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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 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 dependent kinases (CDKs) caused dramatic reduction of switching rate within 6 h. This was associated with less targeting of AID to the Igh locus. Interestingly, ectopically expressed nuclear AID in HeLa cells was dependent on CDK2 activity.

Little is known about how Ig class switching is coordinated with cell cycle control, although cell proliferation is required for Ig class switching (6). It was shown that two to three rounds of cell division was required before switching to IgG and IgA and five to six rounds for IgE (7, 8). This requirement is partly because the AID expression level is upregulated after two cell divisions. Additionally, AID expression levels increase with successive divisions, providing a possible explanation for proliferation-dependent class switching (9). Although there are some early studies suggesting that CSR may occur in the S phase of the cell cycle (10, 11), there is evidence suggesting that AID-dependent DSBs in the Igh locus occur mainly in the G1 phase (12, 13). However, AID is present all through the cell cycle in activated B cells. Because of the existence of the G1/S checkpoint, it would appear unlikely that B cells can pass through the cell cycle checkpoint before CSR is achieved and all the breaks are repaired. Therefore, CSR was postulated to occur in the G1 phase. However, other studies indicate that the G1/S checkpoint is not fully functional in activated B cells and that AID-dependent DSBs can leak into S phase (14–16). This raises the question whether Ig class switching itself is subjected to cell cycle regulation, for example by cyclin-dependent kinases (CDKs).

CDKs are the central players in regulating cell cycle progression. Several CDKs have been identified in mammalian cells with functional redundancy and tissue specificity (17). Recent studies suggest that CDKs may also be involved in the DNA damage response and apoptosis. For example, mammalian CDK2 plays an important role in DNA repair by enhancing the NHEJ pathway (18). So far, it is still unclear how CDKs are involved in these processes. Similar to exogenous DNA damage reagents, class switching also induces DNA damage response and triggers the same set of repair proteins. Instead of faithful repair, these proteins promote a deletional recombination event in switching cells. However, to our knowledge there is no information whether CDKs are also involved in regulating Ig class switching.

In the present study, we examined the early kinetics of Ig class switching in mouse splenic B cells in vitro. We give evidence that Ig class switching ends in the early S phase. Experiments are presented that CDK2 can control access of proliferative AID to the S region. Our data thus provide an explanation for proliferation-dependent AID access.

Materials and Methods

Mice

C57BL/6 mice were purchased from Scanbur and bred in pathogen-free conditions at the animal facility of the Department of Molecular Bio-

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-ethyl-2'-deoxyuridine; eYFP, enhanced yellow fluorescent protein; HR, homologous recombination; LMB, leptomycin B; NHEJ, nonhomologous end joining; S, switch.

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B cell isolation and cell culture

Enriched spleen B cells were cultured by treatment with Abs to CD4, CD8, CD90.2, and CD11b (BD Biosciences) and low-toxic rabbit complement (Cedarlane) followed by Percoll-gradient separation. Cells were cultured at 2–4 × 10^6 cells/ml. Monoclonal rat anti-mouse CD40 (1C10) was purified as described (19) and was used at 10–20 μg/ml. IL-4 (PeproTech) was used at 8 ng/ml. LPS O55:BS (Sigma–Aldrich) was used at 10 μg/ml. RPMI 1640 culture medium was supplemented with sodium pyruvate, penicillin-streptomycin, l-glutamine, 2-ME, and 10% pretested FBS. Cells were treated with various CDK inhibitors 48 h after stimulation with anti-CD40 plus IL-4 for 6, 24, or 48 h. The CDK2 inhibitors used were roscovitine (Sigma–Aldrich) (10 μM) and CVT-313 (Merck Millipore) (10 μM). The CDK4 inhibitor used was NSC 625987 (Calbiochem) (20 μM). The CDK1 inhibitor used was RO 3306 (Sigma–Aldrich) (2 μM). The ATR inhibitor ETP-464 was used at 625 nM and was provided by Dr. Fernandez-Capetillo (20).

5-Ethynyl-2'-deoxyuridine and BrdU double labeling

5-Ethynyl-2'-deoxyuridine EdU (20 μM) was first added to anti-CD40 plus IL-4–stimulated B cells for 30 min at 42 h postactivation and was thereafter removed via washes with complete media. Supernatants from parallel cultures were added to these cells. Incubation continued and BrdU (20 μM) was added 30 min before harvesting the cells, so that each culture only got one pulse of BrdU. Cultures were harvested every hour from 1 to 15 h, the EdU double-labeled cells were fixed in 4% formaldehyde and detected on Alexa Fluor 647 using the Click-IT EdU Alexa Fluor 568 kit (Life Technologies). BrdU was detected with monoclonal anti-BrdU Ab (clone MoBU-1, Life Technologies). Data were acquired with FACSVerse (BD Biosciences) and analyzed with FlowJo.

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 FcεRI 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, IgG3 or isotype controls (BD Biosciences), washed, and labeled with streptavidin–FITC or –allophycocyanin. Data were acquired on FACSCalibur (BD Biosciences) and analyzed using FlowJo.

Cell cycle analysis

Cells were labeled with BrdU (10 μM) for 1 or 6 h and labeled with a monoclonal FITC–conjugated rat anti-BrdU Ab (AbD Serotec). Thereafter, the procedure was performed according to the manufacturer’s protocol using the FITC BrdU flow kit (BD Biosciences).
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

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
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 Sp4. 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 Sp4 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 germ line 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 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,
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-sensitive 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, compared 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 recombinase 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 phase region. Taken together, our data suggest that AID targeting to the switch regions occurs after the restriction point in G1. As
a result, Ig class switching is mainly restrained to the late G1 or early S phase and finishes in the early S phase, providing a fundamental explanation for the division-linked switching.

AID is known to be able to shuttle between cytoplasm and nucleus (24, 27, 28). Owing to its DNA deamination activity, high levels of AID in the nucleus may cause severe genomic instability in B cells. This is a probable reason why >90% of the total AID protein is found in the cytoplasm, regulated through both nuclear export and cytoplasmic retention (28, 29). Recent studies suggest that cytoplastmic AID exists as a 300- to 500-kDa protein complex, containing Hsp90, Hsp40, and eEF1A and probably also other so-far-unknown factors (30–32).

We have found that CDK inhibitors affect class switching, association of AID to the Sp4 region, and the level of nuclear AID. In our minds, there are three possible explanations for these results: CDKs may regulate the release of AID from translocation-inhibitory factors in the cytoplasm; alternatively, they might inhibit nuclear export of AID; third, CDKs may function to stabilize nuclear AID and/or directly regulate its loading to the S regions. However, it is also possible that CDKs regulate this process indirectly by controlling a third factor that performs above suggested functions.

Additionally, previous studies have provided evidence that recruitment of AID to the Ig locus is dependent on the chromatin accessibility (33). An open chromatin structure is created in the downstream S regions after B cell activation, which involves processes similar to histone modification, chromatin remodeling. This can lead to an upregulation of the germline transcription, 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

FIGURE 6. Class switching is partially dependent on DNA synthesis and is negatively regulated by ATR. The inhibitors were added 42 h after activation and cells were harvested at indicated times. (A) Representative flow cytometry plot of class switching to IgG1 with or without treatment with the DNA synthesis inhibitor aphidicolin for 6 or 10 h (left panels) and mean ± SD of nine independent experiments for 6 h treatment and mean value from two experiments for 10 h treatment (right panel); 48 h (without aphidicolin 6 h), p = 0.0013. (B) Representative flow cytometry plot of class switching to IgG1 with or without treatment with the ATR inhibitor ETP-464 for 6 h (left) and quantification of three independent experiments; mean ± SD, p = 0.0113 (right).
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 G1 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 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 Rif1 (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

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