Cyclin-Dependent Kinases Regulate Ig Class Switching by Controlling Access of AID to the Switch Region

Minghui He, Elena M. Cortizas, Ramiro E. Verdun and Eva Severinsson

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Controlling Access of AID to the Switch Region

Minghui He,* Elena M. Cortizas,† Ramiro E. Verdun,*‡§ and Eva Severinson*  

Ig class switching requires cell proliferation and is division linked, but the detailed mechanism is unknown. By analyzing the first switching cells early in the kinetics, our analysis suggested that proliferating B cells had a very short G1 phase (<3.5 h), a total cell cycle time of ∼11 h, and that Ig class switching preferentially occurred in the late G1 or early S phase. Inhibition of cyclin-dependent kinases (CDKs) caused dramatic reduction of switching rate within 6 h. This was associated with less targeting of activation-induced cytidine deaminase (AID) to the IgH locus. Interestingly, ectopically expressed nuclear AID in HeLa cells was preferentially found in the early S phase. Furthermore, in CDK2 hypomorphic cells there was reduced nuclear AID accumulation. Thus, our data are compatible with the idea that division-linked Ig class switching is in part due to CDK2-regulated AID nuclear access at the G1/S border. The Journal of Immunology, 2015, 194: 4231–4239.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; A-EJ, alternative end joining; AID, activation-induced cytidine deaminase; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; C-NHEJ, classical nonhomologous end joining; CSR, class switch recombination; DSB, double-strand break; EdU, 5-ethylthyl-2’-deoxyuridine; eYFP, enhanced yellow fluorescent protein; HR, homologous recombination; LMB, leptomycin B; NHEJ, nonhomologous end joining; S, switch.

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Cyclin-dependent kinases in Ig class switching

Dr. Fernandez-Capetillo (20).

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A protocol using the FITC BrdU flow kit (BD Biosciences). After, the procedure was performed according to the manufacturer's protocol using the Click-iT EdU Pacific Blue kit (Life Technologies). BrdU was detected with monoclonal anti-BrdU Ab (clone MoBU-1, Life Technologies). Data were acquired with FACSVerse (BD Technologies).

Live cell imaging

Primary B cells were first activated with LPS (10 µg/ml) for 40 h to induce proliferation and then with anti-CD40 (20 µg/ml) plus IL-4 (8 ng/ml) for 16 h to induce switching to IgG1. Cells were seeded in anti-CD44–coated Ibidi µ-Slide (eight wells) with 50,000 cells/well. Cells were first incubated with the FcR blockage anti-CD16 for 20 min. At the same time anti-IgG1-biotin (BD Biosciences) was incubated with streptavidin-Alexa Fluor 568 (Life Technologies) for 30 min on ice and then added to cells at a final concentration of anti-IgG1-biotin at 0.5 µg/ml and of streptavidin-Alexa Fluor 568 at 20 µg/ml. Time-lapse images were captured with Zeiss Cell Observer microscope.

Flow cytometry

Activated B cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin in PBS. Cells were incubated with biotinylated anti-mouse IgG1, IgG2b, and IgG3. Six hours later, there was a first detectable round of class switching occurred after 42 h for IgG1, IgG2b, and IgG3. Six hours later, there was a >2-fold increase of the switching (Supplemental Fig. 1A), which must reflect truly new switching events, because one cell division time of mouse B cells is >6 h (22). To analyze the relationship to S phase among the first switching cells, activated B cells were given BrdU at 42 h and harvested 6 h later. More than 80% of IgG1 cells were BrdU-, whereas among total activated B cells, only 30–50% were BrdU+ (Supplemental Fig. 1B). This indicated that class switching either occurred in the S phase of the cell cycle or in the late G1 phase, but that after switching cells immediately entered into S phase. To assess the cell cycle distribution of newly switched cells, they were given a 1-h BrdU pulse before collection and harvested at 42, 45, or 48 h after stimulation of class switching. 7-Aminoactinomycin D (7-AAD) was added to the fixed and permeabilized cells to label the DNA content. Sixty-six to 68% of the IgG1+ cells were in S/G2/M phases at each tested time point, whereas among the total activated B cells there were only 23–25% (Fig. 1). About 25% of the IgG1+ cells were in G1 phase at all tested time points. This population had higher mean fluorescence intensity level of IgG1 staining (IgG1high) than the one in S/G2/M phases (data not shown), indicating that they result from earlier switching events.

Western blot

Total cellular protein extract (100 µg) was separated by SDS-PAGE and analyzed using monoclonal rabbit anti-actin (Sigma-Aldrich) or monoclonal rat anti-mouse AID (Active Motif) and HPR-conjugated swine anti-rabbit Ig (Jackson Immunoresearch Laboratories).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described (21). In short, activated B cells were cross-linked with 1% formaldehyde for 20 min at room temperature and the reaction was stopped by addition of glycine to 125 mM final concentration. Cells were washed twice with cold PBS and then resuspended in RIPA buffer (150 mM NaCl, 1% [v/v] Igepal CA-630, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, 50 mM Tris-HCl [pH 8], 5 mM EDTA, protease and phosphatase inhibitors) and sonicated on a Bioruptor (Diagenode). For immunoprecipitation, 0.5 mg (2 µg/µl) of protein extract was precleared for 2 h with 30 µl 50% protein G-Sepharose slurry before addition of Ab. Immune complexes were eluted and cross-linking was reversed by adjusting to 200 mM NaCl, 1 mM EDTA, 1 mM DTT, and incubating overnight at 65°C in the presence of 5 µg proteinase K. DNA was purified using a QIAquick PCR purification kit and quantified with real-time PCR with SYBR Green.

HeLa cells, plasmid transfection and immunofluorescence microscopy

HeLa cells were cultured at 37°C, 7% CO2 in high-glucose DMEM medium supplemented with 10% FBS. Plasmid transfection was carried out using a Lipofectamine transfection reagent (Life Technologies) following the manufacturer’s protocol. Twenty-four hours after transfection, cells were left untreated or pulsed with roscovitine (20 µM) for 2 h, after which leptomycin B (10 ng/ml) was added for an additional 4 h. Cells were fixed with 4% formaldehyde, stained with Hoechst 33258, and analyzed using a Zeiss LSM 780 confocal microscope. HeLa cells were synchronized to Gi phase by completely removing serum for 72 h or to early S phase by double thymidine treatment (2 mM thymidine was added to cells for 18 h, followed by 9-h release, and 2 mM thymidine was added again for another 17 h).

Results

Cell cycle distribution of newly switched cells

To determine the earliest time point that Ig class switching could be faithfully documented, naive mouse B cells were stimulated in vitro for different periods with either anti-CD40 plus IL-4, for switching to IgG1, or LPS, for switching to IgG2b and IgG3. The first detectable round of class switching occurred after 42 h for IgG1, IgG2b, and IgG3. Six hours later, there was a >2-fold increase of the switching (Supplemental Fig. 1A), which must reflect truly new switching events, because one cell division time of mouse B cells is >6 h (22). To analyze the relationship to S phase among the first switching cells, activated B cells were given BrdU at 42 h and harvested 6 h later. More than 80% of IgG1 cells were BrdU-, whereas among total activated B cells, only 30–50% were BrdU+ (Supplemental Fig. 1B). This indicated that class switching either occurred in the S phase of the cell cycle or in the late G1 phase, but that after switching cells immediately entered into S phase. To assess the cell cycle distribution of newly switched cells, they were given a 1-h BrdU pulse before collection and harvested at 42, 45, or 48 h after stimulation of class switching. 7-Aminoactinomycin D (7-AAD) was added to the fixed and permeabilized cells to label the DNA content. Sixty-six to 68% of the IgG1+ cells were in S/G2/M phases at each tested time point, whereas among the total activated B cells there were only 23–25% (Fig. 1). About 25% of the IgG1+ cells were in G1 phase at all tested time points. This population had higher mean fluorescence intensity level of IgG1 staining (IgG1high) than the one in S/G2/M phases (data not shown), indicating that they result from earlier switching events in vitro or in vivo. To further investigate this question, cells were harvested at 48 h poststimulation, analyzed for both IgM and IgG1 expression, and gated for activated single cells. Among IgG1+ cells, there was a population with intermediate IgG1 levels, which also expressed IgM. These cells probably represented the most newly switched cells, with remaining production of IgM (Supplemental Fig. 1C, left lower panel). This population was almost all in S/G2/M phase (Supplemental Fig. 1C, right panel).

To analyze whether switching is also dependent on the cell cycle after AID has accumulated to sufficient levels, B cells were first activated with LPS for 2 d to allow induction of AID, and thereafter IL-4 was added to allow switching to IgG1. The aicda expression level increased 14-fold after 48 h LPS stimulation (Supplemental Fig. 1E) and this is most likely sufficiently high for switching. The first detectable switching to IgG1 started ~15 h after IL-4 was...
added. At this time, cells were given a BrdU pulse for 6 h. Most of the new IgG1-producing cells were BrdU + (Supplemental Fig. 1D). Altogether, these data show that Ig class switching is regulated by cell cycle phases and suggest that it preferentially ends in the S phase of the cell cycle.

**Ig expression in relationship to cell cycle phases**

To further investigate the correlation between Ig class switching and cell cycle phases, double labeling with both EdU and BrdU was employed to estimate the length of S phase and G2 + M + G1 phases. A 0.5 h EdU pulse was given to the activated B cells at 42 h poststimulation and washed away thereafter. This was followed by a 0.5-h BrdU pulse added every hour after the EdU pulse during an 11-h period, so that all cultures got one pulse of each EdU and BrdU (Fig. 2A). Cells were harvested after the BrdU pulse and analyzed for the incorporation of both EdU and BrdU. Because activated B cells constitute an asynchronous cell population, the EdU + population represents cells in any stages of the S phase (Fig. 2Ba). These cells continuously went through the cell cycle in the following 11 h. In theory, when the cells exit the S phase, they will lose the capability to incorporate BrdU. Therefore, among the EdU + population, cells also labeled with BrdU (EdU +BrdU+) will decrease as time passes after the EdU pulse. If the length of S phase equals or is shorter than that of G2, M, and G1 phases together, all EdU + cells would become BrdU at a certain time point (Supplemental Fig. 2G). In this case, there would be no EdU +BrdU + (0%) population at this time point. However, according to our results this did not occur and the EdU + BrdU + population reached the lowest level (60%) 4 h after the EdU labeling (Fig. 2C). Thus, the S phase must be longer than the G2 + M + G1 phases. Furthermore, the increase after 4 h suggested that some of the EdU + cells that had finished the first cell cycle started to enter into the next S phase after this time point (Fig. 2Bb, Supplemental Video 1). These EdU + cells probably represented those that were at the end of S phase during the EdU pulse. Thus, our data imply that from the end of the S phase to the beginning of the next S phase (G2 + M + G1) it took ∼4 h. From 4 to 7 h after the EdU pulse, some of the divided EdU + cells started entering the next S phase, which would lead to an increase in the EdU +BrdU + population. At the same time, other EdU + cells that were at the early S phase during the EdU pulse exited the S phase, which would lead to a decrease in the percentage of double-labeled cells. Therefore, the slow increase in Fig. 2C represents the net increase out of these two changes (Fig. 2C, 4–7 h, Supplemental Video 1). Based on the model, when the EdU + cells that were at the beginning of the S phase during the EdU pulse exited the S phase, there would be a pure increase of the EdU +BrdU + population resulting from the re-entering of EdU + cells into the next S phase. This increase would be much sharper than the one from 4 to 7 h, because at this point all the EdU + cells will have passed beyond the first S phase. Indeed, the EdU +BrdU + population increased sharply from 7 to 8 h after EdU pulse, compared...
with levels at 4–7 h (Fig. 2Bc, 2C, Supplemental Video 1). Consistently the slopes of the curves at the different parts were quite different; from 1 to 4 h it was −11.8, from 4 to 7 h it was 1.9, and from 7 to 8 h it was 9.4. This means that the EdU+ cells at the beginning of the S phase during the EdU pulse took 7–8 h to finish the S phase, suggesting that the S phase took 7–8 h in these cells. From these data, we calculated that length of G2 + M + G1 at 4 h and the S phase at 7–8 h. Therefore, the average time of a cell cycle in activated B cells is ∼11–12 h.

To monitor when Ig class switching occurs in a cell cycle, the activated B cells were seeded on anti-CD44–coated Ibidi μ-Slide. Time-lapse images were captured with live cell microscope for 20 h. Cells were able to proliferate rapidly with this experimental setup, and two successive divisions were captured for many cells during the 20 h. A proportion of the dividing cells also underwent Ig class switching between these two divisions (Supplemental Videos 2, 3). However, we never observed a nondividing cell that switched. Among the cells that divided twice, the cell cycle time was variable in different cells, ranging from 8 to 16 h. The average cell cycle time of nonswitching B cells was 11.1 h (n = 169), whereas that of switching cells was 11.5 h (n = 41, Supplemental Table I). This is similar to the result from the double-labeling experiment with EdU and BrdU, shown in Fig. 2 (11–12 h). The time from the first division to the appearance of IgG1 on cell surface ranged from 3.3 to 9.8 h, with an average of 5.3 h (Fig. 2D, Supplemental Table I). By analyzing the morphology of the cells, we also found that it took ∼0.5 h from metaphase to division, suggesting that the M phase is slightly longer than half an hour. Taken together, these data show that the time of G2 + G1 must be <3.5 h in proliferating B cells and that they further support the idea that class switching ends in the early S phase.

**CDKs are involved in Ig class switching**

The correlation of class switching with cell cycle phases suggests that it may be subjected to regulation by CDKs. To test the involvement of CDKs during switching, cells were treated with the specific CDK inhibitor roscovitine at 42 h poststimulation for different periods. A 6-h treatment caused a >50% reduction of class switching to IgG1 (Fig. 3A, 3B). Ten- and 24-h treatments caused 60 and 47% reduction, respectively. Similarly, a 6-h treatment with a specific CDK2 inhibitor, CVT-313, resulted in 44% reduction of Ig class switching, whereas 10- and 24-h treatments caused 77 and 88% reduction (Supplemental Fig. 2A). Cell proliferation rate and cell cycle profile, as judged by labeling with CFSE and 7-AAD, respectively, were both comparable between 6 h roscovitine-treated and nontreated cells (Supplemental Fig. 2B). Cell viability was not influenced by a 6-h treatment (Supplemental Fig. 3F, 3G). However, longer treatments (24–48 h) with roscovitine arrested cells in G1 and G2/M phases (data not shown). Interestingly, it was mainly the IgG1+ cells in the S/G2/M phases that were reduced after a 6-h roscovitine treatment, whereas the proportion of switched cells in the G1 phase was unchanged (Fig. 3D). Therefore, blocking CDK activity probably inhibited the ending of class switching in the S phase. The IgG1+ cells in the G1 phase may have remained from earlier switching events in vitro or in vivo, and therefore remained unaffected by the treatment. In conclusion, these data suggest that CDKs are involved in the regulation of Ig class switching.

**FIGURE 3.** CDKs are involved in Ig class switching and association of AID to Sγ. The CDK inhibitor roscovitine was added at 42 h after stimulation and cells were harvested at indicated times. (A) Representative flow cytometry plot of IgG1+ cells in control and in cells treated for 6, 10, or 24 h with roscovitine. (B) Relative level of the numbers of IgG1+ cells, where controls without roscovitine are set to 100%, mean ± SD of five independent experiments; roscovitine 6 h, p = 0.0024; roscovitine 10 h, p = 0.0007; roscovitine 24 h, p = 0.0025. (C) ChIP to detect AID association with Sγ region in controls and 6, 10, or 24 h roscovitine-treated cells. Mean ± SD of three experiments; 0 h, nondetectable; 48 h (with/without roscovitine 6 h), p = 0.012; 52 h (with/without roscovitine 10 h), p = 0.0072; 66 h (with/without roscovitine 24 h), nonsignificant. (D) Influence of roscovitine on DNA content/cell. Left panels, Representative histogram plot of control and 6 h roscovitine-treated cells. Black line, IgG1+ cells; gray line (filled), total cells; x-axis, 7-AAD labeling. Right panel, Quantification of the IgG1+ cells in G1 or S/G2/M phase with or without roscovitine treatment for 6 h, expressed as the percentage of IgG1+ cells in roscovitine-treated as compared with control cells. Mean ± SD from three experiments; p = 0.0049 for IgG1+ cells in S/G2/M. Rosc, roscovitine.
Roscovitine is suggested to be a selective CDK inhibitor for CDK2 and CDK1 (23). To investigate the possibility that the reduced Ig class switching upon treatment was instead due to inhibition of CDK4, the CDK active in the G1 phase, cells were treated with a highly selective inhibitor of CDK4 (NSC 625987, 20 μM) for 6–48 h. Forty-eight-hour treatment with CDK4 inhibitor dramatically inhibited the cell proliferation (Supplemental Fig. 2H). However, 6-h treatment did not cause a significant reduction of switching to IgG1. Combined treatment with both CDK4 inhibitor and roscovitine caused a 52% reduction, which was similar to the effect by roscovitine alone (Supplemental Fig. 3H). Therefore, there was no additive effect when inhibiting both kinases, suggesting that the reduced Ig class switching upon roscovitine treatment was not due to inhibition of CDK4. Interestingly, a 6-h treatment with 2 μM of the specific CDK1 inhibitor, RO 3306, caused 44% reduction of Ig switching (Supplemental Fig. 3H). This suggested either that both CDK1 and CDK2, but not CDK4, are involved in class switching or that the CDK1 inhibitor was not very selective between these two kinases.

CDKs regulate AID access to Sμ region

To reveal the underlying mechanism of reduced class switching upon roscovitine treatment, we analyzed whether key regulators of CSR were influenced by the treatment. Levels of aicda, ung, and γ1 germline transcripts were comparable to control cells after 6-, 10-, and 24-h treatment (Supplemental Fig. 2C). Moreover, total AID protein levels were unchanged after 6-h roscovitine treatment (Supplemental Fig. 2D). Similarly, 24-h treatment with roscovitine and CVT-313 had no negative influence on the total AID protein levels (Supplemental Fig. 2E). We next performed ChIP to investigate association of AID to the Sμ region. Interestingly, treatment with roscovitine for 6 or 10 h caused ~42 and 67% reduction of AID association with the Sμ region, respectively. A 24-h treatment seemed to completely abolish the association (Fig. 3C). Consistently, 6- and 10-h treatments with CVT-313 resulted in 42 and 38% reduction of AID–Sμ association, respectively (Supplemental Fig. 2F). This suggests that access of AID to the Sμ region is regulated by a CDK, probably CDK2.

CDKs regulate the level of nuclear AID

To further investigate the underlying mechanism for cell cycle regulation of Ig class switching, we analyzed the effect of roscovitine treatment on the level of nuclear AID. To this end, HeLa cells were transfected with AID fused to enhanced yellow fluorescent protein (eYFP). Twenty-four hours after transfection, one group of cells was treated first with roscovitine for 2 h, and the other had no treatment. After 2 more hours, leptomycin B (LMB) was added to both groups and AID nuclear accumulation was analyzed 4 h later. In the control group, most cells had accumulation of AID in the cytoplasm, as reported previously (Supplemental Fig. 3D and Ref. 21). LMB treatment alone caused a strong increase of nuclear AID accumulation, in agreement with a previous report (24) (Fig. 4A). However, pretreatment with roscovitine completely abolished this increase. To confirm the above result, a similar experiment was performed in the mouse B cell line CH12, transfected with AID-eYFP and then activated with anti-CD40 plus IL-4 plus TGF-β. LMB treatment alone caused increased nuclear levels of AID-eYFP. Pretreatment with roscovitine for 2 h partly impaired this increase (Supplemental Fig. 3A).

Based on the above results, we hypothesize that the level of nuclear AID is regulated in a cell cycle–dependent manner. To test this, AID-eYFP–transfected HeLa cells were either arrested in G1 phase by serum starvation or early S phase by double thymidine block (Supplemental Fig. 3B, 3C). When arrested in G1 phase, AID subcellular localization

![FIGURE 4.](http://www.jimmunol.org/)

**FIGURE 4.** Subcellular localization of AID. (A) HeLa cells were transiently transfected with AID-eYFP. Twenty-four hours after transfection, cells were left untreated or treated with roscovitine for 2 h and then with LMB for 4 additional hours. Left, Representative images of control-, LMB-, or roscovitine plus LMB–treated cells. Right, Quantification of numbers of cells with AID nuclear accumulation, with mean ± SD of three independent experiments. The data are presented as percentage of cells with the LMB-treated group set as 100%. Nontreated versus LMB treated, nonsignificant; LMB- versus roscovitine plus LMB–treated, p = 0.0243, paired t test). (B) Subcellular localization of AID-eYFP in synchronized cells. Left, Representative images of serum-starved cells or double thymidine arrested cells (lower, fluorescence image; upper, merge of bright field and fluorescence images). Right, Quantification of the percentage of AID-eYFP* cells with AID accumulated in cytoplasm, nuclei, or evenly distributed; three independent experiments (cytoplasmic, p = 0.0092; nuclear, p = 0.0145). A Plan-Apo 40×/1.3 oil objective was used. Rosc, roscovitine.
78% of the transfected cells showed cytoplasmic and 7% nuclear AID accumulation. Among early S phase–arrested cells, 39.7% had AID mainly in the cytoplasm and 39.1% showed nuclear accumulation (Fig. 4B). In nonarrested cells, there were 56% of transfected cells with cytoplasmic accumulation and 25% with nuclear accumulation (Supplemental Fig. 3D). These data indicate that the level of nuclear AID is regulated by CDKs, either by controlling its translocation to the nucleus or by regulating its stability.

**CDK2 regulates nuclear AID levels**

To uncover the possible role of CDK2 in regulating the nuclear level of AID, we took advantage of a cell line in which the wild-type CDK2 is replaced by an analog-susceptible molecule (CDK2AS) (25). CDK2AS is hypomorphic compared with the control RPE-hTERT cells, and its activity can be restored or further inhibited by the adenine analogs 6-BAP or 3-MB-PP1, respectively. AID-eYFP was transiently transfected into control or CDK2AS cell lines and subcellular localization was analyzed after 24 h. Whereas AID was predominantly cytoplasmic in HeLa cells, most of the control RPE-hTERT and CDK2AS cells expressed AID in the nucleus (Fig. 5B). Among the control RPE-hTERT cells, 70% of the transfected cells had AID accumulated predominantly in the nucleus, but only 36% among the transfected CDK2AS cells (Fig. 5A, 5B). Correspondingly, there were 53% of the CDK2AS cells with AID distributed equally in cytoplasm and nucleus, comparing to the 24% among the wild-type ones (Fig. 5B). Consistently, the mean fluorescence intensity level of nuclear AID-eYFP in control cells was also higher than that in the CDK2AS cells (Fig. 5C). Similarly, 48 h after adding adenine analogs in AID-eYFP–transfected CDK2AS cells, cells treated with the CDK2-inducing 6-BAP had higher level of nuclear AID, as compared with the ones treated with CDK2-inhibitory 3-MB-PP1 (Fig. 5D). The reduced level of nuclear AID in CDK2-compromised cells might result from decreased translocation of AID to the nucleus. Another possibility is that CDK2 may function to stabilize nuclear AID, and as a consequence result in shorter half-life of AID in the CDK2AS cells as compared with wild-type ones.

**DNA synthesis is involved in CSR**

Our results suggest that B cells need to enter the S phase to finish the recombination process during class switching. Therefore, we tested whether DNA synthesis was required for switching. The specific DNA polymerase inhibitor aphidicolin was added to the cell culture 42 h after stimulation, and cells were harvested after 6 or 10 h. A 6-h treatment reduced switching by 44% without affecting the cell viability (Supplemental Fig. 3G), whereas a 10-h treatment caused >70% reduction (Fig. 6A). Transcription levels of *aid* remained unchanged, but there was a significant increase of *ung* level after 6 h treatment. Therefore, the reduced class switching is probably due to compromised activity of DNA polymerase. At 10 h, both *aid* and *ung* expression levels were reduced (Supplemental Fig. 3E), which probably contributed to the further reduction of switching. Because aphidicolin is a specific inhibitor for polymerase α and δ, which are mainly involved in DNA replication, it is possible that DNA synthesis is needed for CSR of a subpopulation of cells and that part of CSR events finished after onset of replication in IgH locus. Another possibility is that these polymerases are involved in the DNA repair necessary for CSR, and thus that treatment with aphidicolin affected the end joining stage of CSR, leading to reduced class switching.

Although our data indicate that part of the switching events occur in the early S phase, it is not likely that CSR can occur in the late S phase, owing to the fact that faithful repair mediated by homologous recombination (HR) is predominant at this stage. This prompted us to ask whether ATR can negatively regulate class switching, because recent evidence demonstrated that ATR could activate the HR pathway by promoting extensive DNA end resection (26). To this end, we used a highly specific ATR inhibitor identified recently, called ETP-464 (20). Cells were treated with 625 nM of the inhibitor from 42 to 48 h post-stimulation, and this resulted in a 2-fold increase of switching to IgG1 (Fig. 6B). This suggests that ATR negatively regulates CSR in activated B cells, possibly by maintaining genome stability and thereby restricting CSR to the early S phase.

**Discussion**

The process of Ig class switching is tightly regulated at multiple levels. Although cell proliferation is necessary, the underlying mechanism is partly unknown. Our results reveal a new level of regulation, that CDKs control the level of AID to the nucleus and to the S<sub>p</sub> region. Taken together, our data suggest that AID targeting to the switch regions occurs after the restriction point in G<sub>1</sub>. As...
which in turn facilitates formation of the S/S synaptosome. These above-mentioned processes are probably variable in different cell cycle phases, which in turn might influence AID recruitment. However, our result showed that the germline transcription was not influenced by roscovitine treatment, suggesting that the reduced association of AID to the S region is not due to compromised accessibility to the IgH locus.

Analysis of primary sequence shows that there is only one potential CDK target site in AID, which is at positions 85 and 86 (SP) in the human and murine sequences. Based on the structure analysis of AID homolog APOBEC-3G, this residue is buried inside of the molecule (data not shown) and is probably not accessible for CDKs. Additionally, it has been shown that mutation of the serine residue abolished catalytic activity (34). Many of the known cofactors of AID, which have been demonstrated being important for switching, contain multiple S/TP sites. Hsp90, Hsp40, and eEF1a have been identified in the cytoplasmic AID complex. They function to stabilize AID and prevent its entering to nucleus. However, according to their primary sequences, they contain only one S/TP site each. Therefore, CDK2 may target a yet unknown factor that regulates translocation of AID to the nucleus. Alternatively, 53BP1, Rif1, and Spt5 could be CDK targets because they contain clusters of S/TP sites. Studies in yeast have shown that phosphorylation of 53BP1 by CDKs inhibited its binding to DSBs, thereby promoting HR. Rif1 was shown recently to play a role downstream of 53BP1. Because in our experiments CDKs appear to inhibit a more upstream event, these two factors are not likely targets. Interestingly, Spt5 is a possible candidate, because it seems to facilitate association of AID to RNA polymerase II and S regions. Additionally, CDKs may also be involved in the steps following AID initiating the CSR. Previous studies have suggested that UNG activity is regulated by CDK-dependent phosphorylation (35). Therefore, inhibition of CDK activity may compromise UNG activity and in turn result in reduced class switching. CDKs were recently suggested to be involved in DNA repair (36, 37). Also, CDK2 seems able to regulate NHEJ through Ku70 (18). Thus, it is possible that blocking CDK activity can also compromise the end-joining stage of CSR. Further studies are needed to elucidate the CDK targets in class switching.

As a specific CDK inhibitor, roscovitine mainly targets two CDKs sharing high sequence homology: CDK1 and CDK2. CDK2 has its highest activity from late G1 to early S phase. This is followed by increased CDK1 activity as cells are going into late S and G2/M phases. Results from roscovitine and CVT-313 treatment suggest that CDK2 is a likely candidate responsible for the regulation of class switching. Investigation of AID levels in the RPE-hTERT cell lines (wild-type and CDK2AS) further supports this idea. However, as opposed to HeLa cells, most of the wild-type cells had nuclear accumulation of AID even without LMB treatment. This could be due to the more rapid proliferation of the RPE cell lines as compared with that of HeLa cells, because our result suggests that AID levels increase in the nucleus after the restriction point. Remarkably, AID-eYFP was equally distributed in nucleus and cytoplasm in more than half of the CDK2AS cells (Fig. 4B). This is distinguishable from the wild-type cells, because most of them had a higher level of AID-eYFP in the nucleus than in the cytoplasm. Moreover, the overall level of nuclear AID was lower in CDK2AS cells than in wild-type ones. This differential pattern is possibly caused by the different activity of CDK2. Interestingly, the mean fluorescence intensity level of AID-eYFP in entire CDK2AS cells was lower than the wild-type ones. It is possible that the half-life of AID-eYFP is shorter when CDK2 activity is compromised. Collectively, our data are consistent with involvement of CDK2 as a regulator of CSR. We cannot exclude that the regulation of AID expression is different in B cells and

![Image](http://www.jimmunol.org/DownloadedFrom/4237.jpg)
non-B cells, and that other CDKs may also play a role. However, the CDK2AS cell lines provide a system to investigate a true correlation of CDK2 function to nuclear AID level, instead of a possible nonspecific effect by inhibitor treatment or a redundancy with CDK1 in the CDK2 knockout cells.

Our result suggested that the average time of a cell cycle for an actively proliferating B cell is ∼11 h, and that it can be as short as 9 h. This is much shorter than previously reported (15–25 h dependent on the cell generation) (22). Our conditions differ from those of Duffy et al. (22) in several respects. We used wild-type mice, whereas they used GFP reporter mice. They used CTV-labeled cells and performed cell sorting, which might slow down the cell cycle, but we used nonlabeled cells. Furthermore, Duffy et al. studied cells later in the kinetics. Our results suggested that among those activated proliferating B cells the G1 phase was very short (<3.5 h) and the S phase very long (7–8 h). To our knowledge, this is the first report estimating the length of cell cycle phases in proliferating B cells. Although the length of the G1 phase seems very short, it has been known that the G2 phase is strongly variable among different cells. Moreover, the very long S phase might be a prerequisite for B cells to faithfully repair all the DNA damage induced by AID through HR. Interestingly, we also found that between the twin daughter cells, the switching one had a longer cell cycle time than did the nonswitching one (Supplemental Table II, Supplemental Video 2). This further supports the idea that B cells need to repair all of the DNA damage before moving on to division.

Additionally, our results also showed that most events of Ig class switching occurred in a narrow time window of ∼4–6 h after cell division. Probably it takes at least 20 min and not >2 h for the switched isotype to be expressed after CSR. If accumulation of nuclear AID is limited to after the restriction point, this would mean that at least in part of the cells recombination occurs shortly after onset of the S phase. Previous studies have shown that the C region of the IgH locus is replicated from the middle to late S phase in mature B cells and that replication preferentially initiates from origins within or downstream of the 3′ enhancer region and upstream of the rearranged V region (38). Therefore, it is likely that in a proportion of B cells recombination occurs before the locus has replicated. This could explain why aphidicolin treatment for 6 h only partially reduced the percentage of switching cells. Consistently, our result showed that ATR, a factor mainly coordinating DDR response in the S phase, could negatively regulate class switching. It probably functions to promote HR, thereby faithfully repairing the DSBs that do not lead to productive CSR.

It is known that factors from the canonical NHEJ pathway are involved in regulation of CSR. Such factors are, for example, Ku70/80, 53BP1, and Rad17 (39–42). Many classical HR factors are also implicated to be required for CSR, for example the Mre11, Rad50, and Nbs1 complex, RPA, and possibly CIP (43–46). In the absence of some c-NHEJ factor, CSR is still able to occur, although with reduced efficiency and with increased microhomology in the S–S junctions, a process called alternative end joining. Furthermore, Cortizas et al. (21) recently showed that factors of the C-NHEJ and A-EJ have the same kinetics of recruitment to the S regions. Therefore, CSR may occur at the G1/S border possibly because this is the only time that factors involved in NHEJ, A-EJ, and HR pathways can simultaneously participate.

Accumulating data suggest that AID-dependent DNA damage seems to be one of the major driving forces for variable types of mature B cell–derived lymphomas (47–49). In the absence of factors from the HR pathway, off-target activity of AID seems to be able to generate numerous non–Ig locus DNA DSBs, dramatically increasing genome instability and B cell cytotoxicity (14).

Therefore, it may be greatly beneficial that class switching ends in early S phase, because the remaining off-target activity of AID can be quickly and faithfully repaired through HR, diminishing the risk for genomic instability triggered by mechanisms such as A-EJ.

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Disclosures
The authors have no financial conflicts of interest.

References
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**Supplemental Figure 1.** Analysis of Ig class switching in relation to cell cycle. (A) Percentage of IgG1⁺, IgG2b⁺ or IgG3⁺ cells at 42, 48 and 66 h after stimulation in vitro, as detailed in Materials and Methods. Mean±SD of 5 independent experiments for IgG1, 4 for IgG2b and IgG3. Significance values comparing the 42 and 48 h time points were for IgG1, p=0.0009; IgG2b, p=0.0048; IgG3, p=0.012 using a paired t-test. (B) Representative flow cytometry plot of cells cultured for a total time of 48 h, given BrdU for the last 6 h (left) and percentage of BrdU⁺ cells among total or switched B cells (right), average of 3 independent experiments; comparison between total and IgG⁺ cells: IgG1, p=0.0038; IgG2b, p=0.001; IgG3, p=0.0243. (C) Gating strategy (upper panels) and cell cycle distribution (lower panel) of 48 h activated single B cells among total cells (black), IgG1^hi^ (gray) and IgM^low^IgG1^medium^ (gray, dotted) population. (D) B cells were first stimulated for 48 h with LPS and then IL-4 for additional 15 h, BrdU pulse was from 15 to 21 h after adding IL-4. Left, Representative flow cytometry plot. Right, percentage of BrdU⁺ cells among total and IgG1⁺ B cells and (E) mRNA levels of *aid* and *ung* at indicated times after stimulation.
**Supplemental Figure 2.** CDKs are involved in Ig class switching and association of AID to the Sm region. B cells were activated with anti-CD40 + IL-4 for 42 h before inhibitors were added. Cultures were harvested different times thereafter, as indicated. (A) IgG1 class switching after CVT-313 treatment for 6, 10 or 24 h, average from 3 experiments, p<0.01 for all time points. (B) Cell proliferation rate of 48 h activated B cells after CFSE labeling (left) and cell cycle profile (right) with 7-AAD labeling. Gray dotted, non-treated cells; Black, 6 h Roscovitine treated cells. (C) Levels of *aid*, *ung* and γ1 germline transcripts after 6, 10 or 24 h Roscovitine treatment. Roscovitine was added at 42 h. Average of 3 independent experiments, values were not significantly different from each other. (D) Western blot of AID protein level after 6 h Roscovitine treatment (upper) and quantification (lower), average from 2 experiments. (E) Western blot of AID protein level in control, 24 h Roscovitine or CVT-313 treated cells (upper) and quantification (lower). (F) Chromatin IP of AID association to the Sµ region after 6, 10 and 24 h CVT-313 treatment. Result from one experiment. (G) Modeling of kinetic changes of the EdU⁺BrdU⁺ population if the length of S phase equals or is shorter than that of G2+M+G1 phases. *a*, When BrdU is added immediately after the EdU pulse, all EdU⁺ cells will also be BrdU⁺; *b*, when all EdU⁺ cells have exited the S phase, they will be BrdU⁻. (H) Cell proliferation rate after CFSE labeling, control cells (black) and cells treated with CDK4 inhibitor (grey) for 48 h.
Supplemental Figure 3

A. Nuclear AID-EYFP in CH12

B. % of total transfected

C. Supplemental Figure 3

D. AID subcellular localization

E. RNA expression level

F. % of dead cells among total

G. % of dead cells among total

H. CSR to IgG1
Supplemental Figure 3. Nuclear AID is regulated in a cell cycle dependent manner. (A) Mean fluorescent intensity (FI) level (arbitrary values) of nuclear AID-EYFP transfected into CH12 cells. Each dot represents the intensity of AID-EYFP in a single cell. Mean value with SEM: p<0.0001 for control vs LMB, p=0.0134 for LMB vs Rosc+LMB. (B) Cell cycle distribution of serum starved or double thymidine treated cells, using FUCCI live cell indicators. G1: Fucci-G1^+ cells; G1/S: Fucci-G1^+ and Fucci-S/G2/M^+ cells; S/G2/M: Fucci-S/G2/M^+ cells (C) Cell cycle profile of serum starved or double thymidine treated HeLa cells. X-axis: 7-AAD labeling of DNA content, Y-axis numbers of cells. Black line: serum starved, gray dotted line: double thymidine treated. (D) AID subcellular localization in control, transiently transfected HeLa cells with no cell cycle arrest. (E) Relative transcription levels of aid and ung after 6 or 10 h aphidicolin treatment of activated B cells. B cells were harvested at 48 h (non-treated and 6 h treated with aphidicolin) or 52 h post stimulation (non-treated and 10 h treated with aphidicolin); aid: 91% relative to non-treated for 6 h and 49% for 10 h; ung: 185% for 6 h and 77% for 10 h. (F) Gating strategy of live and dead cells, confirmed by 7-AAD labeling. (G) Percentage of dead cells among total cultured cells. Mean ± SD of four independent experiments, values were non-significant between inhibitor-treated and untreated for all groups. (H) Level of switching to IgG1 upon 6 h treatment with CDK inhibitors. Cells were stimulated with anti-CD40+IL-4 for 42 before inhibitors were added. Cultures were harvested at 48 h. Paired t-test, Mean±SD from at least 6 independent experiments. Cdk4i, NSC 625987, ns, non-significant; Cdk2i, roscovitine treated, p<0.0001; Cdk2i+Cdk4i, p<0.001; Cdk1i, RO 3306, p<0.0001. All p-values are compared to controls.
### Supplemental Tables

**Table I. Ig switching between two divisions**

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**Table II. Ig switching in one of twin cells**

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<th>Twin cells after a division</th>
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<td>14.0</td>
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<tr>
<td>IgG1-</td>
<td>&gt;12.0</td>
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<tr>
<td>IgG1+</td>
<td>&gt;12.6</td>
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**Supplemental Table I.** Monitoring of individual cells that undergo Ig class switching between two successive divisions, as judged by time-lapse photography. A total of 89 cells were observed that divided and switched. No cells switched without division. 13 cells divided first, then switched, with an average time between these events of 5.5 h, but did not divide again during the 20 h period. Thirty-four cells first switched and then divided, with an average time between these events of 6.0. Forty one cells divided twice and switched in between. These are shown in the Table. First column, capture positions. Second column, time from 1st division to appearance of IgG1 on cell surface. Third column, time from appearance of IgG1 to the 2nd division. Forth column, total cell cycle time. Each row represents an individual cell.

**Supplemental Table II.** Twin daughter cells that undergo two successive divisions. Only one of the daughter cells switched between these two divisions. Length of cell cycle time.
**Supplemental Video Legend**

Video 1. Cell cycle progression of EdU$^+$ cells. If the G2+M+G1 phases together are shorter than the S phase, at no time will all of the EdU$^+$ cells become BrdU$^-$. 

Video 2. Ig class switching between two successive divisions on only one of the daughter cells from the first division. Bright and fluorescent field. IgG1 was labeled with anti-IgG1-biotin-Streptavidine-Alexa 568. An arrow marks the cell on the first image and four arrows mark the four daughter cells on the last image.

Video 3. Ig class switching between two successive divisions on both daughter cells. An arrow marks the cell on the first image and four arrows mark the four daughter cells on the last image.