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Research Article

Activation-induced cytidine deaminase (AID) is localized to subnuclear domains enriched in splicing factors ☆

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ABSTRACT

Activation-induced cytidine deaminase (AID) is the mutator enzyme in adaptive immunity. AID initiates the antibody diversification processes in activated B cells by deaminating cytosine to uracil in immunoglobulin genes. To some extent other genes are also targeted, which may lead to genome instability and B cell malignancy. Thus, it is crucial to understand its targeting and regulation mechanisms. AID is regulated at several levels including subcellular compartmentalization. However, the complex nuclear distribution and trafficking of AID has not been studied in detail previously. In this work, we examined the subnuclear localization of AID and its interaction partner CTNNBL1 and found that they associate with spliceosome-associated structures including Cajal bodies and nuclear speckles. Moreover, protein kinase A (PKA), which activates AID by phosphorylation at Ser38, is present together with AID in nuclear speckles. Importantly, we demonstrate that AID physically associates with the major spliceosome subunits (small nuclear ribonucleoproteins, snRNPs), as well as other essential splicing components, in addition to the transcription machinery. Based on our findings and the literature, we suggest a transcription-coupled splicing-associated model for AID targeting and activation.

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Introduction

Antibody (Ab) diversification in stimulated B cells, by somatic hypermutation (SHM) and class switch recombination (CSR), is dependent on activation-induced cytidine deaminase (AID) [32,47]. SHM generates point mutation in the immunoglobulin (Ig) variable regions while CSR exchanges the Ig heavy chain constant region, giving rise to antibodies with enhanced affinity and new effector functions, respectively [59]. AID initiates these processes by deaminating cytosine to uracil in Ig variable and switch gene regions. The resulting U:G mismatches are usually processed by the uracil-DNA glycosylase UNG2 [10,25,44] and a

Abbreviations: AID, activation-induced cytidine deaminase; CB, Cajal body; CTNNBL1, catenin-β-like protein 1; CSR, class switch recombination; Fib, fibrillarin; LeptB, leptomycin B; NLS, nuclear localization signal; SC35, 35 kD serine/arginine-rich splicing factor 2; SHM, somatic hypermutation; SMN, survival of motor neuron; Sm protein, Smith protein; snRNP, small nuclear ribonucleoprotein; WB, Western blot; U2AF65, U2 auxiliary factor 65 kD subunit

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pathway that requires the mismatch recognition factors MSH2 and MSH6 [42]. In the next steps, error-prone DNA polymerases and factors involved in non-homologous end-joining (NHEJ) are used to achieve SHM and CSR, respectively [49,59].

AID is a mutator protein and AID off-target activity is responsible for many of the mutations and translocations involved in B cell lymphomagenesis [30,36,46,48]. Thus, understanding its detailed function, targeting and regulation is important. Transcription is required for both SHM and CSR [12,41]. AID physically interacts with the transcription apparatus, likely via the transcription elongation factors, SPT5 [40] and PAF1 [56]. In addition, AID interacts with the spliceosome-related factor CTNNBL1 (catenin-beta-like protein 1) [7], the splicing regulator PTBP2 (polypyrimidine tract binding protein 2) [35], as well as several other factors involved in splicing [56] and RNA processing [2]. However, the role of these factors in Ig diversification is still unclear.

AID is a nucleocytoplasmic shuttling protein that displays a predominantly cytoplasmic localization [43]. The shuttling in and out of the nucleus is driven by an NLS-dependent active nuclear import mechanism [17,39] and exportin1-dependent nuclear export [20], respectively. CTNNBL1 is a NLS-binding spliceosome-associated protein that has been implicated in subcellular trafficking of AID [14], and recently we showed that CTNNBL1 and AID colocalize in nucleoli [17].

In this study, the subnuclear localization of AID and CTNNBL1 and their association with splicing components were examined in more detail. We found that AID and CTNNBL1 accumulate in distinct nuclear domains enriched in spliceosome-associated factors. Furthermore, we identified physical interactions between AID and the major spliceosome subunits. Moreover, our results indicate that activation of AID by PKA phosphorylation [1,6,37] may be linked to the splicing machinery as well. Based on our findings and the literature we suggest a model where a transcription-coupled splicing complex may target AID to its locus and regulate its function.

Materials and methods

Plasmid constructs

Cloning of human AID cDNA (Image clone 4853069) into pECFP/pEYFP-N1 and –C1 vectors (Clontech) and generation of constructs encoding YFP/CFP-tagged AID with C terminal truncations were described previously [17]. The construct encoding the AID splice variant, lacking exon 4, AIDΔ4-YFP (AID1-142-APV-YFP) was made by site-directed mutagenesis of three codons (AID-D143A-Y144P-F145V, 144P-145V generated an internal *Age*I site) in pAID-YFP followed by *Age*I digestion and religation. Plasmid encoding untagged AID was generated from the pAID-YFP construct by excision of the YFP gene (*Age*I-*BsrG*1 fragment), blunting of ends by T4 DNA polymerase, ligation and reintroduction of AID stop codon. AID-Cherry and AID1-186-Cherry were generated by cloning AID or AID1-186 cDNA (from AID-YFP, or AID1-186-YFP) into the pCherry-N1 vector as *AgeI-Not*I fragments. (pCherry-N1 and pCherry-C1 were made by replacing the YFP gene in the pEYFP-N1 and -C1 vectors with the gene encoding Cherry). pEGFP-CTNNBL1 [7] was a generous gift from Dr. Cristina Rada and Dr. Michael Neuberger and the subcloning into pEYFP-C1 and pECFP-C1 vectors was described previously [17]. pGFP-SMN was kindly provided by Dr. Jianhua Zhou. SMN cDNA was subcloned into the pECFP-C1 and pCherry-C1 as *Bsp*E1-*Bam*H1 fragments to generate pCFP-SMN and pCherry-SMN. pGFP-U2AF65 was a generous gift from Dr. Maria Carmo-Fonseca (Lisbon, Portugal) to Dr. Marit Otterlei in our laboratory. Mutagenesis was carried out using the Quick-Change™ mutagenesis kit (Stratagene) and confirmed by sequencing.

Cell culture, transfection and confocal microscopy

HeLaS3, HEK-293T and U2OS were cultured in DMEM with 10% fetal calf serum (FCS) and CH12F3 was maintained in RPMI, supplied with 10% heat-inactivated FCS, 50 μM β-mercaptoethanol, 1 mM Na-pyruvate. In addition, all cultures were supplied with 0.03% L-glutamine, 0.1 mg/ml gentamicin or 1 × PenStrep and 2.3 μg/ml fungizone. Cells were transfected with FuGENE HD or X-tremeGENE HP (Roche) according to the manufacturer's instructions and analyzed 24 h post transfection. When indicated, cells were incubated with 10 ng/ml Leptomycin B (LeptB) (Sigma/LC-labs) for 2–3 h if not specified otherwise. Permeabilization of cells with digitonin was performed as previously described [17]. Cells were examined in a Zeiss LSM 510 laser scanning microscope (1 μm thickness) with a Plan-Apochromat 63 × /1.4 oil immersion objective. CFP was excited at 458 nm and detected at 470–500 nm, YFP was excited at 514 nm with detection between 530 and 600 nm (YFP was excited with 488 nm and detected at 505–550 when cotransfected with Cherry), and Cherry was excited at 543 nm and detected above 615 nm.

Cell fixation and immunofluorescence

Cajal bodies were identified by immunostaining of coilin. Cells were fixed in 2% paraformaldehyde (freshly made in PBS) for 10 min on ice and permeabilized with ice cold methanol (−20 °C, 20 min). Cells where then rinsed in PBS, blocked in PBS containing 2% FCS and labeled with rabbit polyclonal anti-coilin antibody (ab74739, Abcam) (1:300, 1 h at 37 °C) followed by Alexa Fluor 647 nm goat anti-rabbit antibody (#A-31633, Invitrogen).

For detection of nuclear speckles cells were fixed and permeabilized in ice cold methanol (−20 °C, 20 min). PBS with 0.3% TritonX-100 and 5% BSA was used as blocking and staining buffer. Cells were stained with mouse monoclonal anti-SC35 antibody (#S4045, Sigma-Aldrich) (1:2000, overnight at 4 °C), followed by Alexa fluor 532 nm goat anti-mouse (#A-11002, Invitrogen) or Alexa Fluor 647 nm goat anti-mouse (#A-21235, Invitrogen). Double staining of coilin and SC35 (or AID) were performed on methanol fixed cells using the rabbit polyclonal anti-coilin and mouse monoclonal anti-SC35 (or anti-AID #39-2500, Invitrogen, 1:200) primary antibodies at 4 °C overnight, followed by Alexa Fluor 647 nm goat anti-rabbit and Alexa fluor 532 nm goat anti-mouse secondary antibodies.

Immunofluorescence staining of PKA was performed by the methanol fixation protocol using PKA catalytic subunit-α rabbit polyclonal antibody (#SC-903, Santa Cruz) (1:100 dilution, overnight at 4 °C) and secondary antibody Alexa Fluor 647 nm goat anti-rabbit. Cells were analyzed by confocal microscopy. Alexa Fluor 532 nm was excited with 543 nm and detected between 560 nm and 615 nm while Alexa Fluor 647 nm was excited with 633 nm and detected above 650 nm.

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