Intracellular Localization of Differentially Regulated RNA-specific Adenosine Deaminase Isoforms in Inflammation*

Received for publication, August 5, 2003, and in revised form, August 27, 2003
Published, JBC Papers in Press, September 3, 2003, DOI 10.1074/jbc.M308612200

Jing-Hua Yang‡§, Yongzhan Nie‡, Qingchuan Zhao‡¶, Yingjun Su‡, Marc Pypaert‡, Haili Su‡, and Reuven Rubinovici‡

From the Departments of ¶Surgery and ‡Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06520

Adenosine-to-inosine (A-to-I) RNA editing is a post-transcriptional process that amplifies the repertoire of protein production. Recently, the induction of this process through up-regulation of the editing enzyme RNA-specific adenosine deaminase 1 (ADAR1) was documented during acute inflammation. Here we report that the inflammation-induced up-regulation of ADAR1 involves differential production and intracellular localization of several isoforms with distinct RNA-binding domains and localization signals. These include the full-length ADAR1 (p150) and two functionally active short isoforms (p80 and p110). ADAR1 p80 starts at a methionine 519 (M519) due to alternative splicing in exon 2, which deletes the putative nuclear localization signal, the Z-DNA binding domain, and the entire RNA binding domain 1. ADAR1 p110 is the mouse homologue of the human ADAR1 110-kDa variant (M246), which retains the second half of the Z-DNA binding domain, all RNA binding domains except the dsRNA domain. Additional variations are found in the third RNA binding domain of ADAR1; they are differentially regulated during inflammation, generating isoforms with different levels of activities. Studies in several cell types transfected with ADAR1-EGFP chimeras demonstrated that the p150 and p80 variants are localized in the cytoplasm and nucleus, respectively. In agreement with this observation, endogenous ADAR1 was identified in the cytoplasm and nucleus of mouse splenocytes and HeLa cells. Since the ADAR1 variants are differentially regulated during acute inflammation, it suggests that the localization of these variants and of A-to-I RNA editing in the cytoplasm, nucleus, and nucleolus is intracellularly reorganized in response to inflammatory stimulation.

A-to-I RNA editing is catalyzed by RNA-specific adenosine deaminase (ADAR), which converts adenosine to inosine and leads to the production of mRNA and protein variants. This process is ubiquitous and widely conserved; it has been identified in multiple species including mammals (1–5), Xenopus (6), Drosophila (7), and zebrafish (8). Four A-to-I RNA editing enzymes, termed ADAR1, ADAR2, ADAR3, and ADAT1, have been cloned so far from mammals (1–5). ADAR1 and ADAR2 are widely expressed in a variety of cells and tissues (2, 4), with the highest expression in the brain and spleen. ADAR3 was identified solely in the brain, and its deaminase activity has not yet been established. ADAT1 targets tRNA and has been cloned from humans (5), mice (9), and yeast (10).

ADARs are conserved in their adenosine deaminase domain but differ in their RNA binding domains. ADAR1 and ADAR2 contain two or three dsRNA binding domains (dsRBD) in addition to an adenosine deaminase domain. These editases are capable of both nonspecific editing of dsRNA and substrate-specific editing of glutamate receptor subunit B (gluR-B) mRNA and serotonin receptor mRNA (11, 12). ADAR2 selectively edits gluR-B at the Q/R site and serotonin at the D site; ADAR1 preferentially targets gluR-B at the hot spot and serotonin at the A and C sites (12). Q/R site editing requires the formation of a base-pairing structure around the editing site for specific substrate recognition by ADAR2. The importance of the secondary structure in substrate recognition was confirmed in a study in which deletion of the stem-loop structures around the Q/R editing site abolished site-specific editing (11). ADAR3 and ADAT1 do not seem to edit these substrates.

A nuclear localization signal (NLS) and a Z-DNA binding domain are present near the N-terminal region of ADAR1 and are conserved in all species. This NLS is also called a nuclear export signal, since the human ADAR1 was initially localized to the cytoplasm and was found in the nucleus after nuclear export was blocked (13). The human ADAR1 also has an atypical NLS within its dsRBDIII and thus displays the characteristics of a shuttling protein (14). In contrast, the Xenopus ADAR1 contains a distinct NLS, which leads this enzyme to the nascent ribonucleoprotein matrix on Xenopus lambrush chromosomes, where it is specifically associated with active transcriptional sites. Thus, it is conceivable that the editing activity of Xenopus ADAR1 is coupled with transcriptional events or that it targets newly synthesized RNAs (15).

Functional consequences of A-to-I RNA editing have been observed in the central nervous system. In the mammalian brain, editing of gluR-B pre-mRNA by ADAR2 has been shown to alter the calcium permeability of excitatory neurons (16, 17).
The role of ADAR2 in the nervous system was further studied in mice homozygous for a targeted functional null allele. In ADAR2−−/− mice, A-to-I RNA editing of diverse mRNAs is substantially reduced; seizure activity and early death occur (18). In the Drosophila brain, disruption of the dADAR gene (a homologue of ADAR2) abolishes sodium, calcium, and chloride channels (19–21). Mutants lacking dADAR exhibit extreme behavioral defects and neurodegeneration (21). Furthermore, an oxygen-sensitive dADAR mutant suffers prolonged recovery from anoxic stupor, vulnerability to heat shock, and increased O2 demands (20). Thus, editing of ion channel pre-mRNAs by dADAR appears to be critical for the integrity and function of the central nervous system. Studies in ADAR1 chimeric mouse embryos demonstrated that this editing enzyme affects embryonic erythropoiesis (22). In these studies, most embryos died before the 14th embryonic day due to hematopoietic defects, suggesting that editing in this developmental stage is critical for normal erythrocyte proliferation and/or differentiation.

Several observations suggest that A-to-I RNA editing plays a role in the immune system. First, ADAR1 can be induced by interferon (IFN) in human aminon U cells (23, 24) and pulmonary macrophages (25). Second, IFN-induced enzymes such as dsRNA-dependent protein kinase (PKR), 2′,5′-oligo(A) nuclease, and ADAR1 all interact with dsRNA (23, 26) either as a substrate or an activator. Third, ADAR1 and ADAR2 can destroy dsRNA or mRNA with dsRNA stretches, indicating that they may indirectly regulate the activity of dsRNA-binding proteins such as PKR. Fourth, a C-to-U editing enzyme has been reported to play a role in the specific immune response by modulating class switch recombination and somatic hypermutation in B-lymphocytes (27–31). Introduction of the C-to-U editing enzyme triggers hybridoma cells to generate somatic hypermutations in immunoglobulins (32).

We have recently reported that A-to-I RNA editing by ADAR1 is also involved in both local (25) and systemic (33) acute inflammation. In the lung, A-to-I RNA editing is up-regulated following endotoxin stimulation due to up-regulation of ADAR1, which precedes the development of pulmonary edema and leukocyte accumulation. Similarly, editing activity and ADAR1 expression are induced in cultured alveolar macrophages (MH-S cells) stimulated with endotoxin or IFN. In systemic inflammation produced by endotoxin in mice, inosine-containing mRNA is markedly induced to -5% of adenosine in total mRNA (33). This induction results from up-regulation of A-to-I RNA editing, since both dsRNA editing activity and ADAR1 expression are increased in the spleen, thymus, and peripheral lymphocytes of endotoxin-treated mice. Up-regulation of ADAR1 has been confirmed in vitro in T lymphocytes and macrophages stimulated with a variety of inflammatory mediators including tumor necrosis factor-α and IFN-γ. Late induction of RNA editing occurs in ConA-activated splenocytes stimulated with IL-2 in vitro. Taken together, these data suggest that during local and systemic inflammation, ADAR1-mediated RNA editing is increased, which may affect the inflammatory response through modulation of protein production.

Acute inflammation is the underlying process of many critical illnesses, including the systemic inflammatory response syndrome, multiple organ failure, sepsis, adult respiratory distress syndrome, and ischemia/reperfusion injury (34). One fundamental event that occurs in all of these stress situations is the intense production of multiple pro- and anti-inflammatory proteins. Thus, additional insight into the regulation of protein production during inflammation could shed light on the pathogenesis of this condition. It is within this context that we aimed to further examine the role of A-to-I RNA editing in acute inflammation. Specifically, following the identification of ADAR1 as a key player in local and systemic inflammation (25, 33), we examined and characterized ADAR1 production, localization, and regulation during acute systemic inflammation in vitro and in vivo.

The present study aims to further delineate the A-to-I RNA editing response to acute inflammation, with special emphasis on the regulation through alternative splicing of ADAR1 variants and their distinct functional domains. In addition, this study investigates the regulation and translocation of the inflammation-induced ADAR1 variants in the cytoplasm, nucleus, and nucleolus.

**EXPERIMENTAL PROCEDURES**

**Animal Model of Acute Inflammation**—The model used in the present study was described previously in detail (35). In brief, endotoxin at a dose of 15 mg/kg (LD60) was injected into the peritoneal cavity of conscious adult (6-week-old, 25- to 35-g) male C57Bl/6 mice (Charles River Laboratories). The mice were anesthetized (pentobarbital; 30 mg/kg) at various time points, and tissues were harvested and processed for analysis, as described below. The Yale Animal Care and Use Committee approved all animal protocols. Five mice were used per group, and a mixture of five similar organs from these animals was used for each analysis.

**DNA and Protein Isolation**—Total RNA and mRNA at each time point (n = 5) were isolated using Trizol (Trizol, Inc.) and OligoTex (Qiagen) separately for Northern blotting, RT-PCR, and ADAR1 cloning. Total protein was isolated by homogenizing mouse splenic cells in four volumes of an editing buffer (containing 20 mM Hepes, pH 7.9, 100 mM KCl, 5 mM EDTA, 0.5% Noutet P-40, and 1% glycerol). The lysate was sonicated for 30 s and centrifuged for 30 s at 4,000 × g. Protein concentration in the supernatant was determined using a Bio-Rad protein kit and adjusted to 10 mg/ml for Western blotting or editing analyses.

**Northern Blotting, RT-PCR, and Western Blot Analysis**—Equal amounts (-2 μg) of mRNA from mouse tissues (n = 5) were used for Northern blotting. ADAR1 was detected by hybridization of the blots with 32P-labeled antisense probes (positions 1305–1265 and 3004–2966; GenBank accession number AF291050). Membranes were hybridized at 65 °C overnight and then subjected to a final wash was with 0.1× SSC at 55 °C for 30 min. For RT-PCR, 2 μg of total RNA were used for reverse transcription primed with poly(dT)12– 18. ADAR1 mRNA was determined by RT-PCR using primers that flank exons 6–8 (positions 1975–2003 and 2436–2408; GenBank number AF291050) or the entire coding region (positions 1–24 and 3459–3435; GenBank number AF291050). The relative expression of ADAR1 mRNA was estimated in comparison with GAPDH. For Western blotting, 80 μg of total protein from splenic cells was resolved on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The blots were detected using rabbit antisera against the C-terminal of recombinant mouse ADAR1 expressed in E. coli (positions 2765–3459; GenBank number AF291050) or the N terminus of a synthetic mouse ADAR1 peptide (Santa Cruz Biotechnology).

**Cloning and Sequencing of ADAR1 Variants**—Full-length ADAR1 from mouse splenic cells harvested 24 h after endotoxin stimulation (n = 5) was amplified by RT-PCR. The PCR products were cloned into pCRII (Invitrogen), and the diversity of ADAR1 cDNAs was analyzed by EcoRI digestion. Alternative splice junction sites were mapped by sequencing and analyzed by sequence alignment. Four typical ADAR1 cDNAs with open reading frames, ADAR1s (GenBank number AF291875), ADAR1b (AF291877), ADAR1La (AF291050), and ADAR1Lb (AF291876), were subcloned for further analysis.

**In Vitro Translation and Expression of ADAR1 Isoforms in a Bacteriovorus System**—The cDNAs of ADAR1s, ADAR1s, ADAR1La, or ADAR1Lb in the pCRII vector were translated in vitro using a TNT T7 quick translation system following the manufacturer’s protocol (Promega). For recombinant proteins, these variants were subcloned into pFastBac (Invitrogen) in frame with a His tag at the N termini to generate recombinant ADAR1-Bacmids. After transfection of Bacmids into S99 insect cells, recombinant ADAR1 isoforms were expressed, and proteins were isolated using nickel columns following the manufacturer’s protocol (Amersham Biosciences).

**RNA Editing Assay Using dsRNA**—RNA editing activity was evaluated by measuring A-to-I conversion of synthetic dsRNA (36). In a typical dsRNA editing assay, 10 μl of cell extracts (100 μg of total protein) or 0.098 μg of recombinant ADAR1 isoforms were mixed with
A restriction enzyme site (BamHI) was cleaved with BamHI and directly subcloned into the pEGFP-N1 vector (Clontech) in frame with the N-terminal of EGFP. Different cells, including mouse fibroblasts (3T3), neuroblastoma cells (N18), monocytes (RAW2.17), or human HeLa and 293 cells, were transfected (n = 3) with pEGFP-ADAR1 DNAs; fluorescence was observed under a microscope 6 h after transfection. Editing activity of the transiently expressed ADAR1-EGFP chimeras was tested in 293 cells and proved able to convert adenosine to inosine on synthetic dsRNA.

Electron Microscopy—HeLa cells were fixed in 4 and 8% paraformaldehyde solutions in 0.25 mM Hepes (pH 7.4) at 4°C for 1 h and overnight, respectively. Cells were washed in PBS, scraped, and pelleted in a 10% solution of gelatin in PBS. Pieces of the pellet were infiltrated overnight at 4°C in a 2.3 M sucrose solution in PBS and then mounted on aluminum studs and frozen in liquid nitrogen. Frozen sections were cut on a Leica ultramicrotome at −108°C. They were collected with a 1:1 mixture of 2.3 M sucrose and 2% methylocellulose solutions, thawed, and transferred onto Formvar- and carbon-coated nickel grids. For immunolabeling, grids were incubated with a solution of 0.1% NihCl in PBS for 10 min, followed by 0.5% fish skin gelatin solution in PBS for 20 min at 22°C. They were then incubated with anti-C-terminal ADAR1 antibody (1:50) for 30 min, followed by 10-nm protein A-gold complex (Department of Cell Biology, Utrecht University, The Netherlands) also for 30 min at 22°C. After several washes in PBS, the sections were fixed in 1% glutaraldehyde in PBS, washed in water, and incubated with 1.8% methylcellulose and 0.5% uranyl acetate solution before being air-dried. Sections were examined in a Tecnai 12 Biotwin electron microscope (FEI) at 80 kV. For quantification, 20 random micrographs of nuclei were taken and printed at a final magnification of ×34,000. Labeling densities over the nucleus or nucleolus were calculated by dividing the number of gold particles over these structures by the surface of profiles estimated by point counting using a lattice grid with 20 mm between lines.

RESULTS

Two ADAR1 mRNA Transcripts Are Expressed in a Tissue-specific Manner—To determine whether ADAR1 has splice variants and whether its expression is tissue-specific, ADAR1 transcripts in a variety of mouse tissues were analyzed by Northern blotting. An antisense sequence complementary to the dsRNA-binding domain of ADAR1 cDNA from positions 1305–1265 (GenBank number AF291050) was selected as the hybridization probe to reduce the nonspecific signal from mouse ribosomal RNA. Two ADAR1 transcripts measuring ~7 and 5 kb were detected in all tested tissues (Fig. 1A). The total ADAR1 mRNA level showed tissue-specific differences, with the highest signal in the brain and spleen and the lowest in the liver. The ratio between the 7- and 5-kb transcripts also varied in a tissue-specific manner, with the highest value observed in the spleen and the lowest in the brain. Similar results were noted when antisense probes complementary to the deaminase domain or to full-length ADAR1 were used (data not shown).

Multiple ADAR1 Variants Are Induced in Response to Inflammatory Stimulation—Northern blot analysis of splenic tissue from endotoxin-challenged mice demonstrated up-regulation of both the 7- and 5-kb ADAR1 transcripts (Fig. 1B). To characterize the sequence variations, the coding region of ADAR1 cDNA in spleens harvested 4 h after endotoxin stimulation (analysis by Northern blotting). A short (−2.0 kb, S) ADAR1 variants are induced in spleens harvested 4 h after endotoxin stimulation. RT-PCR was performed using primers covering the entire coding region. A short fragment III has three different sizes. In endotoxin-challenged tissues (lower panel), fragment III is similar in all clones. Fragment II has four sizes, and fragment I has two sizes. v, pCRII vector.

Fig. 1. Diverse ADAR1 variants are produced and up-regulated in immune organs after endotoxin stimulation. A, two ADAR1 transcripts are expressed in an organ-specific manner in all tested sham-injected mouse tissues (analysis by Northern blotting). B, two ADAR1 transcripts are induced in spleens harvested 4 h after endotoxin stimulation (analysis by Northern blotting). C, long (~3.4 kb, L) and short (~2.0 kb, S) ADAR1 variants are induced in spleens harvested 4 h after endotoxin stimulation. RT-PCR was performed using primers covering the entire coding region. D, a variety of long ADAR1 isoforms are induced by inflammation. Individual cDNAs that were cloned from a mix of spleen and kidney homogenates of control (0 h) or endotoxin-stimulated (4 h) mice were analyzed by EcoRI enzyme digestion. Note that in the unstimulated tissues (upper panel) fragments I and II each have a uniform size, whereas band III has three different sizes. In endotoxin-challenged tissues (lower panel), fragment I is similar in all clones. Fragment II has three sizes, and fragment III has four sizes. v, pCRII vector.
lenged mice demonstrated equal levels of ADAR1L and ADAR1S due to a selective induction of ADAR1S. It should be noted that the 1.4-kb difference between ADAR1L and ADAR1S may not reflect the difference between the 7- and 5-kb ADAR1 transcripts observed in Northern blotting (Fig. 1, B and C). In addition, the PCR products of ADAR1L and ADAR1S displayed diverse variants, which slightly differed in size. To identify these variants, RT-PCR products of the ADAR1L fragments from a mixture of spleen and thymus tissues of normal or endotoxin-stimulated mice were recovered and cloned. Diversity of the selected individual clones was analyzed by restriction enzyme mapping (Fig. 1D). In agreement with the sequencing analysis, three fragments (I, II, and III) and a vector were identified. In sham-injected mice, fragment III had three major variations in 14 clones, whereas only a single size was identified for fragments I and II. In stimulated mice, fragments II and III were found to have three and four variants in 16 clones, respectively, whereas fragment I remained unchanged, with a similar size in all clones. As described in the legends to Figs. 1C and 2, variations in fragment I could be detected only when the mapping of ADAR1L and ADAR1S was compared. In conclusion, diverse ADAR1L and ADAR1S variants were generated in response to inflammatory stimulation.

**ADAR1 cDNA Variants Are Generated through Inflammation-induced Alternative Splicing**—ADAR1 cDNA variants were sequenced. Alignment analysis confirmed that all variants were identical at their 5'- or 3'-ends. The difference between ADAR1L and ADAR1S resulted from alternative selection of the 3' splice site of intron 1, which deleted the entire exon 2, comprising ~1.4 kb of ADAR1 mRNA (Fig. 2A). Variations in fragment III resulted from alternative splicing of exon 7 and selective usage of a few cryptic 5'-splice sites (Fig. 2B). Three ADAR1 variants with slightly different sizes, termed a-, b-, and c-forms, were generated. The b-form matched the previously identified mouse cDNA sequence (25) and was dominant in sham-injected mice (9 of 14 clones). The a-form, which had an additional 78 bp in exon 7 due to a cryptic 5'-splice site within intron 7, occurred less frequently (3 of 14 clones). A similar insertion was previously reported in human ADAR1, which altered the specificity of A-to-I RNA editing on glutamate and serotonin receptor pre-mRNAs (37). The c-form, which deleted the entire exon 7, was rare. Interestingly, these variations occurred within the dsRNA-binding domain III (RBDIII). In one clone, alternative splicing skipped the entire exon 3, which may cause deletions in dsRBDII. A scheme of the alternative splicing in mouse ADAR1 is presented in Fig. 2C.

To avoid errors during PCR and cloning, the identified ADAR1 cDNA variants in total mRNA were further verified by RT-PCR using primers that cover the alternative splicing junctions in exon 2 (Fig. 3A) or exons 5–8 (Fig. 3B). PCR products matching ADAR1L and ADAR1S (Fig. 3A) or a-, b-, and c-forms (Fig. 3B) were identified, and splicing junctions were confirmed by sequencing.
Several ADAR1 Proteins Are Produced and Differentially Regulated in Inflamed Splenic Cells—ADAR1S contains an open reading frame starting at position 1555 (GenBank number AF291050) that corresponds to a short ADAR1 protein lacking the N-terminal NLS, the Z-DNA binding domain (38), and the entire dsRBD. Theoretically, mouse ADAR1L and ADAR1S mRNAs should generate 140-kDa (M1) and 76-kDa (M519) proteins, respectively. To analyze ADAR1 protein variants, antibodies against the C- or N-terminal regions of the protein were used. The anti-C-terminal antibody was expected to detect both the long and short variants, whereas the anti-N-terminal antibody was expected to identify the long form only. As anticipated, a protein of ~150 kDa (p150; Fig. 4A) was detected using the anti-N- and anti-C-terminal antibodies, indicating that it is the full-length ADAR1 also observed in humans (39). Two small protein bands measuring 110 and 80 kDa (p110 and p80) (Fig. 4A) were also detected using the anti-C-terminal antibody, suggesting that they are ADAR1 variants lacking the N-terminal sequences. The p80 protein variant is probably the product of ADAR1S (M519), whereas p110 is similar to the human M246, the product of ADAR1 mRNA from another promoter previously reported in amnion-U cells (40, 41).

Both p80 and p100 consist of two slightly different variants, termed p80a and p80b or p100a and p100b, respectively (Fig. 4, A and B). Remarkably, the expression of these short variants was up-regulated in cultured splenic lymphocytes following inflammatory stimulation with ConA or IL-2 (Fig. 4, B and C). Whereas both a- and b-variants of p80 and p110 were up-regulated, the induction of b-variants was dramatic and consistent after ConA and IL-2 stimulations. The up-regulation of p80 is consistent with the RT-PCR result (Fig. 1C); however, previous studies reported that the human 110-kDa ADAR1 (M246) is not responsive to inflammatory stimulation by IFN-γ (39). In contrast, the expression of the full-length ADAR1 protein, p150, was down-regulated by ConA but up-regulated by IL-2. Although the transcript of the full-length ADAR1 mRNA (ADAR1L) might be induced, the differential expression of p150 after ConA and IL-2 stimulations may reflect a balance between induction of ADAR1L mRNA and alternative splicing of the same RNA to produce ADAR1S (Fig. 2). Taken together, these findings indicate that different ADAR1 variants such as p150, p110a, p110b, p80a, and p80b were differentially regulated after inflammatory stimulation. Since p80 and p110 lack NLS, the Z-DNA binding domain, and dsRBDI, it is conceivable that their function differs from that of p150.

Since the a- and b-forms encode ADAR1 isoforms with...
Regulation and Localization of ADAR1 Variants in Inflammation

slightly variable sizes, variations within p110 and p80 might be generated by alternative splicing in the dsRBDIII region during inflammation. To study the regulation of the a-, b-, and c-forms, total RNA was isolated from mouse spleens after endotoxin stimulation and analyzed by RT-PCR using primers designed to amplify exons 5–8 (Fig. 4D). Whereas the b-form was dominant in normal mice and gradually diminished 6 h after endotoxin stimulation, the a-form was progressively induced and sustained at a high level during the entire experiment. Thus, the a- and b-forms were inversely regulated. This observation is in agreement with the cloning studies, in which only the b-form was identified in healthy mice (Fig. 1). The c-form, however, was not detected in normal mice and was generated only after endotoxin stimulation. Therefore, in acute inflammation, the a-, b-, and c-forms also were differentially regulated. Further investigation is needed to confirm whether these alternatively spliced forms actually generated the identified variations within p110 or p80 proteins and whether these variations affect the specificity or localization of ADAR1.

**Inflammation-induced ADAR1 Variants Are Functionally Active**—To evaluate the contribution of each variant to A-to-I RNA editing in the inflamed cells, four different ADAR1 cDNAs were characterized in vitro and in transfected 293 cells (Fig. 5A). The differentially regulated p150 and p80 variants with alternatively spliced a- and b-forms identical in their deaminase domain, termed La, Lb, Sa, and Sb, were translated in vitro, and the sizes of their protein products were determined to correlate with the predicted sequences (data not shown). To produce sufficient quantities, the La, Lb, Sa, and Sb proteins were produced in insect cells using a baculovirus expression system. The recombinant proteins, which contained a tag with 6 histidines at their N termini, were purified and tested for editing on [32P]adenosine-labeled dsRNA. As predicted, the editing activity of all proteins varied and was the highest in Lb, followed by La, Sb, and Sa (Fig. 5B). Thus, the activity of the short and a-forms was lower than that of the long and b-forms. This conclusion was further confirmed in 293 cells by transient expression (Fig. 5C). By normalizing to the levels of protein expression (Fig. 5C, bottom), the editing activities of Lb, La, Sb, and Sa in whole-cell extracts were found to follow the same order as that with the recombinant isoforms (Fig. 5C, right).

When the activity and the protein level of each variant are considered, the total apparent editing can be estimated in acutely inflamed tissues. In a comparison of the a- and b-forms, editing activity was predominantly contributed by the a-form, although the b-form was slightly more active (<4-fold; Fig. 5B), its expression was significantly decreased (>-5-fold; Fig. 4D). Of the long and short ADAR1 isoforms, the short seemed to be the predominant contributor to editing, since the short form was significantly more expressed than the long, especially during stimulation with ConA (Fig. 4B); the short form was only 2–3-fold less active (Fig. 5B). Because these variants differ in their RNA binding domain and localization signal, it is conceivable that the selectivity of editing and the intracellular localization of these variants may be different in the inflamed cells.

**ADAR1 Variants Are Differentially Localized in the Cytoplasm and Nucleolus**—Since the variations between ADAR1 p150 and p80 include the presence or absence of the NLS, it is possible that these forms are transported to different intracellular compartments. To test this notion, La, Lb, Sa, and Sb variants were fused with EGFP at their C termini and first tested for dsRNA editing in transient transfection. The activity of these chimeras was maintained despite a previous report that the C terminus of human ADAR1 is very sensitive to mutations and deletions (42). Next, the localization of different ADAR1-EGFP chimeras was determined in transfected 3T3 or HeLa cells by fluorescence microscopy. The long ADAR1 variants were found in the cytoplasm, whereas the short variants were localized in the nucleus and formed many bright particles (Fig. 6). Since these particles vary in size and number, the short ADAR1 variants appeared to be localized in the nucleolus. Immunohistochemistry studies supported this observation.
The present study demonstrates that several short but functionally active ADAR1 variants, including those lacking the entire Z-DNA binding domain, the NLS, and the dsRBDI, are generated through alternative splicing during acute inflammation. Since the full-length ADAR1 p150 is localized in the cytoplasm and the short ADAR1 p80 predominantly in the nucleolus, it is conceivable that the short ADAR1 isoforms are produced during inflammation and transported into the nucleolus. These observations point to a possible regulatory mechanism of both ADAR1 isoform production and intracellular localization during inflammation. Several important points should be emphasized.

**Alternative Splicing of ADAR1 Variants with Different Functional Domains in Acute Inflammation**—The demonstration that diverse ADAR1 variants with distinct functional domains are produced during acute inflammation is a key finding of this study. More than a dozen different ADAR1 cDNAs were found in spleens harvested from endotoxin-challenged mice, classified as “long” or “short” based upon their sizes. The short variants are produced from the full-length ADAR1 by alternative splicing that deletes the entire exon 2, comprising 1.4 kb of sequence.

At the protein level, three major variants, termed ADAR1 p150, p110, and p80, were detected in mouse splenocytes, thymocytes, and lymph nodes as well as in human HeLa cells. ADAR1 p150 is the full-length protein that was previously reported in humans (44). ADAR1 p110 is probably the homologue of the human 110-kDa ADAR1 (M246) (39) that starts at methionine 246; retains the second half of the Z-DNA motif βZ, all three dsRBDs, and the catalytic domain; and is constitutively expressed and localized in the nucleus. ADAR1 p80, in neuronal and inflammatory cells derived from the mouse and human. The nucleolar localization of the short ADAR1 isoforms is consistent with several observations. First, using differential staining of HeLa cells with anti-N-terminal and anti-C-terminal antibodies, endogenous ADAR1 was also identified in the nucleolus (Fig. 8, A and B). Second, ADAR1 was previously detected by antibodies specific to p110 in the nucleolus of human U-cells (39). Third, nucleolar localization of the human short ADAR1 M246 has been recently implicated in 3T3 cells (41). It should be noted that a cytoplasmic signal of short ADAR1 isoforms was also identified. This may reflect saturation of the nucleolus with ADAR1S due to artificial overexpression, with spillover into the cytoplasm.

**Endogenous ADAR1 Is Localized in the Nucleolus and Cytoplasm**—To confirm the nucleolar and cytoplasmic localization of ADAR1, the localization of endogenous ADAR1 was evaluated in mouse RAW 264.7 and human HeLa cells using immunofluorescence and confocal microscopy. Cells were fixed on a glass slide coated with poly-L-lysine and stained with an antibody against the N or C termini of ADAR1. Positive signals were detected in the cytoplasm of HeLa cells when the N-terminal antibody was used (Fig. 8A) and in the cytoplasm as well as the nucleolus when the C-terminal antibody was applied (Fig. 8B). A similar result was observed when RAW cells were examined (data not shown). To further support nucleolar localization of endogenous ADAR1, HeLa cells were stained with the C-terminal antibody followed by a protein A-gold complex. Cell sections were examined in a Tecnai 12 Biotwin electron microscope (FEI) at 80 kV. The average densities of gold particles in nuclei and nucleoli were measured as 2.05 ± 0.84 and 4.68 ± 1.21 particles/μm², respectively (Fig. 8, C and D). Thus, these data demonstrated that the endogenous ADAR1 was relatively concentrated in the nucleoli of HeLa cells (probability >99.99%, p < 0.0001, n = 20).

**DISCUSSION**

In these studies, 3T3 or HeLa cells transfected with ADAR1Sα-EGFP were fixed on glass slides and hybridized with antibodies against the nucleolar protein nucleolin (43). Visualization by fluorescence microscopy demonstrated co-localization of ADAR1-EGFP (green) and nucleolin (red), which confirmed the localization of the short forms mainly in the nucleolus. Since these forms are induced during inflammation, it is conceivable that ADAR1-mediated RNA editing shifts from the cytoplasm to the nucleolus in inflammatory conditions.

The localization of ADAR1 p150 and p80 was examined in several cell types including monocytic (RAW 264.7), neuronal (N18), and human HeLa cells using EGFP as described above (Fig. 6B). In all cell types, the long form was found in the cytoplasm, whereas the short form predominantly translocated into the nucleolus. The differential localization of the long and short ADAR1 variants occurred not only in fibroblasts but also...
The mechanisms determining both the substrate specificity and the functional significance of the variable ADAR1 isoforms are still obscure. Nevertheless, ADAR1 isoforms containing variable dsRBDS may target different RNA substrates with distinct specificity. This is supported by the selective activity of ADAR1L and ADAR1S with α- or β-forms on dsRNA substrates. ADAR1L is usually more active than ADAR1S; the β-form is usually more active than the α-form. Because the natural substrates for ADAR1 are largely unknown, however, it still remains a question whether these isoforms are selective for different RNA substrates. Previous studies demonstrated that alteration of dsRBDI, II, or III affects the editing activity as well as the specificity of ADAR1 (45). A similar alternative splicing in the dsRBDIII of human ADAR1 was reported to affect the site specificity of serotonin receptor pre-mRNA editing (37, 46, 47). Furthermore, ADAR1 and ADAR2 are known to edit glur-B and serotonin pre-mRNA selectively at distinct sites without requiring corefactors (4, 48, 49). Taken together, these observations suggest that a wider spectrum of mRNA substrates is targeted for editing during acute inflammation.

**Differential Regulation of ADAR1 Variants in Acute Inflammation**—Another key finding of this study is that inflammation triggers differential regulation of the various ADAR1 isoforms. Spleens from endotoxin-treated animals displayed predominant expression of ADAR1S mRNAs (Fig. 1C). In agreement with this observation, studies in cultured splenic lymphocytes in vitro demonstrated that inflammatory stimulation with ConA and IL-2 preferentially induces the short ADAR1 protein variants p110b and p80b (Fig. 4B). In contrast, the full-length ADAR1 protein was up-regulated by IL-2 but down-regulated by ConA. Variations in the dsRBDIII produced by alternative splicing of exon 7 were also differentially regulated, with preferential production of the α- and c-forms of ADAR1 and down-regulation of the sham-injected mouse dominant β-form. Thus, ADAR1 is switched from the β-form to the α- and c-forms during acute inflammation. Since the induced ADAR1 isoforms vary in their RNA binding domains, differential regulation of these isoforms during acute inflammation could be a novel modulatory mechanism of A-to-I RNA editing activity and selectivity.

Although the mechanisms responsible for the differential regulation of ADAR1 variants are still unknown, it is possible that alternative splicing factors are produced during inflammation to promote the skipping of the entire exon 2 and/or to activate the multiple 5′ splice sites of exon 7. As the induction by endotoxin of the α-form is associated with down-regulation of the β-form, it is likely that these forms are generated through a competitive selection of two different 5′ splice sites of intron 7. The induction of the less dominant c-form during inflammation suggests that other mechanisms should be con-
tempered as well. For example, it is possible that the c-form is generated through induction of independent splicing factors or by cell-specific alternative splicing. Another potential regulatory mechanism of ADAR1 isoform production in inflammation could be autoregulation by ADAR1. The possibility that ADAR1 regulates the diversity of its own gene products is supported by previous reports that ADARs can edit their own mRNA (50), are associated with spliceosomes (51), and can regulate pre-mRNA splicing (12). Thus, the coupling of inflammation-induced RNA editing and alternative splicing could be one unique feature of the ADAR-editing enzymes.

We have previously demonstrated that A-to-I RNA editing in mouse thymic and splenic RNA is dramatically increased in endotoxic-induced acute inflammation (33). The data from this study support the hypothesis that the increased editing observed during inflammation is due to the induction of multiple ADAR1 isoforms and the increased diversity in RNA binding domains. It still remains a question what RNAs in the inflamed tissues are edited? We found that the endogenous mouse thymic and splenic RNA is dramatically increased in inflamed tissues (33). The data from this study support the hypothesis that the increased editing observed during inflammation is due to the induction of multiple ADAR1 isoforms and the increased diversity in RNA binding domains. It still remains a question what RNAs in the inflamed tissues are edited? We found that the endogenous mouse thymic and splenic RNA is dramatically increased in inflamed tissues (33).

Acknowledgments—We thank Alfred Bothwell and Chou Hung for helpful comments.

REFERENCES

1. Chen, C. X., Cho, D. S., Wang, Q., Lai, F., Carter, R. C., and Nishikura, K. (1990) EMBO J. 9, 755–767.
2. Kim, U., Wang, Y., Sanford, T., Zeng, Y., and Nishikura, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11457–11461.
3. Maas, S., Gerber, A. P., and Rich, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8895–8900.
4. Melcher, T., Maas, S., Herb, A., Sprengel, R., Seeburg, P. H., and Higuchi, M. (1996) Nature 379, 466–468.
5. De Matteis, S., Maas, S., Herb, A., Sprengel, R., Higuchi, M., and Seeburg, P. H. (1996) J. Biol. Chem. 271, 31795–31798.
6. Bass, B. L., and Weintraub, H. (1998) Cell 93, 1089–1098.
7. Palladino, M. J., Keegan, L. P., O’Connell, M. A., and Reenan, R. A. (2000) RNA 6, 1004–1018.
8. Slavov, D., Clark, M., and Gardiner, K. (2000) Gene (Amst.) 250, 41–51.
9. Maas, S., Kim, Y. G., and Rich, A. (2000) Gene (Amst.) 243, 59–66.
10. Gerber, A., Grossman, H., Melcher, T., and Keller, W. (1998) EMBO J. 17, 4780–4789.
11. Yang, J. H., Sklar, P., Axel, R., and Maniatis, T. (1995) Nature 374, 77–81.
12. Bhardwaj, N. M., Chu, H., Rau, J., Hitzlmann, L. K., Canton, H., Sands-Bush, E., and Emeson, R. B. (1997) Nature 387, 303–308.
13. Poulsen, H., Nilsson, J., Damgaard, C. K., Egebjerg, J., and Kjems, J. (2001) Mol. Cell Biol. 21, 7862–7871.
14. Eckmann, C. R., Neunteufl, A., Pfaffstetter, L., and Jantsch, M. F. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1709–1713.
15. Feldmeyer, D., Kask, K., Brusca, B., Kornhauer, H. C., Holbeck, R., Novak, A., Burnashev, N., Jensen, V., Hvalby, O., Sprengel, R., and Seeburg, P. H. (1999) Nat. Neurosci. 2, 57–64.
16. Higuchi, M., Maas, S., Single, P. N., Hartter, J., Rozov, A., Burnashev, N., Feldmeyer, D., Sprengel, R., and Seeburg, P. H. (2000) Nature 406, 78–81.
17. Hanrahan, C. J., Palladino, M. J., Ganetzky, B., and Reenan, R. A. (2000) Genetics 155, 1149–1156.
18. Ma, E., Gu, X., Xu, X., and Haddad, G. G. (2001) J. Clin. Invest. 107, 685–693.
19. Palladino, M. J., Keegan, L. P., O’Connell, M. A., and Reenan, R. A. (2000) Cell 102, 437–449.
20. Wang, Q., Khulil, J., Gadue, P., and Nishikura, K. (2000) Science 289, 1765–1768.
21. Patterson, J. B., Thomis, D. C., Hans, S. L., and Samuel, C. E. (1995) Virology 210, 508–511.
22. Weiser, H. U., George, C. X., Greulich, K. M., and Samuel, C. E. (1995) Genomics 30, 372–375.
23. Rabinovich, K., Kahal, M., Chen, Y., Zhang, D., Luo, X., and Yang, J. H. (2001) Circ. Res. 88, 1066–1071.
24. Samuel, C. E. (1998) Curr. Top. Microbiol. Immunol. 233, 125–145.
25. Lifshitz, A., and Stuer, U. (2000) Cell 100, 541–554.
26. Neuberger, M. S., and Scott, J. (2000) Science 289, 1705–1706.
27. Muratovska, M., Sankaranandan, V. S., Anant, S., Sugio, M., Kinoshita, K., Davidson, N. O., and Honjo, T. (1998) J. Biol. Chem. 273, 18470–18476.
28. Muratovska, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinoki, Y., and Honjo, T. (2000) Cell 102, 533–536.
29. Rey, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sano, O., Catalan, N., Foreville, M., Dufourcq-Laloue, B., Gennevy, A., Tazian, I., Erazo, P., Kayserili, H., Ugaz, A. G., Brousse, N., Muratovska, M., Notarangelo, L. D., Kinoshita, K., Honjo, T., Fischer, A., and Durandy, A. (2000) Cell 102, 565–575.
30. Martin, A., Bardwell, P. D., Woo, C. J., Fan, M., Shulman, M. J., and Scharff, M. D. (2002) Nature 415, 802–806.
31. Yang, J. H., Loo, X. C., Ye, Y. Z., Yu, J., Zhao, C. G., Kahal, M., Zhang, D. X., and Rabinovici, R. (2000) Immunity 10, 15–22.
32. Hudson, L. D., and Steinberg, K. P. (1999) Chest 116, 74–82.
33. Kaber, K., Gelinas, J. F., Chen, M., Chen, D., Zhang, D., Luo, X., Yang, J. H., Carter, D., and Rabinovici, R. (2002) Shock 17, 300–303.
34. Yang, J. H., Sklar, P., Axel, R., and Maniatis, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4354–4359.
35. Liu, X., George, C. X., Patterson, J. B., and Samuel, C. E. (1997) J. Biol. Chem. 272, 4419–4428.
36. Herbert, A., Alfifien, J., Kim, Y. G., Min, I. S., Nishikura, K., and Rich, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8421–8426.
37. Patterson, J. B., and Samuel, C. E. (1995) Mol. Cell Biol. 15, 5376–5388.
38. George, C. X., and Samuel, C. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4621–4626.
41. Herbert, A., Wagner, S., and Nickerson, J. A. (2002) Mol. Cell 10, 1235–1246
42. Lai, F., Drakas, R., and Nishikura, K. (1995) J. Biol. Chem. 270, 17098–17105
43. Li, Y. P., Busch, R. K., Valdez, B. C., and Busch, H. (1996) Eur. J. Biochem. 237, 153–158
44. George, C. X., and Samuel, C. E. (1999) Gene (Amst.) 229, 203–213
45. Maas, S., Melcher, T., Herb, A., Seeburg, P. H., Keller, W., Krause, S., Higuchi, M., and O’Connell, M. A. (1996) J. Biol. Chem. 271, 12221–12226
46. Liu, Y., Emeson, R. B., and Samuel, C. E. (1999) J. Biol. Chem. 274, 18351–18358
47. Liu, Y., and Samuel, C. E. (1999) J. Biol. Chem. 274, 5070–5077
48. Hurst, S. R., Hough, R. F., Aruscavage, P. J., and Bass, B. L. (1995) RNA 1, 1051–1060
49. Dabiri, G. A., Lai, F., Drakas, R. A., and Nishikura, K. (1996) EMBO J. 15, 34–45
50. Rueter, S. M., Dawson, T. R., and Emeson, R. B. (1999) Nature 399, 75–80
51. Raitskin, O., Cho, D. S., Sperling, J., Nishikura, K., and Sperling, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6571–6576
52. Polson, A. G., and Bass, B. L. (1994) EMBO J. 13, 5701–5711
53. Srivastava, M., and Pollard, H. B. (1999) FASEB J. 13, 1911–1922
54. Schwab, M. S., and Dreyer, C. (1997) Eur. J. Cell Biol. 73, 287–297
Intracellular Localization of Differentially Regulated RNA-specific Adenosine Deaminase Isoforms in Inflammation

Jing-Hua Yang, Yongzhan Nie, Qingchuan Zhao, Yingjun Su, Marc Pypaert, Haili Su and Reuven Rabinovici

J. Biol. Chem. 2003, 278:45833-45842.
doi: 10.1074/jbc.M308612200 originally published online September 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308612200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 26 of which can be accessed free at http://www.jbc.org/content/278/46/45833.full.html#ref-list-1