Activation-induced Cytosine Deaminase (AID) Is Actively Exported out of the Nucleus but Retained by the Induction of DNA Breaks

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B lymphocytes fine-tune the specificity of their receptors to foreign antigen by hypermutating the variable (V) regions of the immunoglobulin (Ig) genes, followed by selection for Ig receptor variants that have acquired affinity-enhancing mutations (1–3). Our understanding of the molecular basis of somatic hypermutation (SHM) of immunoglobulin genes was greatly enhanced by the discovery of a novel cytosine deaminase (AID) (4), a molecule that proved essential for SHM, class switch recombination (CSR), and immunoglobulin gene conversion (5–7). Given its homology to the RNA-editing enzyme Apobec-1, it was speculated that the substrate for deamination by AID may be cytosine in the RNA transcript encoding a molecule critical to SHM, CSR, and gene conversion. Instead, recent data suggest that the target of AID is the DNA encoding the V and switch regions of immunoglobulin genes. In a study by Neuberger and co-workers (8), it was demonstrated that in mice deficient in uracil DNA glycosylase, there is a highly altered pattern of mutation in Ig genes that points to uracil in the DNA of Ig V regions as an intermediate product of the SHM reaction. Because cytosine deamination produces uracil, these data strongly suggest that the substrate of AID is the DNA of Ig V and switch regions.

Although AID likely deaminates cytosine in the DNA of Ig V and switch regions, it cannot deaminate cytosine in double-stranded DNA. Instead, in vitro deamination by AID can be accomplished with single-stranded DNA or with double-stranded DNA undergoing transcription, suggesting that accessibility of DNA-cytosine residues to AID is hindered in duplex DNA (9–14). Recent evidence strongly suggests that AID interacts directly with the transcription machinery during CSR (15). However, the presence of AID-dependent and -independent DNA breaks in IgV regions of B cells undergoing SHM (16–23) raises the possibility that a DNA break, or the DNA repair patch that is initiated as a result, may also generate a substrate for AID deamination. The lack of strand bias at G:C mutations in Ig V genes (24), in particular the equally altered G and C mutation pattern in the absence of uracil DNA glycosylase (8), argues that AID has equal access to both strands, a scenario that appears unlikely with a transcription-mediated process. Thus, although a direct role for transcription-mediated AID deamination appears likely in CSR, it is difficult to reconcile a direct role for transcription in SHM with the absence of mutational strand bias at G:C base pairs.

With the exception of the BCL6 gene in humans, SHM in hypermutating B cells is targeted to Ig V regions (25). However, ectopic expression of AID in non-lymphoid tissues and in Escherichia coli leads to accumulation of mutations at G:C base pairs in non-Ig genes (26, 27). Transgenic mice that overexpress AID develop T cell lymphomas (28), and in humans, certain subsets of germinal center-derived B cell lymphomas display untargeted AID-mediated hypermutation in multiple protooncogenes such as C-MYC and PAX5 (29, 30). Therefore, when expressed outside of the context of targeted SHM in germinal center B cells, AID can act alone as a general mutator of G:C bases. These data suggest that although AID may have a preference for local DNA sequence (31–33), it requires cofactors for its proper targeting to the Ig V and switch regions. Furthermore, the data suggest that in the absence of cofactors, expression of AID leads to generalized mutagenesis and perhaps neoplasia. The existence of these cofactors and their identification remain the subject of intense investigation.

Given its properties as a general mutator of G:C base pairs, we wondered whether AID is actively kept away from DNA by a nuclear export mechanism and then specifically transported back into the nucleus by targeting cofactors. AID has a molecular weight of 24,000 and thus would be expected to diffuse passively through the nuclear membrane because the upper threshold of the nuclear pore in eukaryotic cells is ~50 kDa (34). In searching for evidence of nuclear traffic regulation of

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¶ The abbreviations used are: V, variable; SHM, somatic hypermutation; AID, activation-induced cytosine deaminase; CSR, class switch recombination; NES, nuclear export sequence; NLS, nuclear localization signal(s); UVC, ultraviolet C; SSB, single-strand break; DSB, double-strand break; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; RFP, red fluorescent protein.

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Intracellular Trafficking of AID and DNA Damage

AID, we aligned the AID amino acid sequences of various species and identified a highly conserved putative nuclear export sequence (NES). NES regions in proteins are binding sites for the exportin CRM1 (34). This ubiquitous mechanism of nuclear export is commonly associated with molecules of small size that function in the cytoplasm or with molecules whose entry into the nucleus must be regulated, such as transcription factors that translocate to the nucleus when activated (34). In addition, we detected by proteomic analysis putative nuclear localization signals (NLS) in human AID, but not in mouse or chicken AID. Indeed, when we tested various human AID constructs that lacked either the putative NES or NLS, we found strong evidence that the NES sequence in the AID C terminus is active via a CRM1-mediated export mechanism. However, the putative NLS appears to be non-functional. We also examined the impact of DNA-damaging agents on nuclear retention of AID. We reasoned that if AID has affinity to DNA breaks, exposing transfected cells to DNA-damaging agents would result in the accumulation of AID in the nucleus. Strikingly, we found significant retention of AID in the nucleus following exposure to bleomycin, ionizing radiation, and hydrogen peroxide but not to UVC radiation, the only agent in the group that did not induce DNA breaks. Together, these results suggest that AID passively diffuses into the nucleus but that it is actively transported out of the nucleus by an NES-mediated mechanism. These results also suggest that in the presence of DNA damage, AID accumulates in the nucleus by binding to DNA single- (SSBs) or double-strand breaks (DSBs) either directly or indirectly through the ensuing DNA repair process.

EXPERIMENTAL PROCEDURES

Cell Culture—293 fibroblast cells were obtained from the American Type Culture Collection (ATCC) and maintained in 5% CO₂ atmosphere at 37 °C in minimum Eagle’s medium (MEM). The medium was supplemented with heat-inactivated 10% horse serum, 10,000 units/ml penicillin, 10,000 µg/ml streptomycin, and 25 µg/ml amphotericin (Invitrogen).

Plasmid Constructs—Human AID cDNA was subcloned into the pD-sRED2-N1 vector (Clontech, BD Biosciences; Palo Alto, CA) using the EcoRI, BamHI, and KpnI sites in the vector. In this vector, the multiple cloning site is upstream of the DSRed gene, resulting in a recombinant protein that consists of AID with the red fluorescent protein fused to the cloning site. pD-sRED2-N1 constructs that lack either the putative NES or NLS, we found strong evidence that the NES sequence in the AID C terminus is active via a CRM1-mediated export mechanism. However, the putative NLS appears to be non-functional. We also examined the impact of DNA-damaging agents on nuclear retention of AID. We reasoned that if AID has affinity to DNA breaks, exposing transfected cells to DNA-damaging agents would result in the accumulation of AID in the nucleus. Strikingly, we found significant retention of AID in the nucleus following exposure to bleomycin, ionizing radiation, and hydrogen peroxide but not to UVC radiation, the only agent in the group that did not induce DNA breaks. Together, these results suggest that AID passively diffuses into the nucleus but that it is actively transported out of the nucleus by an NES-mediated mechanism. These results also suggest that in the presence of DNA damage, AID accumulates in the nucleus by binding to DNA single- (SSBs) or double-strand breaks (DSBs) either directly or indirectly through the ensuing DNA repair process.

Flow Cytometry—The H2A.X phosphorylation assay (Upstate Cell Signaling Solutions, Lake Placid, NY) was performed per the manufacturer's instructions. In brief, exponentially growing cells were treated with bleomycin, hydrogen peroxide, γ-rays, or UVC as done for the confocal studies. Cells were harvested, washed twice with 1× PBS, and fixed (37% formaldehyde in 10–15% methanol) on ice for 20 min to stop any DNA repair. Washed cells were incubated with anti-phospho H2A.X-FITC conjugate antibody in the presence of permeabilization buffer and analyzed on a BD Biosciences FACSsort.

Comet Assay—The comet assay was a modified version of that described by Singh et al. (35). Briefly, following exposure to NA-damaging agents under the same conditions as done for the confocal studies, cells were suspended in 100 µl of 1% (w/v) low melting point agarose in PBS, pH 7.4, at 37 °C and immediately pipetted onto a frosted microscope slide that had been precoated with 1% (w/v) normal melting point agarose. Slides were cooled at 4 °C for ~10 min before a second layer of 100 µl of low melting point agarose was placed on top of the previous layer. After cooling the slides as above, they were immersed in lysis solution (2.5 x NaCl, 100 mM Na₂EDTA, 10 mM Tris, and 1% (v/v) Triton X-100, NaOH to pH 10.0) at 4 °C overnight. After they were neutralized for 5 min in 0.4 M Tris-HCl, pH 7.5, slides were subjected to electrophoresis in a horizontal tank containing 0.3 M NaOH and 1 mM Na₂EDTA at pH 13 for 20 min to unwind DNA before electrophoresis at 25 °C constant for 40 min. The slides were rinsed three times for 5 min each with 0.4 M Tris-HCl, pH 7.5, placed in cold ethanol for a minimum of 30 min, dried, and stained with 20 µg/ml ethidium bromide. A total of 25 comets/slide were scored for amount of DNA damage using the Komet 5.0 software (Kinetic Imaging Ltd., Liverpool, UK). Each treatment was done in duplicates.

Statistical and Proteomic Analysis—Putative NLS and NES motifs were detected utilizing the PSORT II for eukaryotic sequences or NES Finder programs, respectively, available at psort.nibb.ac.jp (36) and www.cbs.dtu.dk/services/NET. Chi-square tests were used to compare the fraction of cells with a particular subcellular distribution given various treatments/genetic constructs. The α-level for each set of comparisons was adjusted by the Bonferroni method (37) to p = 0.0127 to account for increased probability of type 1 errors by multiple comparisons.
RESULTS

AID Is Actively Transported out of the Nucleus by a CRM1-mediated Mechanism—A putative NLS in the N terminus and NES in the C terminus of human AID were detected by proteomic programs. To investigate their functionality, we aligned the amino acid sequence of AID across several species and found that although the critical residues associated with the NES were highly conserved, the NLS sequence is only detectable in human AID (Fig. 1) by the PSORT II program. We generated various genetic constructs wherein wild type AID, AID lacking the putative NLS, and AID lacking the putative NES were fused to red fluorescent protein (RFP). We then visualized by confocal microscopy the intracellular locations of the various versions of the AID protein in transiently transfected fibroblast cells (Figs. 2 and 3). While 87% of the cells transfected with wild type AID displayed a cytoplasmic distribution of the fusion protein, 90% of the cells transfected with NES-lacking AID displayed either a diffuse or nuclear distribution, with the majority of the cells having the AID protein in the nucleus (Fig. 2, a and c, and Fig. 3). These results imply that AID is actively shuttled out of the nucleus via its NES.

Surprisingly, the majority of the cells expressing the NLS-lacking AID protein, while containing most of the AID molecules in the cytoplasm, clearly displayed significant levels of AID in the nucleus (Figs. 2b and 3). A result that is not consistent with an NLS in the N terminus. The prediction is that if the NLS is important for AID entry into the nucleus, its removal would result in the disappearance of the class seen in cells transfected with the wild type AID that displayed a diffuse pattern (~13% of wild type cells). Instead, this class of cells increased to a majority with the removal of the putative NLS. Various reports place the size limit for passive diffusion at 50 kDa with a gray area between 40 and 50 kDa (34). The NES-lacking construct was designed to be the same size as the NLS-lacking construct. However, the distribution of the NES-lacking construct was significantly different from the NLS-lacking protein; namely, the majority of the cells displayed a strictly nuclear distribution (Figs. 2c and 3, p < 0.001). To further confirm that the nuclear distribution of AID lacking the NES was the result of its NES region, we treated cells transfected with the full-length AID-RFP fusion with leptomycin B, a specific inhibitor of the CRM1 exportin, the molecule most frequently associated with NES-mediated nuclear export. Indeed, nearly 50% of the cells treated with leptomycin B displayed either a diffuse pattern or a nuclear distribution of the AID-RFP molecules (Fig. 2d), a distribution that is significantly different from untreated cells transfected with the same length construct (Fig. 3). Together, these results demonstrate that the highly conserved NES in the C terminus of AID is
indeed functional through a CRM1-mediated mechanism, whereas the poorly conserved NLS is most likely not functional. These results also suggest that the small AID molecule is able to passively diffuse through the nuclear pore but is then shuttled out of the nucleus primarily by the CRM1 exportin.

DNA Damage-inducing Agents Caused Significant Retention of AID Molecules in the Nucleus—The above results strongly suggest that AID transiently moves into the nucleus via passive diffusion but is actively shuttled out by exportins. Given this finding, we wondered whether this process of export could be interrupted if AID binds another molecule such as DNA in the nucleus prior to being exported. Given recent findings that DNA breaks are associated with the IgV regions of hypermutating cells (16–23), we wondered whether DNA breaks or the DNA synthesis patch that is generated during the repair of those breaks may be a substrate for AID binding. Therefore, we treated fibroblasts transfected with full-length AID with the DNA-damaging agents bleomycin, hydrogen peroxide, ionizing radiation (γ-rays), or UVC radiation and visualized the intracellular location of the AID-RFP molecule by confocal microscopy. Bleomycin and γ-rays produce DNA breaks, particularly DSBs; hydrogen peroxide produces base damage, DSBs, and SSBs; and UVC is primarily associated with pyrimidine dimers (38–42). A sizeable fraction of the transfected cells treated with bleomycin, hydrogen peroxide, or ionizing radiation displayed significant nuclear retention of AID. Various confocal views of the cells treated with these agents are depicted in Fig. 4. We noticed that among the cells with nuclear retention of AID, the distribution of AID in the nucleus varied; some cells displayed discrete AID foci (less than 5% of the cells with retention, Fig. 4, b and d), a patch AID distribution that was confined to a particular region of the nucleus (~35% of cells, Fig. 4a), or a diffuse pattern throughout the nucleus (~60% of the cells, Fig. 4c). The fraction of cells retaining AID in the nucleus after treatment with bleomycin, hydrogen peroxide, or ionizing radiation was significantly higher than in untreated cells, particularly in the case of hydrogen peroxide (Fig. 5). However, treatment of cells with UVC did not result in a significantly higher fraction of cells with nuclear retention of AID (Fig. 5).

To ensure that AID retention in treated cells was not caused by the formation of RFP aggregates getting trapped in the nucleus because of the chemical agents, we treated with bleomycin or hydrogen peroxide the cells that had been transfected only with RFP plasmid and found no evidence of RFP aggregates (data not shown). It was also possible that we biased the population in favor of cells in the S phase of the cell cycle by treating with DNA-damaging agents and that it is this stage of the cell cycle that best correlates with the nuclear retention of AID. However, when we treated transfected cells with aphidicolin, which arrests cells in S phase, the intracellular distribution of AID remained mostly cytoplasmic (data not shown). In addition, nuclear fragmentation or disintegration of the nuclear envelope during apoptosis cannot explain the pattern of AID distribution with the various treatments for the following reasons. (a) Treatments were sublethal both in terms of dosage and time (cells were visualized no more than 1 h following exposure to agents). (b) We were able to observe the chromatin

![Fig. 4. AID-transfected cells exposed to agents that induce DNA damage displayed significant nuclear retention of AID.](image_url)

![Fig. 5. A significant fraction of the cells treated with bleomycin, γ-rays, and hydrogen peroxide but not UVC radiation displayed AID retention in the nucleus. Asterisks depict significant differences from untreated cells. Chi-square tests were used with 1 degree of freedom and an α-level of \( p = 0.0127 \).](image_url)
directly by confocal microscopy, and although we did see evidence of fragmentation and ring formation (from condensed chromatin) near the nuclear membrane in a few cells, these were not considered in our analysis. (c) Cell viability assays with trypan blue following treatments with the various agents revealed that fewer than 1% of the cells were undergoing apoptosis, and (d) apoptotic fibroblasts lose their adherent properties and therefore tend to be washed out during the fixation process (40). Therefore, the significant nuclear retention of AID following treatment with bleomycin, hydrogen peroxide, and ionizing radiation is most likely explained by the impact of these agents on the DNA rather than on RFP, cell cycle, or apoptosis.

**Only Agents That Induced DNA Double-strand and Single-strand Breaks Caused Significant Nuclear Retention of AID**—A cellular response specific to DSBs is the phosphorylation of histone H2AX at the site of DNA damage (43). Therefore, we can measure the presence of DSBs in our cells by examining H2AX phosphorylation. Untransfected cells were treated with bleomycin, hydrogen peroxide, γ-rays, or UVC under identical conditions to those used for the confocal experiments. We found that bleomycin, γ-rays, and hydrogen peroxide induced DSBs, while few cells could be detected to have H2AX phosphorylation in the untreated sample or in the cells exposed to UVC radiation (Fig. 6). To examine the possibility that AID nuclear retention could also correlate with SSBs, we analyzed DNA damage by the alkaline comet assay. In this assay, the DNA of lysed cells embedded in agarose is allowed to unwind under alkaline conditions and is then exposed to an electrical current. The nicked DNA is not supercoiled and thus migrates faster than the supercoiled undamaged DNA so that the size of the "tail" behind the supercoiled chromatin is indicative of the amount of DNA damage, particularly SSBs (see Fig. 7). Again, we treated the cells with the various DNA-damaging agents under the same conditions as done for the confocal microscopy analysis and subjected them to the comet assay. While bleomycin, γ-rays, and hydrogen peroxide induced DSBs, UVC had the same level of SSBs as seen in untreated cells (Fig. 7; DNA damage by hydrogen peroxide treatment was too high for quantification).

Finally, because hydrogen peroxide-induced DNA breaks are repaired rapidly (within 1–2 h of treatment) (38), we were able to examine nuclear retention of AID prior to detectable induction of apoptosis but allowing enough time for significant repair of the DNA breaks. Thus, we allowed the transfected cells to repair their DNA in medium for 30 min after hydrogen peroxide treatment and found a significant drop in the fraction of cells displaying nuclear retention of

![Fig. 6. Detection of DNA double-strand breaks as measured by H2AX phosphorylation in cells treated with bleomycin, γ-rays, or hydrogen peroxide but not UVC radiation. The y axis depicts the fraction of cells with phosphorylated H2AX as measured by flow cytometry.](image)

![Fig. 7. Detection of DNA single-strand breaks as measured by the alkaline comet assay in cells treated with bleomycin, γ-rays, or hydrogen peroxide but not UVC radiation. DNA damage by hydrogen peroxide treatment was too high for quantification. Each duplicate per treatment depicts the average OTM in 25 comets. (OTM is defined as the olive tail moment, and increasing values represent increasing amounts of DNA damage.) The bottom panels depict comet assay cells with DNA damage representative of the kind observed for untreated (0–1 OTM), bleomycin-treated (~6 OTM), and γ-ray-treated (~11 OTM) cells.](image)

![Fig. 8. The fraction of hydrogen peroxide-treated cells with nuclear retention of AID declined when cells were allowed time for recovery. H2O2 low and H2O2 high are defined as a 30-min treatment with either 50 or 100 μM H2O2, respectively, fixed immediately or after a 30-min period of recovery in complete medium.](image)

![Fig. 9. Proposed model of intracellular traffic regulation of AID. The small AID molecule passively diffuses through the nuclear membrane but is actively exported via its NES. Entry of AID into the nucleus is accomplished by the association of AID with an NLS-bearing cofactor that targets it to the Ig V or switch regions.](image)
AID, without a change in cell viability (Fig. 8). The combined results show a strong correlation between the presence of DNA breaks and nuclear retention of AID.

**DISCUSSION**

Herein we demonstrated that AID is transported out of the nucleus by an NES-mediated mechanism, primarily via the CRM1 exportin. We also found that a putative NLS in the N terminus is not important for nuclear traffic of AID. Our results demonstrating the NES in the C terminus are consistent with recent findings by Honjo and co-workers (44), but we were unable to confirm their findings suggesting a functional NLS. This discrepancy cannot be attributed to a difference in cell type because both studies examined human AID localization in fibroblasts. One possible explanation for the discrepancy is the potential presence of a cryptic NLS in the middle of the AID protein, although we were unable to detect additional putative NLS regions by proteomic analysis.

If the function of AID is to deaminate cytosines in the DNA of Ig V and switch regions, why should AID be regulated by an active nuclear export process? We propose that AID alone lacks the proper targeting information that would direct it to the IgV regions during SHM or to the Ig switch regions during CSR. This proposal is backed by data wherein expression of AID outside of the context of B cells undergoing SHM or CSR results in mutation at G:C base pairs in non-Ig genes (26, 27). We further propose that CSR and SHM cofactors associate with AID in the cytoplasm and direct it across the nuclear membrane to the Ig V or switch regions (Fig. 9). This proposal is not without precedent; Apobec-1, a close homolog of AID, lacks the DNA translesion synthesis polymerases (49–53) and, in the case of SHM, for the subsequent components that initiate the reaction by creating a substrate for AID deamination. These results also suggest the intriguing possibility that in B cells transfected with constructs having a deletion or frameshift in the NES-containing C terminus of AID (modeled after similar mutations found in hyper-IgM patients), CSR is impaired (47, 48), but mutations in Ig V and switch regions can be detected (48). The fact that switch mutations are present with C terminus deletion mutants of AID but CSR is impaired (48) is remarkable since it was expected that AID-triggered deamination was the sole trigger for the CSR reaction, inducing the downstream events carried out by normal components of the DNA repair machinery. Because absence of the NES is expected to result in the accumulation of AID in the nucleus, perhaps these mutations are representative of untargeted AID activity. These results also suggest the intriguing possibility that AID-triggered deamination of Ig switch regions is insuffi cient to induce CSR and perhaps normal SHM since the IgV region mutations were confined to mostly G:C bases, a footprint of untargeted AID-mediated mutation. The totality of these results supports the notion that AID cofactors for SHM and CSR help target AID to Ig V and switch regions and perhaps initiate the reaction by creating a substrate for AID deamination and, in the case of SHM, for the subsequent components such as the DNA translesion synthesis polymerases (49–53) and the mismatch repair machinery (54–59). The substrate for AID-mediated deamination during SHM remains unknown. Recent studies have demonstrated that AID can deaminate single-stranded DNA in vitro, and the notion that AID associates directly with the transcriptional machinery is supported by both in vitro and in vivo data in the case of CSR (9–15, 60). However, transcription-facilitated AID deamination may simply reveal the fact that cytosines in the DNA need to be exposed for AID activity, and this accessibility can be accomplished by a variety of processes, including DNA breaks or DNA repair. Furthermore, because transcription is an asymmetrical process, it is difficult to envision a direct role for transcription in SHM when one considers the fact that mutations in G:C base pairs do not reveal any evidence of strand bias. Furthermore, since both G and C bases were equally affected by the absence of uracil DNA glycosylase (8), it is apparent that AID deaminates cytosines in both DNA strands at similar levels. In addition, several investigators have found IgV-specific, AID-independent DNA double-strand breaks in hypermutating B cells (16–23). Because these DNA breaks may lie upstream of AID activity, it is possible that DNA breaks provide an entry site or a DNA repair patch substrate for AID-mediated deamination. Therefore, we examined the possibility that DNA damage or repair may result in the accumulation of AID in the nucleus. We found that agents that induced DNA breaks caused significant retention of AID molecules in the nucleus, whereas UVC, which causes DNA damage but not DNA breaks at significant levels, failed to display an increase of AID accumulation in the nucleus. We cannot distinguish whether this effect was the result of direct binding of AID to broken DNA ends or whether the affinity of AID is to the ensuing DNA repair process. However, these results provide evidence that events associated with DNA breaks may provide a substrate for AID binding. Since repair of DNA breaks can impact both strands, it is reasonable to propose that given these and the previous results on the presence of DNA breaks in hypermutating B cells, a DNA break may initiate the SHM reaction.

**Acknowledgments**—We are grateful to Jan Drake, Michael Resnick, Laurent Verkoczy, and Tom Kunkel for helpful suggestions during these studies and for comments on the manuscript. We also thank Tony Xiao, Mita Ray, Jim Westmoreland, Janine Santos, and Deborah Croteau for assistance with the use of the confocal microscope.

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