Temporal Regulation of Ig Gene Diversification Revealed by Single-Cell Imaging

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Temporal Regulation of Ig Gene Diversification Revealed by Single-Cell Imaging

Ellen C. Ordinario,2,3† Munehisa Yabuki,2* Ryan P. Larson,* and Nancy Maizels4*†

Rearranged Ig V regions undergo activation-induced cytidine deaminase (AID)-initiated diversification in sequence to produce either nontemplated or templated mutations, in the related pathways of somatic hypermutation and gene conversion. In chicken DT40 B cells, gene conversion normally predominates, producing mutations templated by adjacent pseudo-V regions, but impairment of gene conversion switches mutagenesis to a nontemplated pathway. We recently showed that the activator, E2A, functions in cis to promote diversification, and that G1 phase of cell cycle is the critical window for E2A action. By single-cell imaging of stable AID-yellow fluorescent protein transfectants, we now demonstrate that AID-yellow fluorescent protein can stably localize to the nucleus in G1 phase, but undergoes ubiquitin-dependent proteolysis later in cell cycle. By imaging of DT40 polymerized lactose operator-AID cells, in which polymerized lactose operator tags the rearranged λR gene, we show that both the repair polymerase Polη and the multifunctional factor MRE11/RAD50/NBS1 localize to λR, and that λR/Polη colocalizations occur predominately in G1 phase, when they reflect repair of AID-initiated damage. We find no evidence of induction of γH2AX, the phosphorylated variant histone that is a marker of double-strand breaks, and Ig gene conversion may therefore proceed by a pathway involving templated repair at DNA nickas rather than double-strand breaks. These results lead to a model in which Ig gene conversion initiates and is completed or nearly completed in G1 phase. AID deaminates ssDNA, and restriction of mutagenesis to G1 phase would contribute to protecting the genome from off-target attack by AID when DNA replication occurs in S phase.


The V regions of actively transcribed Ig genes undergo physiologically induced and regulated sequence diversification, which expands and modulates the repertoire for Ag recognition and provides a dynamic response to infection by pathogenic microorganisms (1–5). V region diversification produces two distinct mutagenic signatures: somatic hypermutation, which introduces nontemplated single base changes; and gene conversion, which uses upstream pseudo-V (ψV) gene segments as templates for mutagenesis (2, 6–11). Gene conversion is the primary source of diversity in the preimmune Ab repertoire of chicken and other fowl. Somatic hypermutation occurs in Ag-activated human and murine B cells, and also diversifies the preimmune repertoire in other species, such as sheep (12).

The mechanisms of somatic hypermutation and gene conversion are closely related, as first proposed over a decade ago (13). Both processes are initiated by attack of the B cell-specific enzyme, activation-induced cytidine deaminase (AID),5 on actively transcribed Ig genes (14–17). AID deaminates cytosine to uracil in DNA (18–21). Mutagenic repair then depends either on uracil-DNA glycosylase (UNG), which removes uracil to create an abasic (AP) site, or MutSα (MSH2/MSH6), which recognizes U-G mismatches (22). The multifunctional MRE11/RAD50/NBS1 (MRN) complex (23–25) associates with and is enriched at diversifying Ig genes, where it may promote V region gene conversion by using its AP lyase activity to cleave at the AID-initiated AP site (26, 27); by tethering DNA molecules for recombination (24); and/or by carrying out DNA resection necessary for homology-dependent repair (28). The repair polymerase, Polη, participates in both gene conversion (29, 30) and somatic hypermutation (31–35), and can generate the templated mutations characteristic of normally proliferating chicken B cells. Point mutations accumulate if gene conversion is impaired by a variety of strategies, including ablation of critical factors, such as the RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) (36–38), BRCA1 or BRCA2 (39, 40); or deletion of (41) or repressive chromatin modifications at (42) ψV donors, probably as a result of recruitment of this and/or other low fidelity polymerases to sites of AID-initiated DNA damage.

We recently showed that activation of Ig gene diversification depends upon cis interactions of the Igα allele with the regulatory factor E2A that occur in G1 phase of cell cycle (43). Those experiments took advantage of the ability to directly image diversifying λR genes in single DT40 polymerized lactose operator (Poly-LacO)-λR B cells. These cells are derivatives of the chicken bursal lymphoma line DT40, in which PolyLacO has been inserted just upstream of the rearranged and expressed λL chain gene (42, 43). By single-cell imaging, we now demonstrate that AID-yellow polymerized lactose operator; RFP, red fluorescent protein; slgM, surface IgM; Ugi, uracil-DNA glycosylase inhibitor; UNG, uracil-DNA glycosylase; YFP, yellow fluorescent protein.

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5 Abbreviations used in this paper: AID, activation-induced cytidine deaminase; AP, abasic; DAPI, 4,6-diamidino-2-phenylindole; DSB, double-strand break; IR, ionizing radiation; Lac, lactose repressor; LMB, leptomycin B; MRN, MRE11/RAD50/NBS1; NES, nuclear export signal; NHEJ, nonhomologous end joining; PolyLacO, polymerized lactose operator; RFP, red fluorescent protein; slgM, surface IgM; Ugi, uracil-DNA glycosylase inhibitor; UNG, uracil-DNA glycosylase; YFP, yellow fluorescent protein.
fluorescent protein (YFP) can stably localize in the nuclei of cells in G1 phase, but not in S phase or later stages. Colocalizations of AID with the polymerase Polη occur predominately in G1 phase. We find no evidence for induction of γH2AX, the phosphorylated variant histone that is a hallmark for DNA double-strand breaks (DSBs), at any stage of cell cycle. These results suggest that Ig gene conversion involves a DNA intermediate that carries a nick, rather than a DSB, and that gene conversion may initiate and be nearly completed in G1 phase of cell cycle.

**Materials and Methods**

**Cell culture**

DT40 and its derivative cell lines were cultured and transfectected, as previously described (26, 43). Leptomycin B (LMB; Sigma-Aldrich) and MG132 (Z-Leu-Leu-Leu-aldehyde; Sigma-Aldrich) were added at 50 ng/ml and 50 μM, respectively, for indicated times. BrdU (Sigma-Aldrich) was added at 10 μM for 30 min to pulse label cells. Irradiation was conducted at 8 Gy using a 137Cs irradiator. The surface IgM (slgM)-loss assay was conducted, as described (26, 38, 43), and results were compared with the Mann-Whitney U test.

**Plasmid constructs**

An AID-YFP expression construct was generated by cloning an AID cDNA of DT40 into the BglII site of pEYFP-N1 (BD Clontech). AIDΔC, which lacks the C-terminal 16 aa, was generated by amplification with primers 5′-CCAGAGATCTGAGAGAGAACCCAGCT GACATG-3′ and 5′-TTAAGAATCTCCAGAATGGATTCTTGAGAGACCCAGCT GACATG-3′ and similarly cloned into the BglII site of pEYFP-N1. Three phosphorylation-site mutants, AID T27A, AIDS38A, and AIDY184A, were generated by QuikChange site-directed mutagenesis (Stratagene). A Polη-GFP expression construct (44) was provided by J. Cleaver (University of California, San Francisco, CA). A uracil-DNA glycosylase inhibitor (Ugi) expression construct (45) was provided by M. Neuberger (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.).

**Immunofluorescence staining and image analysis**

Immunofluorescence staining was performed, as previously described (43). Primary Abs used were as follows: anti-GFP (3E6, 1:100; Molecular Probes); anti-NBS1 (1:25, provided by P. Concannon, University of Virginia, Charlottesville, VA; or 1:1000, affinity-purified rabbit Ab raised against a peptide corresponding to the C-terminal 20 aa of murine NBS1); anti-γH2AX (JBW3C1, 1:200, Upstate Biotechnology; or BL178, 1:500, Bethyl Laboratories); anti-phenylalanine-glycine repeat-containing nuclear pore proteins (Mabs14, 1:5000; Covance); anti-Golgi 58K protein (58K-9, 1:1000; Sigma-Aldrich); anti-ubiquitin (ab8134, 1:200; Abcam); and anti-SUMO1 (21C7, 1:200; Zymed Laboratories). The BrdU labeling and detection kit 1 (Roche) was used to detect incorporated BrdU. Alexa Fluor 488- or 594-conjugated anti-IgG (Molecular Probes) were used as secondary Abs. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

For imaging of AID-YFP transfectants, we screened multiple independent clones and selected for imaging the two transfectant clones in which the AID-YFP signal was brightest. Results with these two clones were comparable, and detailed analysis of one clone is presented. In that clone, AID-YFP was more than 10-fold overexpressed relative to endogenous AID, as estimated by RT-PCR. Nuclear localization of AID-YFP was examined using a line profile tool of the softWoRx imaging software (Applied Precision) and cells scored as positive if a YFP signal above background was detected throughout the nucleus. Cell cycle stage was determined by BrdU staining or estimated based on the nuclear size, as follows: G1, nuclear radius < 4 μm; G2, nuclear radius ≥ 5.2 μm (43). For experiments in which BrdU staining was used to establish cell cycle stage, DNase treatment (Roche) was used to render DNA single-stranded and expose the newly incorporated BrdU to Abs. This interferes minimally with the YFP signal, but does not create as intense a BrdU signal as denaturation with alkali. Only cells containing five or more distinct BrdU foci were scored as S phase cells, to exclude cells in other stages of cell cycle in which repair DNA synthesis was ongoing. The fraction of S phase cells as determined by BrdU staining was therefore lower than the fraction determined by propidium iodide staining and flow cytometry (36% compared with 56%). To quantitate colocalizations in DT40 PolyLacO-ΔN, and DT40 PolyLacO-ΔN derivatives, fluorescent images were acquired using the DeltaVision deconvolution microscopy system (Applied Precision) and processed and analyzed with softWoRx and Imaris (Bitplane) softwares.

Fluorescence signals were sometimes partially rather than completely overlapping, which may reflect the considerable distance (~17 kb) between the PolyLacO-tag and the VA region, and were scored as colocalizing if the distance of fluorescent peaks was <0.2 μm. Significance of colocalization and cell cycle distribution were analyzed with Pearson’s χ² test.

**UNG assay**

UNG activity in nuclear extracts was assayed, as described (27, 45). The substrate oligonucleotide was 5′-CAGAAAGGAAATATAC AACAAAAAGCACTCAAGCTTCTGGAGAGACA-3′, which was 5′-end labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (PerkinElmer).

**Results**

Regulated localization of AID in the DT40 nucleus

We assayed subcellular localization of AID in the chicken B cell line DT40, which derives from a bursal lymphoma and carries out constitutive Ig gene diversification (10, 11, 46). AID carries a strong nuclear export signal (NES) that specifies export via the nuclear pore, using the CRM1-mediated pathway, and AID has been shown to localize predominantly to the cytoplasm in mammalian cells (47–51). To examine AID localization by single-cell imaging, we constructed a vector expressing chicken AID carrying a C-terminal YFP tag (AID-YFP). Tagging AID at this position has been shown not to interfere with protein function (47, 52). By analysis of slgM-loss rate (43) of 24 independent DT40 AID-YFP transfectants, we determined that stable expression of AID-YFP accelerated the clonal rate of Ig gene diversification 2.5-fold (p = 0.0014, Mann-Whitney U test), comparable to results obtained by others (41, 47). We imaged AID-YFP localization by fluorescence microscopy. AID-YFP localized to the cytoplasm, forming dots or flares just outside the nucleus rather than a diffuse signal (Fig. 1A, left). Others have similarly observed that, in mammalian B cells, AID cytoplasmic signals may be threadlike (47) or concentrated in pockets on the nuclear surface (51). Using quantitation with a line profile tool of the softWoRx imaging software as the criterion for distinguishing nuclear/cytoplasmic distribution of AID, we confirmed the nuclear level of AID-YFP to be very low, indistinguishable from background (Fig. 1A, right). We further verified that cytoplasmic localization of AID is determined by the conserved C-terminal NES, as shown for AID in mammals, by demonstrating that deletion of the NES resulted in nuclear accumulation (AIDΔC-YFP; Fig. 1B, left). This was also confirmed by analysis with the line profile tool (Fig. 1B, right).

AID function is regulated in part by phosphorylation, and mutation of Ser38, a protein kinase A phosphorylation site, has been shown to affect AID function in Ig gene diversification (53–57). To test the possible role of regulatory phosphorylation in nuclear localization, we asked whether localization was affected by mutation at Ser38 as well as two other phosphorylation sites, Thr27 and Tyr184. We generated stable AID T27A-YFP, AID S38A-YFP, and AID Y184A-YFP transfectants, and assayed localization of each. All localized to the cytoplasm, but AID S38A-YFP also formed a few nuclear foci (Fig. 1C), suggesting that regulatory phosphorylation at this site may be important for AID localization.

Cell cycle dependence of AID nuclear localization

To test possible cell cycle dependence of AID-YFP localization, we first attempted to identify the AID-YFP signal in fixed cells sorted following propidium iodide staining, which measures DNA content. The cell cycle distribution was similar in the AID-YFP transfectants compared with the parental DT40 cell line: 23% G1 phase, 56% S phase, and 21% G2/M phase in DT40 AID-YFP transfectants; and 21% G1 phase, 55% S phase, and 24% G2/M phase in the DT40 parent. However, the fixed and sorted cells had
undergone significant loss of the AID-YFP signal. We therefore adopted alternative approaches to determine cell cycle phase. We have previously shown that nuclear radius corresponds closely to cell cycle stage in DT40 and its derivatives, and that nuclear radius \( <4 \mu \text{m} \) provides a conservative criterion for scoring G1 phase cells (43). Cells were cultured with LMB for 30 min, fixed, imaged, and assigned to cell cycle stage based on nuclear radius. A nuclear AID-YFP signal was observed in 21% of cells (\( n = 123 \)); and more than 80% of cells exhibiting a nuclear AID-YFP signal had nuclear radius \( <4 \mu \text{m} \), and were therefore in G1 phase (Fig. 1D, right). G1 phase cells correspond to less than 25% of the cell population, based on either cell sorting or measurements of nuclear radius. Thus, there appeared to be a considerable overrepresentation of nuclear AID-YFP in G1 phase of cell cycle, and underrepresentation in later stages. In particular, these results raised the possibility that AID-YFP might be excluded from the nucleus in S phase.

To specifically assay the presence of AID-YFP in the nuclei of S phase cells, we stained with BrdU to identify cells carrying out DNA replication. Cells were cultured with LMB for 30 min, then

**FIGURE 1.** Regulated nuclear localization of AID. **A**, AID-YFP predominantly localizes to the cytoplasm. **Left**, Representative images of DT40 AID-YFP stable transfectants. Nuclear pore complex (NPC) stained with Abs (red, center), and merged DAPI image (right). Bar, 5 \( \mu \text{m} \). **Right**, Subcellular distribution of AID-YFP analyzed with a line profile tool of the softWoRx imaging software. **B**, AIDΔC-YFP prominently localizes to the nucleus in G1 phase. Left, Representative images of DT40 AID-YFP cells treated with LMB (50 ng/ml, 1 h), then labeled with BrdU (10 \( \mu \text{M}, 30 \text{ min} \)). Bar, 5 \( \mu \text{m} \). Right, Cell cycle distribution of cells containing nuclear AID-YFP (21%; \( n = 123 \) cells), as determined by BrdU staining and nuclear size. **E**, AID-YFP may be degraded in the nucleus in S phase. **Left**, Representative images of DT40 AID-YFP cells treated with MG132 (50 \( \mu \text{M}, 4 \text{ h} \)) and LMB (50 ng/ml, 1 h); Golgi apparatus stained with Abs (red). Bar, 5 \( \mu \text{m} \). **Right**, Cell cycle distribution of DT40 AID-YFP cells treated with MG132 and LMB, which contain nuclear AID-YFP (18%; \( n = 298 \) cells). **F**, AID-YFP may form aggresomes following MG132 treatment. Representative images of DT40 AID-YFP cells treated with MG132 (50 \( \mu \text{M}, 4 \text{ h} \)) and LMB (50 ng/ml, 1 h), and stained with Abs to ubiquitin or SUMO (red, center), and merged DAPI image (right). Arrows indicate potential colocalization of AID-YFP and ubiquitin-staining signal.
BrdU was added for an additional 30 min of culture, after which cells were fixed and DNase-treated to expose incorporated BrdU to Abs, stained with anti-BrdU Abs, and imaged (Fig. 1D, left). We scored as BrdU+ only cells containing five or more nuclear BrdU foci, to exclude cells carrying out repair DNA synthesis, and tabulated BrdU+ cells and cells exhibiting a nuclear AID-YFP signal (Table I). After culture with LMB, followed by addition of BrdU, 19% of cells contained nuclear AID-YFP, but only 2.4% of cells containing nuclear AID-YFP were also BrdU+ (n = 288). When LMB was included after initiation of BrdU labeling, 12% of cells contained nuclear AID-YFP, and only 1.1% of cells contained both nuclear AID-YFP and BrdU+ (n = 353). Thus, there is a considerable underrepresentation of AID-YFP in BrdU+ cells, suggesting that AID-YFP is depleted from the nucleus when DNA replication occurs.

Levels of AID in mammalian cells are regulated by ubiquitin-dependent proteolysis, and the t1/2 of nuclear AID has been reported to be shorter than that of cytoplasmic AID (2.5 vs 18–20 h (52)). This suggested that proteolysis of AID may account for underrepresentation of nuclear AID-YFP in S phase cells, and predicted that culture with MG132, an inhibitor of ubiquitin-dependent proteolysis, would stabilize nuclear AID-YFP and increase the fraction of S phase cells in which it is evident. We tested this by culturing cells with MG132 and LMB for 4 h, then analyzing AID-YFP localization as a function of cell cycle, as determined by measuring nuclear radii of individual cells (43). Nuclear AID-YFP was evident in 18% of these cells (n = 298; Fig. 1E, left), comparable to the fraction of such cells observed following culture with LMB alone (19%). However, the cell cycle distribution of cells in which a nuclear AID-YFP signal was evident was clearly shifted, so only 13% of such cells were in G1 phase, whereas 69% were in S phase and 18% were in G2 phase (Fig. 1E, right; n = 55). This contrasts with the distribution of cells containing nuclear AID-YFP and treated with LMB alone (Fig. 1D, right; p < 0.001, χ2 test). Thus, culture with MG132 appears to stabilize nuclear AID-YFP in S phase cells, without increasing the fraction of G1 phase cells containing nuclear AID-YFP.

Culture with MG132 and LMB also promoted formation of a distinctive cytoplasmic spot of AID-YFP, which was shown to be juxtaposed to the Golgi apparatus by staining with anti-Golgi 58K Abs (red; Fig. 1E, left). This spot is similar to the aggregates of ubiquitinated proteins, or aggresomes that form as consequence of inhibition of the proteasome, as documented for other proteins, including the close relative of AID, APOBEC3G (58, 59). To determine whether these bright cytoplasmic AID-YFP spots might reflect aggregates of modified protein, we asked whether they colocalized with regions stained by Abs to ubiquitin or SUMO. There was some colocalization of the bright AID-YFP spot with regions of ubiquitin, but not SUMO staining (Fig. 1F).

The results above show that whereas AID-YFP localizes predominately to the cytoplasm, it can be found in the nucleus in G1 phase cells. AID-YFP appears to be able to enter the nucleus throughout cell cycle, but to be destabilized by ubiquitin-dependant proteolysis outside of G1 phase.

**E2A preferentially colocalizes with the rearranged λR allele**

Many repair factors and transcription factors function in factories readily identifiable by fluorescence microscopy as distinctive foci within the nucleus, containing 50 or more protein molecules concentrated in a small nuclear volume (60). Single-cell imaging of colocalizations of foci of such factors with specific genes can provide valuable information on temporal regulation of transcription or repair. To image Ig genes in the nucleus, we have used a derivative of DT40, DT40 PolyLacO-λRc, in which PolyLacO has been integrated by homologous gene targeting to the ψVα array just upstream of the rearranged and expressed λR gene (42, 43). In DT40 PolyLacO-λRc cells, the PolyLacO tag enables the diversifying λR gene to be imaged directly in cells expressing green fluorescent proteins or red fluorescent proteins (RFP) fused to lactose repressor (GFP-lactose repressor [LacI] or RFP-LacI), but does not alter proliferation, cell cycle distribution, the clonal rate of Ig gene conversion, or local chromatin structure (42, 43). Colocalizations of factors with the tagged gene can readily be quantitated in these cells by fluorescence microscopy. However, the confined nuclear space means that there will be inevitable background contributed by apparent overlap of the tagged gene and foci of transcriptional or regulatory factors. We have previously shown that the regulatory factor E2A colocalizes with the rearranged λR gene, and that this colocalization correlates with the cis interaction essential to promote Ig gene diversification (43). In addition, by chromatin immunoprecipitation analysis we have shown that E2A specifically associates with the rearranged λR allele in DT40 cells, exhibiting an 18-fold enrichment at that allele relative to the unrearranged λα allele (43). Those results enabled us to ask whether E2A preferentially colocalizes with the rearranged λR allele, as predicted by chromatin immunoprecipitation, and at the same time determine the background level of colocalizations evident, as analyzed by fluorescence microscopy. To do this, we assayed colocalizations of E2A with the unrearranged λα L chain allele, in the DT40 PolyLacO-λαc cell line. In that cell line, the PolyLacO tag is integrated in the ψVα array upstream of the unrearranged and unexpressed λα allele (Fig. 2A). In DT40 PolyLacO-λαc cells, proliferation (Fig. 2B), cell cycle distribution (Fig. 2C), and the clonal rates of Ig gene diversification (Fig. 2D) were comparable to the DT40 parental line, and to the DT40 PolyLacO-λRc cells. However, whereas λRc/E2A colocalizations were evident in 26% of DT40 PolyLacO-λRc cells (n = 227) in asynchronous culture (43), λα/E2A colocalizations were evident in only 6.3% (n = 191) of DT40 PolyLacO-λαc cells (Fig. 2E; p < 0.0001, χ2 test). Thus, quantitation of colocalization can provide very useful information on function of a factor at a gene.

### Table I. Nuclear AID-YFP is absent from S phase cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BrdU &amp; BrdU</th>
<th>BrdU &amp; LMB</th>
<th>Nuclear AID-YFP</th>
<th>Nuclear and BrdU+</th>
<th>Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMB</td>
<td>36%</td>
<td>32%</td>
<td>19%</td>
<td>2.4%</td>
<td>288</td>
</tr>
<tr>
<td>BrdU</td>
<td>12%</td>
<td>11%</td>
<td>12%</td>
<td>1.1%</td>
<td>353</td>
</tr>
</tbody>
</table>

**Notes:**

- a Cells were first treated with LMB (50 ng/ml) or BrdU (10 μM), as indicated, for 30 min (t = 0–30 min).
- b Incubation was continued with both compounds, as indicated, for 30 min (t = 30–60 min).
- c Percentage of cells containing five or more distinct BrdU foci was scored as BrdU+.
- d Nuclear localization of AID-YFP was examined using a line profile tool of the softWoRx software (Applied Precision), and cells scored as positive if a YFP signal above background was detected throughout the nucleus.

**References**

1. **D**

2. **AID-YFP and treated with LMB alone (Fig. 1**

3. This contrasts with the distribution of cells containing nuclear AID-YFP and treated with LMB alone (Fig. 1D, right; p < 0.001, χ2 test). Thus, culture with MG132 appears to stabilize nuclear AID-YFP in S phase cells, without increasing the fraction of G1 phase cells containing nuclear AID-YFP.

4. Culture with MG132 and LMB also promoted formation of a distinctive cytoplasmic spot of AID-YFP, which was shown to be juxtaposed to the Golgi apparatus by staining with anti-Golgi 58K Abs (red; Fig. 1E, left). This spot is similar to the aggregates of ubiquitinated proteins, or aggresomes that form as consequence of inhibition of the proteasome, as documented for other proteins, including the close relative of AID, APOBEC3G (58, 59). To determine whether these bright cytoplasmic AID-YFP spots might reflect aggregates of modified protein, we asked whether they colocalized with regions stained by Abs to ubiquitin or SUMO. There was some colocalization of the bright AID-YFP spot with regions of ubiquitin, but not SUMO staining (Fig. 1F).

5. The results above show that whereas AID-YFP localizes predominately to the cytoplasm, it can be found in the nucleus in G1 phase cells. AID-YFP appears to be able to enter the nucleus throughout cell cycle, but to be destabilized by ubiquitin-dependant proteolysis outside of G1 phase.

6. **E2A preferentially colocalizes with the rearranged λR allele**

This page contains text that may be part of a scientific study or research paper, discussing the localization and regulation of the protein AID in mammalian cells. It references experiments involving the use of fluorescent proteins and related techniques to study the movement and stability of AID within the cell cycle. The text describes how AID-YFP localization changes in response to different treatments and how these changes can be quantified. It also mentions the use of MG132, a proteasome inhibitor, to stabilize nuclear AID-YFP, and discusses the implications of these observations for understanding the regulation of AID and its role in the diversification of Ig genes during B cell development.
lambdaR/Poleta colocalizations occur preferentially in G1 phase

The repair DNA polymerase, Poleta, participates not only in Ig gene diversification, but also in repair of UV damage, and has been shown to form repair foci after UV irradiation (44). To establish when in cell cycle repair of AID-initiated DNA damage occurs, we therefore assayed temporal regulation of localization of the repair factor Poleta to lambdaR in DT40 PolyLacO-lambdaR, and DT40 PolyLacO-lambdaU cells. Exponentially growing cells were analyzed by flow cytometry after staining with propidium iodide. Accumulation of sIgM-loss variants by DT40, DT40 PolyLacO-lambdaR, and DT40 PolyLacO-lambdaU cells. Frequencies of sIgM-loss variants in 24 subclones from each line were quantitated by flow cytometry following 6 wk of clonal expansion. Mean sIgM-loss frequencies were 0.8, 0.9, and 0.6%, respectively, as indicated at the bottom of the panel. C. Comparison of levels of colocalizations of E2A with the rearranged lambdaR allele in DT40 PolyLacO-lambdaR cells or the unarranged lambdaU allele in DT40 PolyLacO-lambdaU cells.

Localization of MRN to lambdaR is evident throughout cell cycle

To ask whether MRN localizations at lambdaR exhibit cell cycle dependence, we imaged lambdaR/NBS1 colocalizations, by staining DT40 PolyLacO-lambdaR GFP-LacI cells with anti-NBS1 Abs. lambdaR/NBS1 colocalizations were evident in 17% of cells in asynchronous cultures (n = 232; Fig. 3C, left). Analysis of cell cycle distribution of lambdaR/NBS1 colocalizations showed that 28% occurred in G1 phase, 48% in S phase, and 24% in G2 phase (Fig. 3C, right). This did not differ significantly from the cell cycle distribution of these cells (G1, 27%; S, 56%; G2/M, 17%; p = 0.13, χ2 test).

DT40 cells do not contain γ-H2AX foci, diagnostic of DSBs

Foci of the phosphorylated variant histone, γ-H2AX, are hallmarks of DSBs (61–63). We therefore stained DT40 cells with Abs to γ-H2AX to determine whether DSBs are an intermediate in Ig gene conversion, but this produced only very faint signals in DT40 PolyLacO-lambdaR GFP-LacI cells, and lambdaR/γ-H2AX colocalizations were not evident (data not shown). We then quantitated γ-H2AX foci in the DT40 parental cell line, to ensure that the strong signal from the tagged lambdaR gene would not interfere with the weak γ-H2AX signal. We found that most cells in asynchronous DT40 cultures contained no γ-H2AX foci (88%, n = 410; Fig. 3D, above left), but a small fraction of cells contained one to three small, faint, and punctate foci (12%; Fig. 3D, above left), similar to those observed in other cell lines proliferating under normal conditions (62, 64). Analysis of cells sorted by cell cycle stage before staining showed that faint γ-H2AX foci were present in 2.3% of G1 phase, 9.0% of S phase, and 7.7% of G2 phase cells (n > 300 in each case; Fig. 3D, above right). In contrast, NBS1 foci were present in 37% of G1 phase, 54% of S phase, and 43% of G2 phase cells (Fig. 3D, above right), but there was no evidence of colocalization of γ-H2AX and NBS1. Following treatment with ionizing radiation (IR), which induces DSBs, brightly staining γ-H2AX foci were induced and colocalized with NBS1, as has been reported for many other cell types: three or more γ-H2AX foci can form in DT40 cells, but γ-H2AX foci do not characterize cells carrying out gene conversion. This suggests
that if a DSB is formed in the course of gene conversion, it is present only very transiently, or does not associate with $\gamma$-H2AX in the course of repair.

$G_1$ phase $\lambda_r/Pol\eta$ colocalizations are UNG dependent

$G_1$ phase is the window in which AID can stably localize to the nucleus (Fig. 1) and in which E2A activates diversification at the Ig genes (43). This suggested that $\lambda_r/Pol\eta$-GFP colocalizations in $G_1$ phase may reflect repair synthesis at the diversifying V region. To test this, we examined the effect of Ugi expression on $\lambda_r/Pol\eta$-GFP colocalizations. Ugi inhibits UNG, and expression of Ugi in DT40 B cells inhibits Ig gene conversion (65). We generated stable DT40 PolyLacO-$\lambda_R$ RFP-LacI Pol$\eta$-GFP Ugi transfectants, and verified inhibition of UNG activity upon Ugi expression by assaying deglycosylation of a uracil-containing, 5’-end-labeled synthetic oligonucleotide by nuclear extracts from the parental cell line and the Ugi-expressing derivative (Fig. 4A). Single-cell imaging showed that $\lambda_r/Pol\eta$-GFP colocalizations were evident in 5.4% of asynchronous DT40 PolyLacO-$\lambda_R$ RFP-LacI Pol$\eta$-GFP Ugi cells ($n = 298$), compared with 11% of parental DT40 PolyLacO-$\lambda_R$ RFP-LacI Pol$\eta$-GFP cells ($p = 0.0035, \chi^2$ test). In DT40 PolyLacO-$\lambda_R$ RFP-LacI Pol$\eta$-GFP Ugi cells, 19% of colocalizations occurred in $G_1$ phase (Fig. 4B; $p < 0.001$ vs 39% in the parental line, $\chi^2$ test), 75% in S phase, and 6.3% in $G_2$ phase.

To better gauge the effect of Ugi, $\lambda_R/Pol\eta$-GFP colocalizations in each phase of cell cycle were graphed as a fraction of the entire cell population. This showed that, in Ugi-expressing cells, the percentage of $G_1$ phase cells containing $\lambda_R/Pol\eta$-GFP colocalizations was reduced from 4.5 to 1.0% (65. $G_2$; $p = 0.0011, \chi^2$ test). In contrast, the percentage of S phase cells containing $\lambda_R/Pol\eta$-GFP colocalizations was reduced from 6.3 to 4.0% ($p = 0.20, \chi^2$ test); and the percentage of $G_2$ phase cells containing $\lambda_R/Pol\eta$-GFP colocalizations was reduced from 0.6 to 0.3% ($p = 0.63, \chi^2$ test). Thus, only in $G_1$ phase did Ugi expression have a significant effect on the fraction of cells containing $\lambda_R/Pol\eta$-GFP colocalizations. Although we cannot eliminate the possibility that
Cytosolic translocation. In addition, mutations created in G1 phase could be initiated mutagenesis or other forms of genomic instability, such as transient DNA denaturation essential for replication would be prevented. Diversification is activated by E2A, initiated by DNA deamination by AID and degragation by UNG, generating an AP site (diamond) that is cleaved by MRN, creating a nick. The nick primes nontemplated (somatic hypermutation, left), or templated (gene conversion, right) repair DNA synthesis by Polβ. Ig V region diversification may be initiated and nearly completed in G1 phase of cell cycle, producing a heteroduplex. Replication in S phase fixes mutations. If initial mutagenesis affects only a single DNA strand, then one mutated and one germline chromatid will segregate in G2 phase.

FIGURE 4. UNG-dependent localizations of Polβ-GFP at rearranged \(\lambda_R\) genes. A, Uracil-DNA excision activity in the nuclear extract of DT40 PolyLacO-\(\lambda_R\) RFP-LacI Polβ-GFP cells or its derivative stably expressing Ugi (Ugi – and +, respectively). B, Cell cycle distribution of \(\lambda_R\)/Polβ-GFP colocalizations in DT40 PolyLacO-\(\lambda_R\) RFP-LacI Polβ-GFP Ugi cells (\(n = 16\); 298 cells analyzed. C, Fraction of \(\lambda_R\)/Polβ-GFP colocalizations in each stage of cell cycle, presented as the percentage of total cells in which colocalizations were evident. Ugi expression diminished total colocalizations by about one-half.

Ugi expression has subtle effects on DNA metabolism throughout cell cycle, the simplest interpretation of these results is that the G1 phase-specific colocalizations reflect repair initiated by AID and UNG. This suggests that G1 phase is the window in which much of the repair synthesis that is induced in response to AID-initiated DNA damage occurs.

Discussion

Regulation of Ig gene diversification in the course of cell cycle

Using single-cell imaging to analyze temporal regulation of Ig gene conversion, we have found that two key factors act primarily in G1 phase of cell cycle. AID-YFP stably localizes to the nucleus in G1 phase, but not other phases of cell cycle; and Polβ localizes to the Igα locus predominately in G1 phase. AID initiates gene conversion, and repair synthesis by Polβ is a late step in the mutagenic pathway. These results complement earlier evidence that G1 phase is key to activation of Ig gene diversification by E2A (43), and raise the possibility that gene conversion may initiate and be nearly completed in G1 phase, before DNA replication in S phase.

AID preferentially deaminates ssDNA (19–21, 66), and the transient DNA denaturation essential for replication would be predicted to create a target for AID. Restricting AID attack to G1 phase would therefore protect the replicating genome from AID-initiated mutagenesis or other forms of genomic instability, such as translocation. In addition, mutations created in G1 phase could be fixed in the subsequent S phase (Fig. 5), and thereby protected from postreplicative repair, which would have an adverse effect on mutagenesis essential to the immune response.

Spatiotemporal regulation of AID localization to the nucleus

To analyze regulation of AID, we generated a snapshot of AID-YFP subcellular localization by treating cells briefly with LMB, which inhibits CRM1-dependent nuclear export, and then distinguishing S phase cells by nuclear size and/or BrdU staining. This analysis showed that AID-YFP was present in the nucleus in early stages of cell cycle, but absent from the nucleus in S and later stages, when DNA replication occurs. Inhibition of ubiquitin-dependent proteolysis altered cell cycle dependence of AID-YFP localization, increasing the fraction of S phase cells in which AID-YFP was observed. Like its close relative, APOBEC3G (59), AID-YFP accumulated in cytoplasmic aggresomes upon inhibition of proteasomal degradation. These experiments analyzed localization of tagged fluorescent protein in stable AID-YFP transfectants, so we cannot preclude the possibility that some of the observed effects were due to overexpression of AID. Nonetheless, this evidence for nuclear instability of AID-YFP in chicken B cells is consistent with analysis of mammalian cells, which demonstrated that the t1/2 of nuclear AID is 8-fold below that of cytoplasmic AID (52). These results therefore suggest that regulatory proteolysis prevents accumulation of AID-YFP in the nucleus at stages of cell cycle later than G1 phase.

Distinct temporal regulation of colocalizations of \(\lambda_R\) with Polβ and the MRN complex

Single-cell imaging showed that both the repair polymerase Polβ and the MRN complex localize to the diversifying \(\lambda_R\) allele in DT40 B cells. The visual evidence provided by analysis of colocalizations complements genetic and biochemical evidence for function of Polβ and MRN in Ig gene conversion (26–30). Imaging further suggests that localizations of Polβ and MRN to the Ig genes are under distinct temporal regulation. Colocalizations of \(\lambda_R\) with Polβ-GFP occurred predominately in G1 phase; and expression of Ugi, which inhibits UNG activity and thereby mutagenic repair downstream of deamination of cytosine to uracil, diminished colocalizations in G1 phase over 4-fold, but had relatively modest effect on colocalizations in other stages of cell cycle.

In contrast, colocalizations of \(\lambda_R\) with NBS1 occurred throughout cell cycle. This may reflect the multiple roles played by the multifunctional MRN complex. Gene conversion depends on factors that promote homology-dependent repair, which occurs primarily in S and G2 phases. Gene conversion may be prolonged into...
S phase to take advantage of elevated levels of those factors in that phase of cell cycle. If so, then MRN may function differently at the V region in each stage of cell cycle, using its AP lyase to initiate diversification in G1 phase and then resecting the DNA or tethering recipient and donor later in cell cycle. This is distinct from MRN function in switching B cells, in which nonhomologous end joining (NHEJ) promotes DSBR repair, and NBS1 colocalizes with the IgH locus predominately in G1 phase (67), where the NHEJ pathway is most active (63, 68–71).

**DNA intermediates for Ig gene conversion may carry nicks rather than DSBs**

In principle, gene conversion can involve a DNA intermediate carrying either a DNA DSB or nick. In one example studied in considerable mechanistic detail, mating type switching in the yeast, *Saccharomyces cerevisiae*, is initiated by a DNA DSB (72). However, DNA nicks have also been shown to be effective in initiating recombinational repair (73, 74). We assayed for DSBRs by staining DT40 cells for foci of γH2AX, diagnostic of DNA DSBs (61–63). We found no evidence of such foci in normally proliferating DT40 cells, although bright γH2AX foci were inducible by IR. This suggests that gene conversion at the Ig loci does not involve DSBRs; or if DSBRs do form, they are present only very transiently.

At the diversifying Ig loci, AID, UNG, and MRN act sequentially to generate a nick, in a series of steps common to gene conversion and somatic hypermutation (Fig. 5). If a nick rather than a DSB initiates recombinational repair, then this suggests a model in which a nick generated in G1 phase after successive action of AID, UNG, and MRN would be repaired in G1 phase by Polη, and by other recombination factors in later cell cycle stages (Fig. 5). This model does not postulate involvement of DSBRs or γH2AX.

Two groups have shown that deficiencies in NHEJ increase the rates of Ig gene conversion (75, 76). This has been interpreted as evidence that gene conversion depends on DSBRs, based on the view that DSBRs not repaired by NHEJ would then be intermediates for gene conversion. However, products of V region gene conversion rarely carry the short deletions typical of NHEJ, making it unlikely that a significant fraction enters this pathway. The absence for evidence of γH2AX induction in DT40 cells suggests that impaired NHEJ may not simply increase available substrates, but have other effects on Ig gene conversion. For example, impaired NHEJ may result in activation of factors that promote homology-dependent repair, and thus increase the rates of gene conversion.

**Gene conversion and somatic hypermutation are closely related γH2AX is not necessary for somatic hypermutation, because mice deficient in γH2AX exhibit normal levels of somatic hypermutation (77). In contrast, γH2AX is required for Ig class switch recombination, a process of regulated DNA deletion that depends upon the NHEJ pathway and involves the formation and rejoining of DNA DSBRs; and γH2AX is evident in distinct foci in B cells carrying out class switch recombination (67). The apparent lack of participation of γH2AX in Ig gene conversion (see above) and somatic hypermutation (77) reinforces the mechanistic relationship between these two processes. Both can result from templated or nontemplated mutagenesis at a DNA nick (Fig. 5). In gene conversion, strand transfer occurs to permit templated mutagenesis, whereas the error-prone polymerases that promote somatic hypermutation can extend directly from a DNA nick. The RAD51 paralogs promote strand transfer, and deficiencies in these factors alter the outcome of repair at the chicken Ig loci, so that hypermutation occurs rather than gene conversion (36–40), providing further support for the model that gene conversion and somatic hypermutation proceed via the same DNA intermediate.

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**Disclosures**

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