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To cite this version:

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Activation-induced cytidine deaminase (AID) initiates all postrearrangement processes that diversify the immunoglobulin repertoire by specific deamination of cytidines at the immunoglobulin (Ig) locus. As uncontrolled expression of AID is potentially mutagenic, different types of regulation, particularly nucleocytoplasmic shuttling, restrict the likelihood of AID–deoxyribonucleic acid encounters. We studied additional mechanisms of regulation affecting the stability of the AID protein. No modulation of protein accumulation according to the cell cycle was observed in a Burkitt’s lymphoma cell line. In contrast, the half-life of AID was markedly reduced in the nucleus, and this destabilization was accompanied by a polyubiquitination that was revealed in the presence of proteasome inhibitors. The same compartment–specific degradation was observed in activated mouse B cells, and also in a non–B cell line. No specific lysine residues could be linked to this degradation, so it remains unclear whether polyubiquitination proceeds through several alternatives sites or through the protein N terminus. The nuclear–restricted form of AID displayed enhanced mutagenicity at both Ig and non–Ig loci, most notably at TP53, suggesting that modulation of nuclear AID content through proteasomal degradation may represent another level of control of AID activity.

The online version of this article contains supplemental material.
The induced lesion triggers an error-prone repair cascade leading to the introduction of mutations in the Ig variable region or to double-strand breaks in switch regions allowing the rejoining of distantly located sequences. To modify bases on DNA, AID must be localized in the nucleus; however, data from several studies using either cell fractionation, microscopic examination of cells expressing AID fused to EGFP or immunohistological staining of lymphoid tissues, showed that AID localizes predominantly in the cytoplasm (8–12). This localization was shown to result, as for APOBEC1, from a constant nucleocytoplasmic shuttling mediated by the CRM1 pathway, in which nuclear export dominates (9–11). Active export has been clearly assigned to a nuclear export signal (NES) located in the C-terminal part of AID. The role of an N-terminal bipartite nuclear localization signal (NLS) remains in debate, but whether directly or indirectly, point mutations in this domain can clearly abolish the nuclear translocation of AID (13).

In a transgenic mouse model, constitutive expression of AID has been associated with the onset of tumors, particularly tumors of thymic origin (14). More recent data have clearly demonstrated the implication of AID in the induction of translocations between the c-myc and the heavy chain locus, similar to those observed in Burkitt’s lymphoma (15–17). Therefore, the identification of AID as a potential tumor inducer has brought to light the importance of its regulation during the germinal center reaction. In this study, we report that the stability of AID is related to its subcellular localization, with the AID protein being destabilized in the nucleus through proteosomal-mediated degradation. Nuclear polyubiquitination of AID thus appears as another level of regulation that participates in controlling the access of this enzyme to its DNA target. The possibility that polyubiquitination may compete or interact with other posttranslational modifications modulating AID function is further discussed.

RESULTS

No cell-cycle regulation of AID expression at either the transcriptional or the posttranscriptional level

To facilitate the analysis of regulatory signals affecting the expression level of the AID protein, knock-in (KI) of the EGFP coding sequence at the AICDA locus was performed by homologous recombination in the BL2 cell line. EGFP was fused in frame with exon 5 at the 3' end of the AID coding region and a hygromycin resistance (hygro<sup>R</sup>) gene flanked by loxP sites. Configuration of the targeted AICDA locus is depicted after Cre-mediated excision of the hygro<sup>R</sup> gene. (A) EGFP KI at the AICDA locus in BL2 cells. The AID–EGFP KI construct includes the EGFP sequence inserted in-frame in exon 5 at the 3' end of the AID coding region and a hygromycin resistance (hygro<sup>R</sup>) gene flanked by loxP sites. Configuration of the targeted AICDA locus is depicted after Cre-mediated excision of the hygro<sup>R</sup> gene. (B) Expression of AID–EGFP throughout the cell cycle. 48 h after IL-4 addition (10 ng/ml), AID–EGFP KI BL2 cells were fractionated according to their cell cycle status using counterflow elutriation. Collected fractions were stained with propidium iodide and analyzed for both DNA content and AID–EGFP MFI. Data for fractions 12, 30, and 39 are shown in C. (C) Cell cycle analysis and AID–EGFP expression level of the BL2 KI cell line with and without IL-4, and of representative elutriated fractions, corresponding to the different phases of cell cycle: G1 (fr.12), S (fr. 30), and G2/M (fr. 39).

Figure 1. Variation of AID expression during the cell cycle. (A) EGFP KI at the AICDA locus in BL2 cells. The AID–EGFP KI construct includes the EGFP sequence inserted in-frame in exon 5 at the 3' end of the AID coding region and a hygromycin resistance (hygro<sup>R</sup>) gene flanked by loxP sites. Configuration of the targeted AICDA locus is depicted after Cre-mediated excision of the hygro<sup>R</sup> gene. (B) Expression of AID–EGFP throughout the cell cycle. 48 h after IL-4 addition (10 ng/ml), AID–EGFP KI BL2 cells were fractionated according to their cell cycle status using counterflow elutriation. Collected fractions were stained with propidium iodide and analyzed for both DNA content and AID–EGFP MFI. Data for fractions 12, 30, and 39 are shown in C. (C) Cell cycle analysis and AID–EGFP expression level of the BL2 KI cell line with and without IL-4, and of representative elutriated fractions, corresponding to the different phases of cell cycle: G1 (fr.12), S (fr. 30), and G2/M (fr. 39).
the collection of cell fractions according to their position in the cell cycle. Each collected fraction was analyzed for both AID-EGFP fluorescence and DNA content by flow cytometry, thus allowing the correlation of the relative abundance of the protein to the various phases of the cell cycle. Fig. 1 (B and C) thus allowing the correlation of the relative abundance of the protein to the cell cycle. Each collected fraction was analyzed for both AID-EGFP fluorescence and DNA content by flow cytometry, thus allowing the correlation of the relative abundance of the protein to the various phases of the cell cycle.

**Differential accumulation of AID mutant proteins according to their subcellular localization**

To obtain BL2 subclones expressing AID-EGFP with various subcellular localizations, we generated tetracycline-inducible expression vectors containing WT human AID tagged with EGFP (WT-AID-EGFP), or AID-EGFP containing mutations in either the NLS (MutNLS-AID-EGFP) or in the NES (MutNES-AID-EGFP), as indicated in Fig. 2 A. The different constructs were transfected in the BL2 cell line and the protein localization was confirmed by fluorescence microscopy, in the presence or absence of LMB (Fig. 2 C). Mutations of three hydrophobic residues within the NES blocked the exit of the fusion protein from the nucleus, which is in agreement with previous studies (9). Mutations in the NLS, performed as described by Shinkura et al. (13), completely prevented its entry into the nucleus as evidenced upon LMB treatment (Fig. 2 C), thus confirming the requirement for precise residues within the putative NLS to drive AID either directly or indirectly into the nucleus. The fluorescence intensity of the expressed AID-EGFP protein was monitored by analyzing three different clones for each mutant or WT construct, a representative example being shown in Fig. 2 B for each of them. We noticed that expression levels varied considerably according to the subcellular localization of the AID-EGFP protein, while being similar between different clones harboring the same construct. Fig. 2 B shows that, compared with WT-AID (mean fluorescence intensity [MFI] = 350), the cytoplasmic AID (Mut-NLS) exhibits a stronger expression (MFI = 1,340), whereas expression of the nuclear AID (Mut-NES) was by far the lowest (MFI = 60). Because all proteins have been expressed using the same expression vector driven by the same promoter, the level of expression of the fusion protein is likely to reflect its accumulation, and consequently its stability, within the cell. Our data therefore suggest that AID displays a different stability depending on the specific cell compartment in which it is located. The percentage of induced cells varied between the constructs as well, always being lower for mutNES-AID clones, a counterselection probably related to the higher mutagenicity of nuclear AID (see section The NES-AID-GFP protein is mutagenic in the BL2 cell line).

To investigate further the relationship between the cellular localization of the AID protein and its stability, we performed [35S]methionine/cysteine pulse-chase labeling of BL2 cells expressing either WT, nuclear, or cytoplasmic AID-EGFP. Protein levels were quantified, after immunoprecipitation with anti-EGFP antibodies, by autoradiography of PAGE-fractionated immunoprecipitated products (Fig. 3, A–C). The half-life of the nuclear AID-EGFP protein was estimated as ~2.5 h, compared with ~8 h for the WT and 18–20 h for the cytoplasmic AID-EGFP (Fig. 3 D). To confirm this observation, the different BL2 subclones were incubated with cycloheximide to block protein synthesis, and the decay of the protein was followed by the decrease of its fluorescence intensity. Similar to pulse-chase data, AID localized in the nucleus displayed the fastest decay after cycloheximide treatment, indicating amino acid positions within the AID protein) or the NES (mutNES, L189A, F193A, and L196A), with both domains being represented as black boxes. (B) FACS analysis of the tetracycline-inducible clones, with values of the MFI of the EGFP-positive population. By comparison with endogenous AID expression levels in BL2 (corresponding to a MFI value of 15 in the KI clone [Fig. 1 C]), AID overexpression in EGFP-positive cells can be estimated at ~4 for mutNES-AID, 24 for WT-AID, and 90 for mutNLS-AID constructs. (C) Confocal images of EGFP signals from inducible clones, with or without 2-h LMB treatment, are shown, together with nuclear staining with propidium iodide (PI) of the same field. Clones with a lower percentage of inducible expression are shown, as indicated by the presence of EGFP-negative cells in the field, a shut-off of expression regularly observed in clones kept in culture in induced conditions.

Bars, 20 μm.
LMB alone did not lead to the appearance of such modified forms of AID, but their detection was markedly increased when LMB and MG132 were combined. To demonstrate that these high MW species correspond to polyubiquitinylated forms of AID, BL2 cells stably transfected with an HA-tagged AID-expressing vector were treated with LMB or MG132, or both, and proteins were extracted after 5 h of incubation and denatured before immunoprecipitation with agarose-conjugated anti-HA antibodies. MG132, total protein extracts were prepared at different time points, and the amount of AID and AID-EGFP proteins was assessed by Western blot using anti-AID antibodies. Fig. 4 A shows that incubation with MG132 has no obvious effect on either form of AID. However, when the cells containing MutNLS-AID-EGFP were incubated with MG132, slower migrating protein species were detected (Fig. 4 B, third lane; see also Fig. 5 C), suggesting that the nuclear fraction of AID is sensitive to inhibition of the proteasome. When the modifications of the endogenous protein were studied in the same conditions, i.e., by first incubating BL2 cells with LMB for 1 h to block AID in the nucleus before MG132 addition, modifications were observed for the endogenous AID protein as well (Fig. 4 B, last lane). As incubation with proteasome inhibitors results in a stabilization that is not easily quantifiable by Western blot, the combined effect of MG132 and LMB was monitored for a WT-AID-EGFP-expressing clone through the evolution of its fluorescence intensity over time; the specific fluorescence decay observed upon nuclear sequestration of AID was prevented by the inhibition of proteasomal activity, whereas the fluorescence of a MutNLS-AID-EGFP clone treated in the same conditions was not affected (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20070950/DC1). These results thus suggest that polyubiquitination as a prelude to proteasomal degradation may specifically take place in the nucleus.

To verify this hypothesis, nuclear and cytoplasmic fractions were extracted from normal BL2 cells after incubation with either MG132 or LMB, or both. High molecular weight (MW) species were already detectable in the presence of MG132 in the minor nuclear AID fraction, whereas they were absent in the major cytoplasmic fraction (Fig. 4 C). Addition of LMB alone did not lead to the appearance of such modified forms of AID, but their detection was markedly increased when LMB and MG132 were combined. To demonstrate that these high MW species correspond to polyubiquitinated forms of AID, BL2 cells stably transfected with an HA-tagged AID-expressing vector were treated with LMB or MG132, or with both inhibitors, and proteins were extracted after 5 h of incubation and denatured before immunoprecipitation with agarose-conjugated anti-HA antibodies.
for both forms of AID expressed from the endogenous loci, whether they were EGFP-tagged or not (Fig. 4E). Modified forms of AID, although less abundant, were detectable in the absence of both inhibitors, a recurrent finding when ubiquitin-expressing vectors are transfected, suggesting that such massive expression of ubiquitin substrates alone compromises the proteasome machinery (Fig. 5C and Fig. 6C).

Although the formation of nonspecific aggregates cannot be ruled out even in the denaturing conditions used, the convergent results obtained using either Ub-HA- or AID-HA-tagged proteins and either antiubiquitin or anti-AID antibodies strongly argues against the fact that the detection of high MW species of AID could be an artifact. Collectively, these observations are thus consistent with an essential role of the ubiquitin–proteasomal pathway in the nuclear degradation of AID.

No specific lysine residue is involved in AID ubiquitination

Polyubiquitination proceeds most frequently through the conjugation of the first ubiquitin molecule to the ε-NH2 moiety of a lysine residue within the substrate protein. Five AID mutants, in which one or several lysines were mutated to arginine, including a lysineless mutant of AID ("Kzero-AID-EGFP";...
depicted in Fig. 5 A), were transfected in the BL2 cell line, and stable clones were isolated. EGFP fluorescence decay in presence of both LMB and cycloheximide was followed for all these mutants, in parallel with WT-AID and mutNLS-AID–expressing clones. In the four clones with one to four lysines mutated, no alteration in the protein decay was observed (Fig. 5 D, top). The lysineless mutant behaved differently, being less destabilized by this treatment (Fig. 5 D, bottom). Moreover, incubation with either inhibitor indicated that the Kzero-AID-EGFP mutant is not efficiently destabilized in the presence of LMB, in spite of its nuclear localization (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20070950/DC1), thus indirectly showing that nuclear destabilization is not an artifact due consequence of LMB addition. Western blot analysis after incubation with LMB, MG132, or both revealed that although higher MW species were observed for the lysineless mutant of AID after inhibition of both nuclear export and proteasome degradation, this occurred at a much lower level than for the WT-AID, thus correlating with the very different impact of LMB addition on the stability of this protein (Fig. 5 B). Accordingly, transfection of Ub-HA, followed by anti-HA immunoprecipitation of the different lysine mutants, confirmed a much lower level of modification of the Kzero–AID-EGFP protein compared with WT-AID, whereas all partial lysine modifications had no quantitative impact (Fig. 5 C). In the control immunoprecipitation performed in the absence of both inhibitors, the much higher level of modifications of the mutNES-AID-EGFP protein should be noted (Fig. 5 C, lane 2), reflecting, as discussed in the previous section, the proteasome saturation effect of massive ubiquitin expression, and which appears specifically pronounced for the sole AID mutant that resides in the nucleus without LMB addition.

No specific lysine was thus shown to be involved in AID destabilization, whereas the total absence of lysines had a stabilization effect that was intermediate between the WT and the cytoplasmic–only AID. The residual ubiquitination observed could explain this partial effect. Two alternative explanations are thus possible: either AID is, indeed, polyubiquitinated at its N terminus and not at internal lysines, with the low efficiency observed resulting from a conformational alteration of the protein induced by the eight mutations introduced. Alternatively, polyubiquitination of AID may proceed through several alternative lysine positions, with artifactual and inefficient conjugation taking place at its N terminus in the absence of internal acceptor sites.

Nuclear degradation of AID occurs in normal mouse B cells and in a human nonlymphoid cell line

We have shown that, in the BL2 Burkitt’s lymphoma cell line, AID is polyubiquitinated in the nucleus, which leads to its rapid degradation via the 26S proteasome. To validate this observation in vivo, we studied the turnover and the ubiquitination of AID in mouse splenic B cells stimulated to undergo CSR. The culture of mouse spleen B cells in the presence of LPS and IL-4 drives cell proliferation and triggers the induction of AID gene expression, together with an increased accessibility and targeting to the SY1 switch region, allowing the cell to undergo CSR in culture from IgM to IgG1 (18). Cells were collected at different time points after LPS/IL-4 addition, and total or nuclear extracts were prepared as described in Materials and methods. As previously reported, AID expression was induced 48 h after LPS/IL-4 addition, with a small fraction of the protein being detectable in the nucleus (Fig. 6 A)(19, 20). 4 d after stimulation, cells were treated with LMB, MG132, or both, followed by total protein extraction. The direct Western blot analysis using anti–mouse AID antibody showed that addition of LMB, together with MG132, resulted in the accumulation of AID with the appearance of slow migrating forms of the protein (Fig. 6 B). Transient transfection of a Ub-HA–expressing vector was performed in LPS + IL-4–activated spleen cells 2 d after stimulation, and MG132 and LMB were added for 5 h, 16 h after transfection. Western blot analysis with an anti–AID antibody, after anti–HA immunoprecipitation performed as described in the previous section, revealed similarly high MW species, thus confirming that polyubiquitination of AID takes place in the nuclei of activated B cells (Fig. 6 C). As observed for the BL2 cell line, modifications were already observed in the control sample, but were quantitatively less important.

We next studied whether nuclear destabilization was restricted to the cells naturally expressing AID, i.e., to activated B lymphocytes, or whether it constitutes a general regulation of AID turnover. To this end, we transiently transfected the human embryonic kidney 293T cell line with the WT-AID-HA
AID seems to occur faster in BL2 (Fig. S4). In the 293T and the BL2 cell lines, the overall degradation of shuttling and the nuclear form of the protein appears similar. The NES-AID-EGFP protein is mutagenic in the BL2 cell line and various measures showed that AID expression level and minimal hypermutation of its immunoglobulin genes constitute a valid model for studying the basal regulation of AID expression of AID was induced by tetracycline removal, and expression of AID was regulated according to the cell cycle. We chose the BL2 Burkitt’s lymphoma cell line for this study. BL2 expresses AID, but it only undergoes minimal hypermutation of its immunoglobulin genes constitutively (21). It is nevertheless likely that this cell line constitutes a valid model for studying the basal regulation of AID because it represents the transformed counterpart of a germinal center B cell.

The NES-AID-EGFP protein is mutagenic in the BL2 cell line. Inducible MutNES-AID-EGFP and WT-AID-EGFP constructs were transfected in the previously described AID-deficient BL2 cell line (21). Individual clones were isolated, expression of AID was induced by tetracycline removal, and EGFP-positive cells were isolated by cell sorting 14 d after induction. Mutations in the functional V4-39 gene were analyzed for three mutNES-AID-EGFP and three WT-AID-EGFP clones. The average mutation frequency for the three clones analyzed was 2.3 times higher for the mutant compared with WT ones, with larger individual variations being observed between the mutant clones (Table S1, available at http://www.jem.org/cgi/content/full/jem.20070950/DC1). The mutNES-16 clone displayed the highest mutation frequency, 3.2-4.5 times higher than any of the WT-AID-expressing clones analyzed. Mutations were almost exclusively targeted at G/C positions. Moreover, G-to-A and C-to-T transitions predominated in the mutant clones (81%), consistent with a high number of C to U deaminations being carried over to replication. The proportion of transversions, essentially G-to-C or C-to-G, was higher in the WT-AID–expressing cell lines (45%; unpublished data), suggesting that saturation (or inhibition) of repair by uracil glycosylase occurs when AID is retained in the nucleus.

Mutations induced by AID overexpression were also analyzed at the Cμ locus, as well as in several non-Ig genes, including the AID cDNA sequence of the expression construct, in one WT (WT-24) and one mutant (mutNES-16) clone. Among non-Ig genes, we selected BCL6 which is targeted by AID and mutated in normal centroblasts (22, 23), PAX5, which is mutated in some B cell lymphomas (particularly diffuse large B cell lymphomas, DLBCL) and has been recently shown to be targeted by AID, but subjected to error-free repair in normal B cells (24), and TP53, which has not been described as mutable in either DLBCL lymphomas or germinal center B cells (24, 25). A low mutation frequency was observed for Cμ, BCL6, and PAX5 genes in the WT clone, while being at background level for TP53 (Table I). Mutagenesis was increased for the BCL6 gene in the mutNES-16 clone, but, more surprisingly, also at the TP53 locus. The highest mutation frequency is observed for the AID transgene, in both WT and mutant clones, indicating that the strength of the transcriptional activation mediated by the tetracycline-regulated CMV promoter can possibly allow the targeting of AID at a level comparable to, if not higher than, the one occurring at the endogenous VH sequence. However, considerable variations in mutagenesis of the AID transgene were observed when all six clones were analyzed (unpublished data), confirming, as previously reported, that the insertion site can influence the accessibility to AID-induced mutagenesis of transgenes displaying equivalent transcriptional activity (26, 27).

**DISCUSSION**

In this work, we studied additional modes of regulation of AID that can impact on the availability of this protein in lymphoid cells, and may thus contribute to restrict access to its DNA target. The first question asked was whether expression and/or accumulation of AID were regulated according to the cell cycle. We chose the BL2 Burkitt’s lymphoma cell line for this study. BL2 expresses AID, but it only undergoes minimal hypermutation of its immunoglobulin genes constitutively (21). It is nevertheless likely that this cell line constitutes a valid model for studying the basal regulation of AID because it represents the transformed counterpart of a germinal center B cell.

KI of EGFP at the AICDA locus was therefore performed in BL2, by fusing EGFP at the end of the last coding exon, and various measures showed that AID expression level and protein accumulation from both endogenous and KI alleles were similar. Fractionation of BL2 by counterflow elutriation and monitoring of AID–EGFP fluorescence in elutriated cells

**Table I.** Mutation frequency at various Ig and non-Ig loci in BL2 clones transfected with WT-AID-EGFP or mutNES-AID-EGFP inducible expression vectors

<table>
<thead>
<tr>
<th>Gene locus (domain sequenced)</th>
<th>WT-24 clone</th>
<th>mutNES-16 clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of mutations/total number of sequences</td>
<td>(mutation frequency)</td>
<td>(mutation frequency)</td>
</tr>
<tr>
<td>V4-39 (390 bp)</td>
<td>13/65</td>
<td>69/74</td>
</tr>
<tr>
<td>Cμ (403 bp)</td>
<td>5/74</td>
<td>6/73</td>
</tr>
<tr>
<td>BCL6 (640 bp)</td>
<td>3/47</td>
<td>12/51</td>
</tr>
<tr>
<td>TP53 (1,064 bp)</td>
<td>1/39</td>
<td>14/43</td>
</tr>
<tr>
<td>PAX5 (885 bp)</td>
<td>6/47</td>
<td>8/46</td>
</tr>
<tr>
<td>AID cDNA (594 bp)</td>
<td>14/22</td>
<td>39/22</td>
</tr>
</tbody>
</table>
showed that AID expression varied approximately within a factor of 2 between the G1 and the G2/M phases, i.e., in proportion to its gene copy number. AID expression is thus not restricted to a specific phase of the cell cycle. Expression of AID and cytidine deamination throughout the cell cycle fit with current models of hypermutation, and we have proposed that mutations at A/T versus G/C bases might result from a differential handling of the deaminated base, according to its occurrence before or during DNA replication (28, 29).

AID has been shown to undergo constant nucleocytoplasmic shuttling, with a net balance toward export, thus resulting in a main cytoplasmic localization (8–12). In effect, incubation of AID-expressing cells with LMB, a specific inhibitor of CRM1, which is the protein responsible for this nuclear export, results in a rapid sequestering of AID in the cell nucleus (9). Alteration of a nuclear export sequence located at the 3′ end of the AID molecule, as well as of a putative NLS, has been shown to result in a protein with exclusive nuclear or cytoplasmic localization, respectively, a localization that is not further altered by addition of LMB (9, 10, 13). We generated such mutants, fused to the EGFP reporter gene, and obtained stable tetracycline inducible clones in BL2. We noticed that, although these mutants were all expressed from the same CMV promoter-driven inducible vector, they displayed markedly different protein expression levels that consistently correlated with their specific subcellular localization, suggesting that such a differential accumulation might mirror their stability within the cell. The protein half-life was determined by either [35S] pulse-chase protein labeling or by incubation with cycloheximide and follow up of the radioactivity or fluorescence decay of the AID protein. When compared with the normal shuttling protein (WT-AID-EGFP), the protein sequestered in the nucleus (mutNES-AID-EGFP) showed a 3.3-fold shorter half-life ($t_{1/2} = 2.5$ vs. $8$ h), whereas the protein retained in the cytoplasm (mutNLS-AID-EGFP) was 3 times more stable ($t_{1/2} = \sim 20$ h), resulting in a 10-fold difference in stability between the 2 cell compartment–restricted mutants of AID. AID nuclear localization thus appears to be linked with increased degradation.

Degradation of most cellular proteins requires tagging of protein substrates by polyubiquitin chains and proceeds via an energy-dependent process involving the 26S proteasome that constitutes the major neutral proteolytic activity in both the cytosol and the nucleus (for review see [30]). Incubation with proteasome inhibitors was shown to result in the appearance of high MW species of AID that were shown to be polyubiquitinated. However, such modifications were essentially observed when AID was retained in the nucleus, either because of the addition of LMB or the mutation in the nuclear export sequence. We did not observe any ubiquitination of the cytoplasmic mutant, which displays the longest half-life (unpublished data). This could be caused by a modification rate that would be below the detection level of our assay, or possibly to a degradation that could be proteasome independent. Alternatively, AID could be complexed in the cytosol with proteins that would protect it from ubiquitin cross-linking and degradation. Specific interaction between cytoplasmic AID and protein factors has indeed been described in the Ramos cell line (31).

Nuclear-specific degradation of endogenous mouse AID was similarly observed in mouse splenic B cells, after its induction by LPS and IL-4 stimulation, indicating that this regulation is an intrinsic part of its physiological turnover. This process is nevertheless not restricted to B cells, as it is similarly observed, albeit with some quantitative differences, upon transfection of the human embryonic kidney 293T cell line.

In proteins destined for degradation by the proteasome, polyubiquitin chains are commonly anchored to the ε-amine group of one or several internal lysine residues. To identify the putative lysine target within the eight candidate amino acids present in the human AID protein, various mutants were generated by replacement of lysines by arginines, including a lysineless form of AID. No single lysine residue could be identified as the internal site of ubiquitin conjugation, as no single mutation (up to four) mutations were able to stabilize the nuclear form of the protein. In contrast, the lysineless AID could be polyubiquitinated upon addition of proteasome inhibitors and sequestration in the nucleus, but at a much lower level, and displayed accordingly a nuclear stability intermediate between the normal and cytoplasmic–only protein. Thus, polyubiquitination of AID may proceed through several alternative internal sites, as has been shown for the IkBα protein (32), the low level of modification still observed in the lysineless mutant representing an N-terminal modification that would be artificially forced. Or, alternatively, N-terminal ubiquitination, observed for a few proteins such as the ARF/INK4a tumor suppressor protein or the MyoD and Id2 transcriptional regulators (33–36), could be the physiological process, its relative inefficiency being indirectly generated by the eight mutations that would alter the targeting of the ubiquitin ligase involved. Stepwise reversion of mutant lysines may allow us to settle this issue.

The fraction of ubiquitinated AID observed appears to always be low in BL2, which may be linked to the intrinsic difficulty of observing such modifications outside the classical readouts that 293T cells or fibroblasts constitute. We cannot exclude on the other hand that an ubiquitin-independent pathway may contribute to AID turnover, as has been recently described for the p21 cell cycle regulator, which is channeled for proteasomal degradation by the REGγ complex in the absence of ubiquitin cross-linking (37, 38). In addition to the proteasome-mediated nuclear degradation that we have described, we do not exclude either that there may be other steps at which AID turnover might be regulated, which would impact its interaction with other protein partners or its assembly as a dimeric or multimeric complex, for example.

The natural target of AID-induced mutagenesis in normal germinal center B cells is the Ig locus, as well as several genes like BCL6, which nevertheless harbor a much lower mutation frequency (22, 23, 39). Moreover, numerous genes, including oncogenes like PAX5, PIM1, MYC, and RhoH/TTF, which undergo a low level of mutagenesis in some B cell...
lymphomas constitutively expressing AID (25, 40), appear to be targeted by AID in normal B cells, but are spared from mutations caused by error-free repair (24). Deliberate overexpression of AID has been shown to increase mutation levels of several transcribed genes in both prokaryotic and mammalian cells (41–45). However, although the nuclear-restricted AID protein induces enhanced mutagenesis both in Escherichia coli and on a transfected substrate in fibroblasts, its mutagenicity in B lymphocytes is not clearly established (9, 10). We obtained inducible clones overexpressing the WT or mutant form of AID in the AID-deficient BL2 cell line, and confirmed that, although different clones show variable mutation frequencies of their endogenous rearranged V\textsubscript{H} gene, higher mutagenicity can be achieved by the nuclear restricted AID compared with the WT one. As nuclear AID is expressed at a lower level than the normal, shuttling protein, the mutagenicity related to the amount of AID protein present in a cell is thus 6–10-fold higher for a protein with an exclusive nuclear residence. When several non-Ig loci were studied in the mutNES-AID–EGFP clone showing the highest mutation frequency, mutagenesis at the BCL6 locus was also found to be increased, as well as, and more surprisingly, at TP53. However, it should be mentioned that, whereas p53 expression is repressed by Bcl-6 in Burkitt’s lymphoma, as well as in germinal center B cells, and is thus logically spared from hypermutation (24, 46), its expression has been shown to be inducible in specific stress conditions in BL2 (47). The TP53 gene could thus become an AID target in cells that failed to repress its expression. Such an AID-induced mutagenesis of the p53 coding sequence has been recently described in human gastric epithelial cells (48), even though in this case mutations were focused in the core p53 coding region and not in the region studied here, which is located downstream from the promoter. Abnormal AID expression in nonlymphoid cells could thus unmask additional AID targets that would become mutated depending on the specific gene expression profile of a given cell type, and would thus increase the number of genes recently described as susceptible to AID deamination (24).

HIGM2 patients with heterozygous or homozygous mutations in the nuclear export sequence of AID do not show markedly different mutation frequencies of their endogenous Ig genes, suggesting that transcriptional regulation and nuclear destabilization during germinal center B cell differentiation are sufficient to keep AID activity under control (49, 50). However, in transformed B cells displaying ongoing AID expression (25, 40, 51), or in conditions of chronic inflammation leading to AID activation (48, 52), factors that would modify the nuclear turnover of AID could aggravate its oncogenic potential and thus contribute actively to aberrant mutagenesis.

In conclusion, our study shows that ubiquitin-mediated nuclear degradation of AID is part of a multilayer control that contributes to the regulation of AID function during hypermutation and CSR. A fraction of AID has been detected in activated B cells as being linked to chromatin, and phosphorylated AID is found enriched in such a chromatin fraction (18, 53). Phosphorylation at serine 38 has also been shown to impact AID activity in vivo (31, 53, 54). It is therefore likely that specific posttranslational modifications, among them phosphorylation, may contribute to sequester AID from the nucleus. Such stabilization in chromatin domains would at the same time prevent AID from being constantly exported from the nucleus, while possibly allowing it to escape the nuclear-specific degradation described in this study. This competition between attachment to DNA, degradation and export could also be central in limiting the magnitude of AID activity, which has been proposed to be mainly nonprocessive (55, 56), a mode of action that would, after each enzymatic round, render this enzyme accessible to destabilization and nuclear exclusion. Whether such a competition between phosphorylation and polyubiquitination, be it direct or indirect, occurs, and whether additional posttranslational modifications, like for example sumoylation, orchestrate the assembly of AID into an active mutasome remain to be addressed.

**MATERIALS AND METHODS**

**Construction and transfection of expression vectors for AID mutants.** Full-length cDNA encoding human AID without stop codon was fused in frame with the sequence coding for 3 repeats of VPDYDVYA, corresponding to residues 99–107 of the influenza virus hemagglutinin (HA), cloned into pIRESpuro vector. Stable clones were obtained by electroporation in the Burkitt’s lymphoma BL2 cell line. HA-tagged ubiquitin-expressing vector was a gift from M. Treier (European Molecular Biology Laboratory, Heidelberg, Germany). Transient transfection was performed using the Amaxa device (program C-009). AID-EGFP fusion sequences were cloned in the pIRESpuro vector or in the tetracycline-inducible pBl expression vector (Clontech Laboratories), in which a blasticidin resistance gene amplified from the pcDNA6/TR plasmid (Invitrogen) was inserted in its AarI site to allow for selection of transfected clones. The EGFP coding sequence was fused at the 5’ end of AID. Mutants were constructed by stepwise PCR, mutagenesis, introducing the following mutations. MutNES: L189A, F193A, L196A; MutNES: V18S, R19V (13); Kzero: all lysines (K10, 16, 22, 34, 52, 120, 142, 166) mutated to arginines (AAA/G to AGA/G); K1–4: first four lysines to arginines; K6–8: last three lysines to arginines; K4, K5: fourth and fifth lysines (positions 34 and 52), respectively, to arginines. Inducible vectors were electroporated in the BT2 cell line (a tetracycline-inducible subclone of BL2, obtained by transfection of the pTet-cTAK vector [Invitrogen] in which a zeocin resistance gene was inserted [vector and cell line provided by M. Muramatsu and T. Honjo, Kyoto University, Kyoto, Japan]). Tetracycline-inducible AID-deficient BL2 clones were obtained by electroporation of the same pTet-cTAK-zeo vector in the 60–129 AID-deficient cell line (21). Selection of transfected clones was performed in the following conditions: 350 ng/ml puromycin, 5 μg/ml blasticidin, 500 μg/ml zeocin, and 1 μg/ml tetracycline. Tetracycline-free serum (Clontech Laboratories) was used for the culture of inducible clones. Inducible 293T cells were similarly obtained after transfection of the pTet-cTAK vector, and inducible WT-AID-EGFP-expressing cells were selected as a pool. Transient transfection in 293T was performed using Amaxa conditions (program Q-001).

**Mouse B cell culture, stimulation, and transfection.** B cells were purified from mouse spleen using the mouse B cell isolation kit (Miltenyi Biotec) and cultured in complete RPMI 1640 medium with 20 μg/ml murine IL-4 (PeproTech) and 25 μg/ml LPS (Sigma-Aldrich). Cells were collected at different days after stimulation. 2 d after stimulation, 4 × 10\(^5\) cells were collected and transfected with Ub-HA–expressing vector using the Test Nucleofector kit 104A1 (Amaxa), by sets of 5 × 10\(^6\) cells and 5 μg of Ub-HA.
DNA per transfection. Increasing the transfection volume to 200 μl had a major incidence on cell viability. Transfection of 2.5 μg EGFP-expressing vector (pMaxGFP, Amaxa) in parallel resulted in 60-70% transfection efficiency and 90% cell viability estimated by 7-amilinoactinomycin-D exclusion. Mouse experiments were performed according to the Institut National de la Santé et de la Recherche Médicale guidelines for laboratory animals and approved by the Scientific Committee of the Neckar Animal Facility.

Immunofluorescence microscopy. BL2 cells transfected with AID-EGFP fusion constructs were spun down on poly-l-lysine-coated glass coverslips for 5 min at 100 g. Cells were fixed in cold methanol for 20 min at −20°C. Coverslips were mounted on slide using Vectashield mounting medium containing propidium iodide (Vector Laboratories). EGFP fluorescence was visualized using an LSM 510 confocal microscope (Carl Zeiss, Inc.).

Flow cytometry, cell sorting, and elutriation. Transfected BL2 clones were analyzed with Cell Quest software on a FACScan apparatus (BD Biosciences). For AID stability estimates, EGFP fluorescence was measured at various time points after cycloheximide, LMB, or MG132 addition. For the determination of mutation frequencies, EGFP-positive cells were sorted on a FACS Ana cell sorter (Becton Dickinson). Fractionation of BL2 cells according to their cell cycle status was performed by counterflow elutriation, and collected fractions were stained and analyzed for DNA content, as previously described (21), after determination of their EGFP fluorescence intensity.

Pulse-chase assay. Exponentially growing BL2 cells were washed twice with methionine and cysteine-free DME medium, and then incubated at 106 cell/ml in the same medium containing 5% of dialyzed FCS. After 1 h at 37°C, 100 μCi/ml of [35S]-L-methionine/cysteine promix (GE Healthcare) was added, and incubated for an additional 60 min, and the supernatants were collected as nuclear extracts. In ubiquitination and incubated under agitation in the cold room for 20 min in RIPA (50 mM PBS during the cell washes and to both the HB and HS buffers.

Total extract preparation, immunoprecipitation, and immunoblotting. The whole-cell protein lysates were washed twice with cold PBS, and incubated under agitation in the cold room for 20 min in RIPA (50 mM Hapes, pH 7.9, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1x protease inhibitor mixture [Roche], and 0.25 mM PMSF). After incubation on ice for 15 min, NP-40 was added to a final concentration of 0.5%. The mixtures were vortexed at high speed for 10 s. The nuclei were recovered by centrifugation at 13,000 rpm for 30 s. The supernatants were collected as cytoplasmic extracts. The pellet was washed once with buffer HB, and proteins were extracted by adding high-salt (HS) buffer (20 mM Hapes, pH 7.9, 420 mM NaCl, 2 mM EDTA, 1 mM DTT, 1x protease inhibitor mixture, and 0.25 mM PMSF), followed by incubation at 4°C for 30 min under gentle agitation. The mixtures were spun at 13,000 rpm for 15 min, and the supernatants were collected as nuclear extracts. In ubiquitination experiments, 10 μg/ml of N-ethylmaleimide (Sigma-Aldrich) was added to PBS during the cell washes and to both the HB and HS buffers.

Total extract preparation, immunoprecipitation, and immunoblotting. The whole-cell protein lysates were washed twice with cold PBS, and incubated under agitation in the cold room for 20 min in RIPA (50 mM Hapes, pH 7.9, 150 mM NaCl, 1.25 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM dithiothreitol, 1x protease inhibitor mixture, and 0.25 mM PMSF). Insoluble material was removed with 30-min centrifugation at 15,000 rpm at 4°C. The protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific). Protein samples were mixed with an equal volume of electrophoresis loading buffer containing 2% SDS and heated at 95°C for 5 min before SDS-PAGE separation and transfer to nitrocellulose membranes. The membranes were then incubated with antibodies, followed by chemiluminescence detection, as previously described (21).

Immunoprecipitation was performed after denaturation in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail) plus 1% SDS, and then heated twice for 5 min at 95°C. Supernatants were diluted 10-fold with immunoprecipitation buffer containing 5 mM N-ethylmaleimide and 0.5% NP-40 before antibody addition and immunopurification.

In addition to our monoclonal anti-human AID antibodies, the following antibodies were used: monoclonal anti-mouse AID (Cell Signaling Technology), monoclonal anti-nucleolin, agaro-conjugated anti-HA (Santa Cruz Biotechnology), rabbit polyclonal anti-actin (Abscam), monoclonal anti-ubiquitin (clone FK2; BioMol), HRP-coupled monoclonal anti-EGFP (Miltenyi Biotec), HRP-coupled anti-mouse and -rabbit IgG (Bio-Rad Laboratories), monoclonal anti-PARP (Boehringer), and 7-amilinoactinomycin-D (BD Biosciences). MG132 and LMB (both from Calbiochem) were used at concentrations of 10 μM and 10 ng/ml, respectively, and cycloheximide (Sigma-Aldrich) was used at either 20 or 50 μg/ml.

Online supplemental material. Supplemental materials and methods describe the production of the anti-human AID monoclonal antibodies used in this study, the generation of the BL2 cell line with EGFP knocked-in at the AICDA locus, and the conditions for the analysis of AID-induced mutations at various loci. Fig. S1 compares the protein turnover of AID and AID-EGFP expressed from the endogenous loci. Fig. S2 shows the specificity of the anti-human AID monoclonal antibodies used. Fig. S3 shows the effect of the MG132 protease inhibitor on AID stabilization. Fig. S4 shows the lack of nuclear destabilization of the lysineless AID mutant. Table S1 describes the mutation frequency at the Vδ1 locus of various AID-expressing BL2 clones. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070950/DC1.

We thank Damiana Leceouche for excellent technical assistance, Meriem Garfa for assistance with confocal microscopy, and Jérôme Mégret for performing cell sorting. We thank T. Honjo and M. Muramatsu for the gift of the pTet-tTAK-zeo plasmid and the BTZ cell line, and Novartis Pharma [Base, Switzerland] for graciously providing recombinant human IL-4.

This work was supported by the Ligue Nationale contre le Cancer (Equipe labellisée) and the European Commission Sixth Framework Program (HYBLIB project, NEST pathway project in Synthetic Biology).

The authors declare no competing financial interest.

Submitted: 11 May 2007
Accepted: 11 April 2008

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