immunoblotting analysis of 14-3-3-y expression in freshly isolated human IgD+ B cells or B cells stimulated with an agonistic anti-CD40 mAb plus IL-4 for 48 h or 72 h (left panels) or in mouse B cells stimulated with CD154, LPS, or TLR ligand R-848 or CpG ODN, alone or in the presence of IL-4 or anti–δ mAb/dex, for 48 h (right panels). (E) QRT-PCR analysis of expression of 14-3-3-y and AID in mouse B cells stimulated with CD154, LPS, or TLR ligand R-848 or CpG ODN, alone or in the presence of IL-4 or anti–δ mAb/dex, for 24 h. Data are expressed as ratios of values in stimulated B cells to those in unstimulated B cells (mean and SEM of data from three independent experiments).

downstream TSS for 33% (Fig. 3B and not shown). These two mouse 14-3-3-y TSSs are only 9 bp apart, and the human 14-3-3-y promoter is suggested to have one TSS around this region (http://tinyurl.com/8m3jhbp; an antisense TSS is also suggested). Thus, the 14-3-3-y promoter falls within the “sharp” class of promoters (i.e., one or few TSSs), which represents less than 30% of eukaryotic promoters (38); however, it contains no classical TATA-box or initiator element that is tightly associated with sharp promoters (38), but instead a high density of 5′-CpG-3′ dinucleotide motifs and a high GC content, the hallmarks of CpG islands (Fig. 3C). CpG islands range from 200 bp to 6 kb in the genome (39). Notably, the 14-3-3-y promoter is highly conserved and displays a high density of the 5′-CpG-3′ motif (e.g., 34 and 30 motifs in −120 to +80 bp of the TSSs, or 34% and 30% of DNA, in the human and the mouse, respectively; Fig. 4). This density of 5′-CpG-3′ is higher than 99.5% of more than 10,000 CpG island-containing promoters (39, 40), which occur in 70% of eukaryotic gene and are mostly broad promoters with multiple and spreading TSSs (38). In addition, 5′-CpG-3′ dinucleotide motifs are enriched in the 14-3-3-y 3′ UTR, but not in the coding region or intron, accounting for 15% of 3′ UTR DNA (Fig. 3C).

Thus, the 14-3-3-y promoter is a unique sharp promoter, as it features a narrow CpG island with one of the highest 5′-CpG-3′ dinucleotide densities, but mediates transcription initiation at only two close TSSs.

14-3-3-y induction is concomitant with enhanced CFP1 and H3K4me3 enrichment in its promoter

Unmethylated 5′-CpG-3′ dinucleotide motifs, as occurring in most CpG island-containing promoters, directly interact with the zinc finger Cys-X-X-Cys (CXXC finger) in proteins such as CXXC finger protein 1 (CFP1, also known as CXXC1) (41). CFP1 is a nonenzymatic (structural) subunit of the COMPASS histone methyltransferase complex, which contains either a mixed-lineage leukemia (MLL) protein (MLL1, MLL2, MLL3 or MLL4) or SET1 (SET1a or SET1b) as the enzymatic subunit and several other nonenzymatic subunits (42). In B cells stimulated by LPS plus anti–δ mAb/dex, CFP1 specifically bound the 14-3-3-y pro-
FIGURE 3. The 14-3-3-γ promoter is mapped to a 5′-CpG-3′-rich region. (A) Two regions (boxed) with conserved sequences (red lines) in the human and mouse 14-3-3-γ locus (as shown by alignment using the MacVector software). (B) Agarose gel analysis of 5′-RACE products corresponding to the two 14-3-3-γ TSSs (clone 1 and 2, the downstream TSS; clone 3, the upstream TSS) and sequencing analysis of clone 1 and 3. Asterisk (*) indicates downstream TSS. (C) High densities of 5′-CpG-3′ motifs around the human and mouse 14-3-3-γ promoter (~120 to +80-bp of the TSS) and 3′ UTR. Each vertical line represents a 5′-CpG-3′ motif (lines are stacked when multiple 5′-CpG-3′ motifs are closely spaced; images were generated by MacVector). Red arrows indicate TSSs; gray bar indicates the promoter; dark blue bars indicate the coding region (the two exons are also indicated).

The 14-3-3-γ promoter DNA upstream and downstream of the TSSs (regions 5 and 6, respectively; Figs. 4, 5A), but not the 5′-CpG-3′-rich 3′ UTR. CFP1 also bound the 14-3-3-γ promoter in purified resting B cells and in B cells cultured without any primary CSR-inducing stimuli, but at lower levels (Fig. 5A and data not shown). The enhancement of the CFP1 recruitment in B cells undergoing CSR was

FIGURE 4. The conserved 5′-CpG-3′-rich 14-3-3-γ promoter contains two κB motifs and one E-box motif. The human, mouse, and rat 14-3-3-γ promoters (within the red frame) were aligned by MacVector (gray marks conserved DNA sequences). 5′-CpG-3′ dinucleotide motifs (in blue) and the promoter DNA upstream (region 5, orange bar) and downstream (region 6, green bar) of the two mouse TSSs (red arrows) to be analyzed in ChIP–quantitative PCR are depicted. Also depicted are two putative κB motifs upstream of the TSSs and an E-box motif downstream of the TSSs, as identified by the BioBase TRANSFAC program (http://www.biobase-international.com/product/transcription-factor-binding-sites).
associated with upregulated CFP1 expression, which increased by 4-fold within 60 min of stimulation by LPS (Fig. 5B).

CFP1 mediates the marking of CpG island-containing promoters by histone 3 lysine 4 trimethylation (H3K4me3), a histone modification that indicates the open chromatin state and marks transcription-competent promoters and first exons (41, 43). The modification that indicates the open chromatin state and marks promoters by histone 3 lysine 4 trimethylation (H3K4me3), a histone modification that indicates the open chromatin state and marks transcription-competent promoters and first exons (41, 43). The regulation of 14-3-3 induction critically depends on NF-κB activation, which is induced in a sustained fashion to mediate AID and CSR induction (8, 23). NF-κB comprises heterodimers activated by the canonical pathway (e.g., the p65/p50 heterodimer) or the noncanonical pathway (e.g., p52/RelB heterodimer). As we have shown, both pathways can be activated by LPS, particularly in the presence of anti-ΔmAb/dextran, or CD40 engagement (8). The NF-κB p65 and p52 subunits were recruited to the 14-3-3 promoter in B cells stimulated with LPS plus anti-ΔmAb/dextran for 24 h, as in (A). Data is representative of three independent experiments.

**FIGURE 5.** Upregulation of CFP1 recruitment to the 5′-CpG-3′-rich 14-3-3 promoter is associated with H3K4me3 induction. (A) ChIP– quantitative PCR analysis of CFP1-binding to the selected 16 conserved regions in the 14-3-3 promoter in B cells stimulated with nil or LPS plus anti-IgG mAb/dextran for 24 h. (B) Kinetics of CFP1 induction in B cells by LPS. Data are expressed as the ratio of expression levels in LPS-stimulated B cells at different time points to those in freshly isolated B cells (0 h, set as 1). (C and D) ChIP–quantitative PCR analysis of enrichment of H3K4me3 (C, region 9 might contain a cryptic gene promoter) and H3K4me1 (D) in the 14-3-3 locus in freshly isolated B cells or B cells stimulated with LPS plus anti-ΔmAb/dextran for 24 h, as in (A). Data is representative of three independent experiments.

NF-κB binds to the 14-3-3 promoter and regulates 14-3-3γ induction

The highest induction of 14-3-3γ among the 20 selected CpG island-containing genes (Fig. 2B) suggested that cis-regulatory elements in addition to CpG islands mediate the rapid 14-3-3γ induction in B cells undergoing CSR. We analyzed putative transcription factor-binding sites using the BioBase TRANSFAC program (http://www.biobase-international.com/product/transcription-factor-binding-sites) and identified two conserved consensus κB motifs (scored at 0.9 and 1.0, respectively) 23 and 88 bp upstream of the TSSs and an E protein-binding site (E-box motif; scored at 0.9) downstream of the TSSs (Fig. 4). As a result, we hypothesized that 14-3-3γ induction depends on NF-κB, which is induced in a sustained fashion to mediate AID and CSR induction (8, 23). NF-κB regulates heterodimers activated by the canonical pathway (e.g., the p65/p50 heterodimer) or the noncanonical pathway (e.g., p52/RelB heterodimer). As we have shown, both pathways can be activated by LPS, particularly in the presence of anti-ΔmAb/dextran, or CD40 engagement (8). The NF-κB p65 and p52 subunits were recruited to the 14-3-3 promoter in B cells stimulated with LPS plus anti-ΔmAb/dextran or CD154 plus IL-4, but were virtually absent in the 14-3-3 promoter in unstimulated B cells (Fig. 6A and not shown). In addition, p65 was associated with CFP1 on the 14-3-3 promoter (Fig. 6B), likely in a macromolecular complex that also contains other components of histone methyltransferases. NF-κB also bound an intronic region (region 10) and a region in the 3′ UTR (region 14), both containing putative κB motifs and displaying enrichment of H3K4me1, but not H3K4me3 (Fig. 5 and not shown).

To address the role of NF-κB in 14-3-3γ induction, we inhibited NF-κB activation by using TPCA-1, an inhibitor of IKK-2 in the canonical NF-κB pathway. Recruitment of p65 to the 14-3-3γ promoter was virtually abolished by TPCA-1 in LPS-stimulated B cells, resulting in more than 80% reduction in 14-3-3γ expression (Fig. 6C, 6D and Supplemental Fig. 1). Induction of 14-3-3γ was also severely impaired in B cells lacking p85α, the major regulatory subunit of PI(3)K (p85α−/−), because of the critical role of PI(3)K in transducing BCR signaling, including that triggered by anti-IgG mAb/dextran or LPS, to activate both the canonical and the noncanonical NF-κB pathways (8). It was completely abolished in TPCA-1-treated p85α−/− B cells (Fig. 6D).

Thus, 14-3-3γ induction critically depends on NF-κB, which is activated by the canonical and noncanonical pathways and then recruited to the 14-3-3γ promoter in B cells undergoing CSR. E2A transcription factors bind to the 14-3-3γ promoter and synergize with NF-κB to sustain 14-3-3γ induction for CSR

The E-box motif located immediately downstream of the 14-3-3γ TSSs suggests a role of E2A proteins (E12 and E47) in 14-3-3γ induction in B cells. E2A proteins belong to the class I helix-loop-helix transcription factor members of the E protein family that are essential for B and T cell development. E12 and E47 regulate a...
network of genes that orchestrate B and T cell fates (46), possibly by mediating DNA demethylation in gene promoters and chromatin remodeling to increase the accessibility to the transcription machinery (47, 48). E2A proteins, which are activated by LPS or BCR crosslinking in mature B cells and play a role in CSR (34), specifically bound the E-box motif in the 14-3-3γ promoter (region 6).

**FIGURE 6.** NF-κB binds to the 14-3-3γ promoter and regulates 14-3-3γ induction. (A) ChIP–quantitative PCR analysis of NF-κB (p65 of the canonical and p52 of the noncanonical NF-κB pathway, as indicated) binding to the 14-3-3γ locus in freshly isolated B cells or B cells stimulated with LPS plus anti-δ mAb/dextran or CD154 plus IL-4 for 24 h. (B) Sequential ChIP–quantitative PCR analysis of NF-κB (p65; first ChIP) and CFP1 (second ChIP; an IgG Ab with irrelevant specificity was used as the control) binding to the 14-3-3γ promoter in B cells stimulated with LPS plus anti-δ mAb/dextran for 24 h. (C) ChIP–quantitative PCR analysis of NF-κB p65 binding to the 14-3-3γ locus in B cells treated with nil (DMSO) or TPCA-1 and then stimulated with LPS plus anti-δ mAb/dextran for 24 h. (D) Kinetics of 14-3-3γ induction in p85α+/+ and p85α−/− B cells treated with nil (DMSO) or TPCA-1 and stimulated with LPS. Data are ratios of expression levels in stimulated B cells harvested at different times to those in freshly isolated B cells (0 h, set as 1). Data is representative of three independent experiments.

**FIGURE 7.** NF-κB and E2A mediate different phases of the 14-3-3γ induction. (A) ChIP–quantitative PCR analysis of E2A-binding to the 14-3-3γ locus in B cells stimulated with nil, LPS, or LPS plus anti-δ mAb/dextran for 24 h. (B) Luciferase reporter assays of the activity of the 14-3-3γ promoter or the 14-3-3γ promoter mutants (mut1-mut3) that lack the κB or E-box motifs, as indicated. Data are expressed as the percentage of expression levels to B cells transfected with wild type 14-3-3γ promoter for 24 h (set as 100; mean and SEM of data from three independent experiments). The p values were calculated by paired Student t test. NS, Not statistically significant.
in B cells stimulated by LPS alone or LPS plus anti-Igδ mAb/dex (Fig. 7A). They also bound this motif in unstimulated B cells, albeit at lower levels.

We next addressed the role of E2A proteins and NF-κB in 14-3-3y induction using luciferase reporter assays in CH12F3 B cells. These transcription factors are induced by CD154, IL-4, and TGF-β to express high levels of AID and undergo CSR to IgA (14). The 14-3-3y promoter was highly activated within 3 h of stimulation by CD154, IL-4, and TGF-β and was fully activated within 24 h (Fig. 7B). A promoter mutant that lacked the two κB motifs (Mut1) or both the κB and E-box motifs (Mut3) displayed little if any promoter activity. In contrast, a promoter mutant lacking the E-box motif (Mut2) displayed virtually normal activity within 3 h of stimulation, but significantly reduced activity after 24 h, suggesting that E2A proteins are important for sustained 14-3-3y expression. To confirm this notion, we preactivated B cells with LPS to induce 14-3-3y and then used retrovirus transduction to express Id3, which lacked a DNA-binding domain but could heterodimerize with E2A proteins to block them from binding to E-box motifs and mediating transcriptional activation (48, 49). Id3 blocked E2A binding to the 14-3-3y promoter, decreased 14-3-3y transcript and protein levels, and inhibited CSR without altering germline Ig1-Cγ1 transcription (Fig. 8A–8E). AID expression was also reduced, which is consistent with a previous report (34).

These results show that E2A proteins are dispensable for the initial induction of 14-3-3y mediated by NF-κB. They are, however, important for sustained 14-3-3y expression.

**FIGURE 8.** E2A sustains 14-3-3y expression for CSR. (A) ChIP-quantitative PCR analysis of E2A binding to the 14-3-3y promoter in B cells transduced with S-003 or S-003-Id3 retrovirus and then stimulated with LPS plus IL-4 (bottom) for 48 h. (B) Intracellular staining analysis of 14-3-3y protein levels in 14-3-3y+/+ and 14-3-3y−/− B cells stimulated by LPS (top), and in B cells transduced with S-003 or S-003-Id3 retrovirus and then stimulated with LPS plus IL-4 (bottom) for 48 h. Representative of three independent experiments. (C and D) Flow cytometric analysis of the proportion of GFP+ B cells that were IgG1+ in B cells transduced with S-003 or S-003-Id3 retrovirus and then stimulated with LPS plus IL-4 for 96 h. Histograms in (C) depicting the mean and SEM of data from three independent experiments. (E) QRT-PCR analysis of levels of germline Ig1-Cγ1, circle Ig1-Cγ1, postrecombination Igα-Cγ1, 14-3-3y, and Aicda transcripts in B cells transduced with S-003 or S003-Id3 and stimulated with LPS plus IL-4 for 48 h. Data are ratios of expression levels in B cells transduced with S-003 or S003-Id3 retrovirus and then stimulated with LPS plus IL-4 for 96 h. Histograms in (C) depicting the mean and SEM of data from three independent experiments. (E) QRT-PCR analysis of levels of germline Ig1-Cγ1, circle Ig1-Cγ1, postrecombination Igα-Cγ1, 14-3-3y, and Aicda transcripts in B cells transduced with S-003 or S003-Id3 and stimulated with LPS plus IL-4 for 48 h. Data are ratios of expression levels in B cells transduced with S-003 or S003-Id3 retrovirus and then stimulated with LPS plus IL-4 for 96 h. Histograms in (C) depicting the mean and SEM of data from three independent experiments.

**FIGURE 9.** NF-κB enhances CFP1 recruitment for H3K4me3 modifications and E2A binding to the 14-3-3y promoter. ChIP-quantitative PCR analysis of CFP1-binding, H3K4me3 enrichment and E2A-binding to DNA in the 14-3-3y locus (regions 3–11 as diagramed in Figs. 5, 6, and 7) in p85α+/+ and p85α−/− B cells treated with DMSO (nil) or TPCA-1 and stimulated with LPS plus anti-κB mAb/dextran for 24 h. Data is representative of three independent experiments.
NF-κB enhances CFP1 and E2A binding to the 14-3-3γ promoter

To gain further insight into the molecular mechanisms underlying the critical role of NF-κB in 14-3-3γ induction, we next addressed the role of NF-κB in regulating the recruitment of CFP1, which can interact directly with NF-κB (50), and E2A, which mediated the sustained 14-3-3γ expression. In B cells (particularly p85α−/− B cells) treated with TPCA-1, which inhibited NF-κB p65 activation and binding to 14-3-3γ promoter (Fig. 5D), CFP1 recruitment to and H3K4me3 enrichment in the 14-3-3γ promoter were impaired (Fig. 9). Likewise, E2A binding to the 14-3-3γ promoter was decreased.

Thus, NF-κB triggers rapid and sustained 14-3-3γ induction by recruiting or stabilizing CFP1 and E2A to or on the 14-3-3γ promoter (Fig. 10).

Discussion

We have shown that 14-3-3γ was induced by primary CSR-inducing stimuli rapidly and at higher levels, as compared with genes reported to be rapidly induced in a B cell differentiation stage-specific fashion, such as Ifn4 and A20. Expression of 14-3-3γ was sustained over the course of CSR induction, consistent with the important role of this adaptor in the targeting of AID. The important role of 14-3-3γ in AID targeting has been shown by our previous ChIP analysis of this adaptor in the targeting of AID. The important role of 14-3-3γ over the course of CSR induction, consistent with the important role in early induction of 14-3-3γ lights the requirement of highly regulated AID targeting for the maintenance of genomic integrity, because overexpression of AID or expression of AID1-190 leads to enhanced mutagenesis in non-Ig genes (15, 52, 53). 14-3-3γ induction preceded germline IκB-S-Cα transcription and the introduction of combinatorial H3K9acS10ph histone modifications in the acceptor region that will undergo recombination (12). 14-3-3γ docking onto H3K9acS10ph-modified chromatin and its subsequent locking to 5′-AGCT-3′ repeats in the target S regions will stabilize the binding of 14-3-3γ and, therefore, AID to those S regions (28, 30, and G. Li et al., submitted for publication). Finally, upon early induction, 14-3-3γ could have a role in regulating gene expression in B cells undergoing CSR, as suggested by its ability to recognize H3S10ph/S28ph and mediate histone crosstalk (54, 55). 14-3-3γ, however, is dispensable for AID expression, germline IκB-S-Cα transcription, and B cell proliferation and viability, because these are largely normal in 14-3-3γ−/− B cells (29).

Like AID and histone-modifying enzymes (8, 30, and G. Li et al., submitted for publication), 14-3-3γ was upregulated in a B cell differentiation stage fashion and induced by T-dependent and T-independent primary CSR-inducing stimuli, which are the main B cell NF-κB-activating stimuli (12). Accordingly, 14-3-3γ induction is critically dependent on NF-κB, as demonstrated by our findings that the (canonical) p65 and (noncanonical) p52 were recruited to the 14-3-3γ promoter, the 14-3-3γ promoter mutants lacking the κB motifs had markedly reduced activities, and 14-3-3γ expression was abrogated in p85α−/− B cells treated with the IKK-2 inhibitor TPCA-1. The canonical NF-κB pathway plays a major role in early 14-3-3γ promoter activation, as suggested by our previous findings that p65 phosphorylation is rapidly induced in B cells (generally within minutes) by CD154 or LPS (8). This NF-κB pathway typically induces rapid but transient gene expression. By contrast, NF-κB activated by the noncanonical pathway mediates sustained gene expression, including 14-3-3γ induction (Fig. 10). Finally, NF-κB can be recruited to an intronic...
enhancer (region 10), which was constitutively enriched in H3K4me1 and was devoid of H3K4me3 (45), to further enhance the 14-3-3-y induction.

Epigenetic modifications are emerging as key regulators of the Ab response, including SHM, CSR, memory B cell differentiation, and plasma cell differentiation (28). The time lag of 14-3-3-y induction and those of AID induction and germline Igκ-S-Cγ transcription in the downstream Igκy, Igε, or Igα sublocus (the Igκ sublocus is constitutively transcribed) would reflect different epigenetic states of these genes in resting mature B cells. In these cells, the 14-3-3-y promoter is already bound by CFP1 and enriched for H3K4me3 at low levels, suggesting that it is in a relatively open chromatin state poised for the recruitment of NF-κB and the transcription machinery (Fig. 10). In contrast, the Aicda gene and Igκ (Iy, Ie, and Io) promoters are in an epigenetically repressed state, as suggested by the DNA hypermethylation and low H3 acetylation, H3K4me3, H3K9ac, or H3S10ph in those regions (30, 56–59, and G. Li et al., submitted for publication). In activated B cells, binding of NF-κB would facilitate the recruitment of the CFP1-containing COMPASS complex for induction of much higher levels of H3K4me3 enrichment (Fig. 10), as indicated by our findings that NF-κB colocalized with CFP1, likely through direct interactions (50), on the 14-3-3-y promoter in activated B cells. Conversely, CFP1 binding and H3K4me3 enrichment were impaired in B cells treated with TPCK-1. This finding is reminiscent of the recruitment of chromatin modifying enzymes, such as the H3K9 demethylase Aof1 and H3K27 methyltransferase Ezh2, by transcription factors, including NF-κB, Pax5, and Stat5, to their target genes (60–62). In addition, NF-κB can also upregulate CFP1 expression (30, and G. Li et al., submitted for publication), thereby further augmenting the CFP1 recruitment to the 14-3-3-y promoter. Finally, knockdown of Cfp1 in CH12F3 B cells by short hairpin RNA significantly inhibits CSR to IgA, as induced by CD154, IL-4, and TGF-β, without affecting AID expression or germline Iκα-κα transcription (27), perhaps resulting from defective AID recruitment or stabilization to ro on S region DNA, possibly because of 14-3-3-y downregulation.

The open chromatin state of the 14-3-3-y promoter would be promoted by the unusually high density of 5′-CpG-3′ dinucleotide motifs (accounting for more than 30% of DNA), resulting in not only CFP1 binding but also virtually complete DNA demethylation and nucleosome destabilization (H. Zan and P. Casali, unpublished observations). Such a high density in the relatively short (~200 bp) promoter region might explain the sharp nature of the 14-3-3-y promoter, in contrast to most CpG island-containing promoters, which average 1 kb in size (40). Notably, the 14-3-3-y promoter, but not the 5′-CpG-3′-rich 3′ UTR, is rich in 5′-CpGG-3′ trinucleotide motifs, which in their unmethylated form are bound by CFP1 with a higher affinity than unmethylated 5′-CpGA/C-T-3′ trinucleotide motifs (63). In contrast, MLL1 and KDM2A, two other proteins that bind to unmethylated 5′-CpG-3′, have not been shown to have a higher affinity for unmethylated 5′-CpGG-3′ (40, 64). Collectively, stabilization by NF-κB and high-affinity binding to 5′-CpGG-3′ trinucleotide motifs might explain the promoter-specific recruitment of CFP1, which would in turn prevent de novo 5′-CpG-3′ methylation (40, 65).

In addition to NF-κB, which is ubiquitously expressed and broadly regulates many genes (66), E2A proteins are necessary for sustained 14-3-3-y expression in B cells undergoing CSR, as shown by our luciferase reporter assays of the 14-3-3-y promoter mutant lacking the E-box motif and Id3 blocking of sustained expression of 14-3-3-y, as already induced by LPS. The location of the E-box motif with the 14-3-3-y promoter is unique, because it is localized immediately downstream of the TSSs and is flanked by 5′-CpG-3′ motifs, and this unique location might contribute to the TSS sharpness by anchoring the RNA polymerase II pre-initiation complex. E2A transcription factors have been suggested to interact with and recruit the p300/CBP histone acetyltransferase to insert the active histone mark H3K27ac, thereby promoting recruitment of RNA polymerase II and unswapping of DNA from histones (67–69). These putative E2A activities together with low levels of H3K4me3 in 14-3-3-y promoter in resting B cells would mediate basal levels of 14-3-3-y expression (Fig. 10). Upon CD40 or TLR engagement, E2A binding to the 14-3-3-y promoter could be significantly enhanced in an NF-κB–dependent fashion, probably through upregulation of E2A expression, enhanced locus accessibility, or interactions with other components in the macromolecular complex assembled on the promoter. Interestingly, it has recently been shown that E2A proteins bind 14-3-3 (70), raising the possibility that E2A proteins can interact with newly induced 14-3-3-y to regulate gene expression.

Overall, our findings on the role of NF-κB and E2A in induction of 14-3-3-y gene expression, together with the previous demonstration of the AID gene activation by a multitude of transcription factors (12), highlight the importance of combinatorial interplay of transcription factors in mediating gene expression in a B cell–specific and B cell differentiation stage-specific fashion. These findings have lent support to two principles in epigenetic regulation of gene expression: 1) 5′-CpG-3′ motifs and their binding protein CFP1 provide an important link of crosstalk between DNA demethylation and histone modifications, and 2) recruitment of histone modifying enzymes is mediated by activated transcription factors. Finally, they emphasize the critical role of epigenetic modifications in the specification of biological information output in response to environmental stimuli, such as Ab class-switching in response to infection by microbial pathogens.

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References


