Chapter

The APOBEC1 Paradigm for Mammalian Cytidine Deaminases That Edit DNA and RNA

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Abstract

Proteins are classified as members of the APOBEC family based on the occurrence of a signature amino acid sequence and its characteristic three-dimensional fold known as a zinc-dependent deaminase domain (ZDD). This domain enables APOBEC proteins to bind nucleic acids and in most cases, deaminate cytidines. The ZDD coordinates a zinc atom necessary for hydrolytic deamination of cytosine or cytidine to form uracil or uridine. The family is named after the founding member Apolipoprotein B mRNA Editing Catalytic Subunit 1 or APOBEC1 that was discovered as the catalytic subunit of a macromolecular complex that carries out a site-specific cytidine to uridine transition at nucleotide position 6666 in *apoB* mRNA. Although eleven additional members of this family have been discovered, APOBEC1 is the only one known to edit RNA. Current data suggest that the function of other members of the APOBEC family is to edit single stranded genomic or viral DNA. However cells may use the intrinsic RNA-binding of APOBEC proteins to suppress coding and noncoding RNAs. Binding RNA has the additional effect of inactivating APOBEC ssDNA editing activity. Within cells these interactions have been observed as the reversible formation of APOBEC homomultimeric complexes and high molecular mass complexes containing numerous other cellular or viral proteins and RNAs. The dynamics in the cell that determine active and inactive APOBEC are key to our understanding of how these enzymes can function without becoming genotoxic. This chapter will focus on factors responsible for *apoB* mRNA editing and their regulation and will draw parallels to systems involving other APOBEC family members. The goal of this chapter is to put into perspective mechanistic themes that continue to provide the foundation for testing new hypotheses. As such this chapter cannot be a comprehensive review and therefore where appropriate, the reader will be directed to other publications for details.

The APOBEC Protein Family

When APOBEC1 was discovered in 1993, there were no obvious homologous sequences listed in the human cDNA database. However the amino acid sequences and structures of prokaryotic cytidine deaminases active on nucleosides/nucleotides were known at that time and these provided a foundation for understanding of the APOBEC proteins[1-5] (Fig. 1). Members of the APOBEC family of metalloenzymes coordinate a zinc atom through three residues (two cysteines and a histidine) that serve as a Lewis acid by positioning a water molecule for hydrolytic deamination

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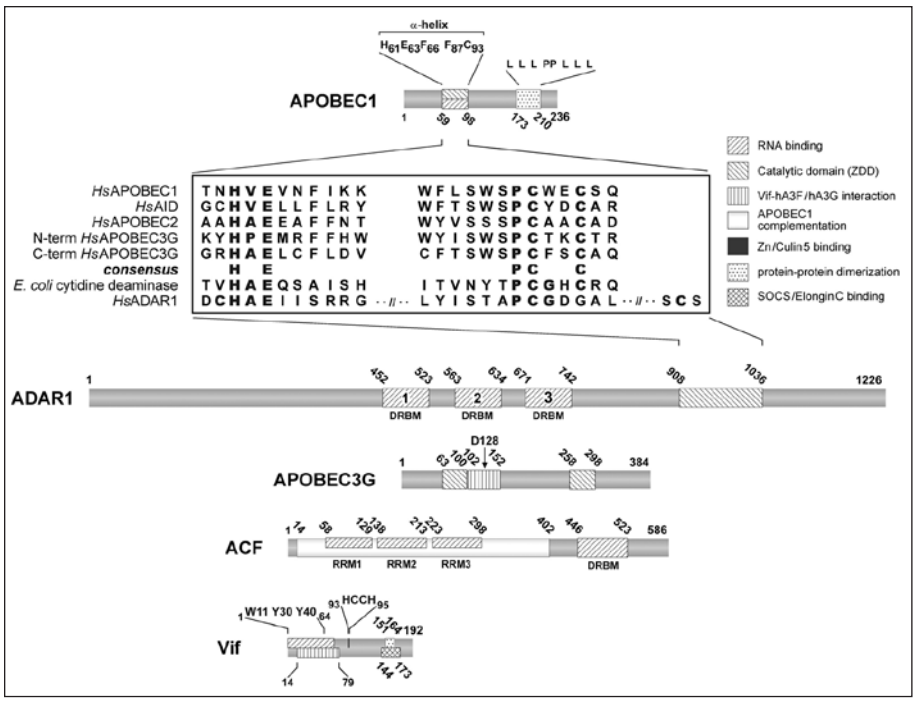

Figure 1. Examples of the functional motifs within editing factors. APOBEC1 functional motifs are represented with an expansion showing essential amino acid residues. The 'consensus' ZDD motif found with APOBEC homologs (see also Chapters 19 and 30), ADAR/ADAT (see also Chapters 19 and 40) and *E. coli* cytidine deaminase are indicated within the central box. In addition, the RNA-binding ZDD in the N-terminal half of APOBEC3G and ssDNA binding and catalytically active ZDD in its C-terminal half are shown. Functional motifs with ACF and Vif are also indicated. Proteins are represented to scale with their respective molecular masses. Functional motifs are color coded and keyed to the right. A color version of this image is available at www.eurekah.com

of cytidine (Fig. 1). The proximity of a conserved glutamic acid residue within the active site ensures that a proton is transferred from the water to the N3 imino group of the pyrimidine ring in the mechanism of hydrolytic cytidine deamination[3,4,6-10] and a conserved proline residue ensures conformational positioning of the reacting moieties within the catalytic pocket[11] (for details see ref. 5 and Chapter 19). This zinc dependent deaminase domain (ZDD) is a defining characteristic of all APOBEC proteins[5,12-14] and of adenosine deaminases active on double stranded RNA (Fig. 1) and tRNAs (referred to as ADAR and ADAT respectively).[15-17]

Phylogenetic modeling suggests that the APOBEC family evolved from a primordial cytidine deaminase active on free nucleosides/nucleotides.[1,3,12,14,18-21] A series of gene mutation events may have given rise to an APOBEC progenitor cytidine deaminase with RNA or ssDNA editing function. Gene duplication, mutation and recombination would have led to the expansion of the APOBEC family to include AID and APOBEC1 on human chromosome 12 and APOBEC2, APOBEC3 and APOBEC4 on human chromosomes 6, 22 and 1 respectively. APOBEC2[22] and APOBEC4[23] are expressed in cardiac/skeletal muscle and testis respectively but have not been ascribed functions. All of the other members of the APOBEC family have been characterized as having functions.

AID deaminase activity on ssDNA within the variable region of immunoglobulin genes results in somatic hypermutation (SHM) that is necessary to produce antibodies with different antigen recognition characteristics[24,25](see Chapter 30). AID expression is also required for immunoglobulin class switch recombination (CSR), a nonhomologous recombination event that is necessary to produce antibodies that will have an appropriate distribution and functionality in the body[24,26] and gene conversion (GC) in which stretches of nucleotide sequences from one of several pseudogene variable regions are recombined to generate immunoglobulin diversity in fowl, rabbits and sheep (reviewed in refs. 24, 27, 28). Evidence for the ancient origin of AID in vertebrate evolution comes from gene sequence comparisons demonstrating immunoglobulin gene SHM emerged in cartilaginous fish.[21,29-31] In contrast, immunoglobulin gene CSR is first evident in amphibians and land vertebrates.[32]

AID was discovered through a search for genes that participate in and regulate CSR and SHM through subtractive hybridization of mRNAs (cDNAs) expressed in B-cell lymphomas with and without induction of CSR.[33] AID–/– knockout mice no longer carried out CSR, were more sensitive to secondary infections but otherwise were healthy. Patients with hyper-IgM syndrome type 2 (HIGM2) that cannot perform CSR were demonstrated to have mutations that linked to the AID gene.[34] HIGM2 patients and AID–/– knock mice were also deficient in SHM. Expression of catalytically active AID was shown to be necessary and sufficient to induce CSR and SHM[35,36] (reviewed in refs. 25, 26). AID expression is also required for GC[37] (see Chapter 19 for structural mapping of AID mutations associated with HIGM2).

AID functions in CSR, GC and SHM as a ssDNA deaminase targeting the transcribed regions of the immunoglobulin locus in B-lymphocytes[38-41] that participate in nonhomologous recombination for CSR and GC and in the variable region of immunoglobulin genes for SHM. The resultant deoxyuridines trigger a repair response involving the removal of uridine bases by uracil DNA glycosylase (UNG)[42,43] and strand break repair of the resultant apyrimidinic sites.[44-46] Although ssDNA deaminase activity of AID is essential for both CSR and SHM, targeting of AID to these specific genomic regions is independently regulated through chaperones and trafficking into the nucleus.[47,48]

APOBEC3 proteins are only expressed in mammals and are largely viewed as having host-defense functions that provide a post-entry block to viral replication (for those viruses with an extracellular phase) and regulate mobile DNA transposable and retrotransposable elements within the genome (reviewed in refs. 49, 50). Mice have a single APOBEC3 gene that encodes a protein with two ZDD[12,14,51] however an expansion of the APOBEC3 gene during evolution into a tandem array of APOBEC3A, 3B, 3C, 3D/E, 3F, 3G and 3H containing either one or two ZDD (Fig. 2) is suggested by the progressive increase in number of APOBEC3 genes from cloven-hoof mammals[52] to nonhuman primates and humans.[20,53,54] The emergence of the APOBEC3 gene cluster may have undergone adaptive evolution in response to the rapid evolution of endogenous retroelements and retroviruses.[12,20,55-60] The genetic variation within the human APOBEC3 gene cluster is extremely high.[20,53,54,61] Perhaps the most overt variation is in the APOBEC3B gene where deletions within this gene are becoming fixed in oceanic human populations.[62]

The function of APOBEC3G as an anti-viral host factor was demonstrated in 2002 by Michael Malim's laboratory through cDNA transfer experiments designed to identify a host cell suppressor of the viral accessory protein known as the virion infectivity factor or Vif.[63] Viruses deficient in Vif have low infectivity if they are produced in cells known as 'nonpermissive', but otherwise exhibit near wild type infectivity levels when produced in cells known as 'permissive'. Several studies have shown ≥1000-fold reduced infectivity of virions produced by Vif-deficient virus compared to wild type virus in nonpermissive cells. The inhibition is due to a defect at the post-entry step of infection arising from reduced reverse transcript production and/or stability.[64,65] Heterokaryons comprising nonpermissive and permissive cells retained the nonpermissive phenotype, demonstrating expression of a dominant inhibitory factor in nonpermissive cells that could be neutralized by Vif.[66,67] Transfection of permissive cells with APOBEC3G cDNA proved necessary and sufficient for conversion to the nonpermissive phenotype when challenged with Vif deficient virus.

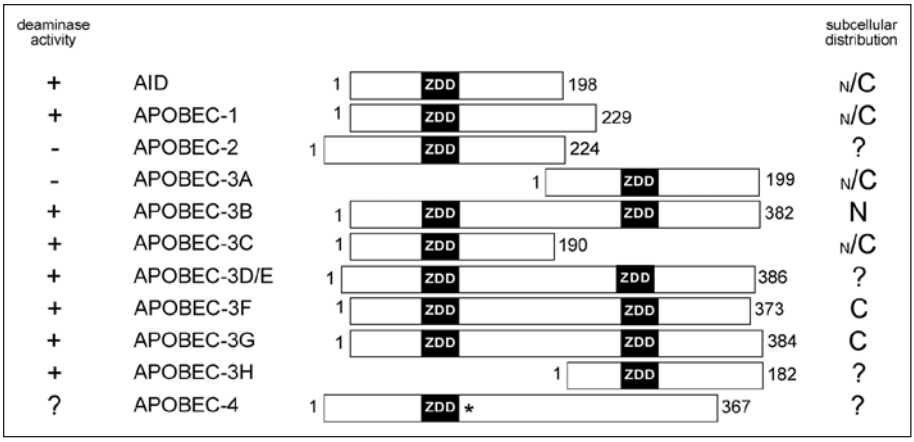

Figure 2. Summary of activity and subcellular localization. APOBEC family members are shown with their ZDD homologies aligned and to scale with their relative primary sequence length. Whether or not each APOBEC has been characterized as having deaminase activity is indicated (+ or –) to the left and subcellular distribution (C, cytoplasmic; N, nuclear) is listed to the right. For proteins with a bipartite distribution, N/C′ indicates the predominant cytoplasmic localization. The * next to the ZDD in APOBEC4 indicates that this sequence is divergent from the consensus.

APOBEC3 proteins deaminate deoxycytidine (dC) to form deoxyuridine (dU) within ssDNA regions of lentiviral proviral DNA that arise during its replication.[68-73] The dC-to-dU transitions lead to deoxyguanosine (dG) to deoxyadenosine (dA) mutations during positive strand HIV replication and these changes occur with a frequency similar to that observed in HIV DNA isolated from T-cells of HIV positive patients.[74-76] APOBEC3G deaminase activity may not depend on additional[68,77,78] host or viral factors as evident by the finding that most APOBEC3 proteins expressed in bacteria readily deaminate ssDNA in actively transcribed genes[79,80] although there is evidence for a cellular cofactor that facilitates the anti-viral activities of APOBEC3F and 3G.[81]

To identify the antiviral deaminase domain of APOBEC3G, point mutagenesis and deletion mutagenesis were conducted on the N- and C-terminal ZDD motifs. Several groups ascribed the C-terminal ZDD motif as the source of antiviral deaminase activity, whereas the N-terminal ZDD motif was deemed necessary for RNA binding, interaction with HIV Gag protein and packaging of APOBEC3G into budding virons[51,82-87] (reviewed in ref. 49). Other groups found that mutation in either ZDD motif abolished deaminase activity but did not ablate APOBEC3G antiviral activity.[85,88,89] This effect has been attributed to an APOBEC3G-dependent physical block to reverse transcription.[90-92] The data remain controversial as the antiviral effect of the catalytic mutant may be due to the experimental system in which APOBEC3G is expressed well beyond physiological levels.[93] A similar controversy exists concerning the mechanism by which APOBEC3G inhibits hepatitis B virus.[94-97] For more complete discussion of this topic the reader is directed to a recent review.[49]

Long terminal repeat (LTR) containing retrotransposons are inhibited by APOBEC3B, C, F and G through both a reduction of the number of copies of reverse transcribed cDNAs as well as hypermutation.[98] Non-LTR retrotransposons (LINE and the L1-dependent SINE, principally Alu elements) are differentially inhibited by APOBEC3 members. There are several mechanism whereby APOBEC3 proteins inhibit these retroelements that include nuclear APOBEC3A, B and C blocking LINE reverse transcription and integration within the genome and APOBEC3B, F and G sequestering essential LINE encoded proteins, L1 RNA[99] and Alu RNA[100] in the cytoplasm[101] (see discussion on following page).

Apolipoprotein B mRNA Editing Opens a New Field

Apolipoprotein B is an integral structural protein of lipopoprotein particles that is required for the assembly of lipids into very low-density lipoproteins (VLDL) in the liver and chylomicrons in the small intestine.[102] This process is essential for mammalian life.[103] ApoB predominantly exists as two variants, a full-length protein (ApoB100) and a truncated protein consisting of the N-terminal 48% of ApoB (ApoB48). Hepatic secretion of lipoproteins into the blood stream and their uptake by tissues is differentially regulated through these ApoB variants. An elevated level of ApoB100 lipoproteins in circulation is positively correlated with a higher risk of developing atherosclerosis as seen in a number of diseases such as Type II diabetes and a variety of hyperlipidemias and obesity.[104-107]

ApoB mRNA editing was discovered simultaneously by the laboratories of Lawrence Chan and James Scott in an effort to determine the molecular mechanism regulating the expression of ApoB 100 and ApoB48.[108,109] Editing occurs at nucleotide position 6666 in *apoB* mRNA through a posttranscriptional cytidine to uridine transition and converts a CAA glutamine codon (that enables ApoB100 to be expressed) to UAA translation stop codon (resulting in the expression of ApoB48). The cells that line the small intestine (enterocytes) of all mammalian species edit ~100% of the *apoB* mRNA that they transcribe.[110] A significant portion (40% to 70%) of *apoB* mRNA expressed in the liver of rodents is edited but this is not true in other species.[111] *ApoB* mRNA is not edited in human and nonhuman primate liver (because the catalytic subunit APOBEC1 is not expressed in this tissue[112]) and this results in a heightened risk of cardiovascular disease in persons consuming a western diet consisting of high fat and high fructose sweeteners.[113,114]

The discovery of APOBEC1 as the enzyme responsible for *apoB* mRNA editing was a significant breakthrough in the field[115] and together with the availability of the human genome sequence, proved to be important in the discovery of the APOBEC protein family[1,12,14] (Fig. 1). Functional characterization of APOBEC1 and in fact its discovery was expedited by pre-existing enabling technologies.[116] Specifically, progress in the field was enabled through the methods for in vitro RNA editing on short recombinant *apoB* RNA reporters in cell or tissue extracts and a rapid quantitative assay for editing activity (known as 'poisoned' primer extension[117]).

APOBEC1 was identified by size fractionating polyA+ mRNA from rat small intestine and microinjecting these RNAs into Xenopus oocytes[115] for expression. Oocyte extracts were screened for in vitro editing in an assay containing an *apoB* mRNA reporter and cell extracts from chicken small intestine (that can support editing activity on human *apoB* RNA in vitro but do not naturally edit chicken *apoB* mRNA in vivo[118]). A cDNA encoding a 229 amino acid open reading frame for APOBEC1 was cloned and shown to induce *apoB* mRNA editing in transfected human liver cells. APOBEC1 was proven to be the sole cytidine deaminase responsible for *apoB* mRNA editing using APOBEC1−/− knockout mice. These mice no longer edited intestinal or liver *apoB* mRNA and produced chylomicrons and VLDL using only ApoB100.[119,120] APOBEC1 gene delivery induced *apoB* mRNA editing activity.[121-125]

Identification of the Minimal Components of Editosome Assembly

The nucleotides flanking cytidine 6666 that are required for editing site recognition had been identified prior to the discovery of APOBEC1.[126-129] The entire editing site consists of tripartite motif: a 5′ enhancer sequence (improves the efficiency of editing site recognition), a four nucleotide spacer 3′ of the editing site and an eleven nucleotide mooring sequence (reviewed in refs. 19, 116). The mooring sequence serves as the principal cis-acting element for editing site recognition. Translocation of the mooring sequence to other RNAs is typically sufficient to direct editing to 5′ cytidines[130,131] provided that the flanking RNA sequences are A-T rich and the cells or cell extracts can support editing activity.

A tripartite motif also supports editing at an additional site within *apoB* mRNA 3′ of cytidine 6666 (nt 6802) whose editing has no functional consequence because these mRNAs are typically edited at nt 6666 as well. The mRNA encoding the NF1 tumor suppressor (a G-protein regulator of Ras signaling), also contains a tripartite motif whose editing may contribute to the dysregulation

of Ras signaling seen in neurofibromas, gliomas and schwannomas.[132,133] While computational methods have identified other mRNAs with mooring sequences in the annotated human, mouse and rat cDNA databases,[19] none of these candidate editing sites supported editing activity when added to editing competent extracts. Although editing of these transcripts in yet-to-be identified cell types or tissues cannot be ruled out, additional constraints in vivo may limit editing. For example, the close proximity of the tripartite motif to premRNA splicing sites (a characteristic of most of the candidate editing sites) can dramatically reduce editing site utilization in the context of reporter RNAs.[134-136]

APOBEC1 does not selectively bind to the mooring sequence. APOBEC1 can bind AU-rich RNA nonspecifically and with low affinity[137] through key residues within its ZDD (Fig. 1). Purified recombinant APOBEC1 alone cannot edit RNA unless the in vitro reaction is incubated at 45˚C.[138] ssDNA editing activity of most members of the family, including APOBEC1, will take place at 30˚C to 37˚C s when purified recombinant proteins are added to ssDNA substrates that are partially or completely single stranded.[71,72,139-142] APOBEC1 requirement for elevated temperatures to edit RNA stems from a requirement for a single stranded RNA substrate that is ensured by heat denaturation of the AT-rich RNA sequence surrounding the *apoB* editing site.[143] In this regard, the next major advance in the field was the discovery of an RNA binding protein that could recruit APOBEC to the mooring sequence and facilitate site-specific editing.

A role for RNA binding proteins in editing activity was first suggested by glycerol gradient sedimentation studies. Reporter RNAs containing the mooring sequence assembled as 11S complexes that progressed to 27S complexes with longer incubations. Both complexes contained RNA binding proteins that selectively bound to the mooring sequence.[48,144] The 27S complexes were proposed to be C to U editosomes because: (1) they did not form on RNAs lacking the mooring sequence,[145] (2) their assembly only occurred in cell or tissue extracts that supported *apoB* mRNA editing,[144] (3) in vitro editing activity commenced following their assembly[145] and (4) edited RNA and editing factors were recovered from these complexes.[145,146]

Donna Driscoll's laboratory was first to identify and clone the mooring sequence RNA binding protein responsible for site-specific editing. They used a combination of *apoB* RNA affinity chromatography of baboon kidney extracts and peptide sequencing to obtain a human EST clone to screen a human cDNA library.[146] The newly identified clone encoded a ~64 kDa protein (dubbed as APOBEC1 Complementation Factor (ACF)) that proved to be necessary and sufficient to complement APOBEC1 in site-specific *apoB* mRNA editing. Immunodepletion of ACF from extracts resulted in a marked inhibition of in vitro editing activity. These studies brought closure to the controversy over whether *apoB* mRNA editing involved more than one protein by showing that ACF interacted with APOBEC1 to form the 'minimal editosome' and gave credence to the proposed role of RNA binding proteins in the editosome assembly process.[144,145,147,148]

A number of alternatively spliced variants of the ACF were subsequently identified by several labs through biochemical and bioinformatics analyses.[149-152] An alternatively spliced variant of ACF[153] known as APOBEC1 Stimulatory Protein, ASP[151] was discovered in the same time frame as ACF. Although expression of ASP in rat liver is >10-fold lower than ACF,[153] on a per mass basis, ASP is as good as ACF in complementing APOBEC1 editing activity.[151,153] Although alternatively spliced ACF variants identified subsequently[19,152] contained the same three RNA Recognition Motifs (RRM) in tandem followed by Nuclear Localization Signal (NLS) found in ACF and ASP (Fig. 1 and reviewed in refs. 19, 116, 154), they did not have the same ability to bind to APOBEC1 or the mooring sequence nor do they complement editing with the same efficiency.[19,152] In addition, these ACF variants were expressed at different levels in various tissues. The mechanism ACF variants serve in editosome assembly and function remains to be determined.[144,148,155]

Historically, the process of searching for a factor that could complement APOBEC1, lead to the discovery of several RNA-binding proteins (some containing three RRMs) that had the ability to bind APOBEC1, *apoB* mRNA and/or ACF (156-158 and reviewed in ref. 19). In contrast to ACF, introduction of these RNA-binding proteins into cells through transfection or addition of

recombinant proteins to in vitro editing assays inhibited editing activity. It has been proposed that the function of these 'candidate' auxiliary proteins may be to suppress the activity of the C to U editosome by interacting with ACF and/or APOBEC1.[156,157] In fact, complexes containing ACF and APOBEC1 that do not supporting editing in situ have been isolated from the cytoplasm of cells[144,159] (see further discussion below) and immunoprecipitation analysis suggested that ACF and APOBEC are not directly associated with each other in these complexes.[160]

The ability of ACF to selectively bind to the mooring sequence and position APOBEC1 for site-specific editing has focused attention on ACF as an RNA editing factor. However, ACF is likely to have other functions because it is an essential gene product that is required at or before the time of blastocyte implantation.[149] This is in contrast to APOBEC1 which is not an essential gene product[119,120] as well as ApoB that becomes a requirement at the time of yolk sack development and thereafter.[103] It is not known whether ACF binds to other APOBEC family members however these proteins are either not essential (e.g., APOBEC2 and APOBEC3[161]) or only required later in life for a fully functional immune system (e.g., AID[34,162]). Structural analyses of ACF and its interactions with the mooring sequence and APOBEC1 will hopefully be forthcoming and provide insight for future studies of ACF function(s) during cell growth and tissue development.

In contrast to the sequence requirements for APOBEC1 editing of RNA, ssDNA editing activity by APOBEC family members is lax. With rare exception (APOBEC2,[161] APOBEC4[23]) all members of the APOBEC family will bind to and edit several genomic sequences when transformed into *E. coli*.[77,79,80,163] The cis-acting sequence requirement for ssDNA editing is not well characterized but there are 5′ nearest neighbor preferences. These are for example: GTC for APOBEC1;[79] (A/T)(A/G)C for AID,[71,79,163,164]; TTC for APOBEC3F and GCC for APOBEC3G,[68,70,73,77-79,165-167] (where the edited C is underlined). AID prefers to edit ssDNA within unpaired regions (bubble) of otherwise duplex DNA[71] such as is predicted to be present in transcribed regions of the genome. APOBEC3 proteins may have similar preferences but in general, bind and edit ssDNA as it becomes exposed during reverse transcription of the viral RNA genomes.[72,73,168] Once bound to a ssDNA substrate, both AID and APOBEC3G have been shown to be processive enzymes with 3′ to 5′ polarity of their catalytic activities.[72,164,168]

Subcellular Distribution of Editing Factors Determines Their Access to Substrates

RNA sequence analysis by Lawrence Chan's laboratory demonstrated that *apoB* mRNA editing activity occurred on nuclear RNA. Editing took place subsequent to polyadenylation and coincident with or immediately after premRNA splicing.[169] Even though APOBEC1 and ACF are distributed throughout the cell, 27S editosomes are only recovered from nuclear extracts.[159] Metabolic activation of *apoB* mRNA editing does not require de novo protein synthesis[170] but rather can be accomplished through nuclear import of pre-existing cytoplasmic ACF and APOBEC1.[160] In addition, access to nuclear premRNA within the time frame of transcription, processing and nuclear export requires precise timing. Localization of sufficient editing factors to ensure efficient editosome assembly must therefore involve regulation at the temporal and spatial level as proposed in the 'gating hypothesis'.[134] Taken together these findings underscore the importance of intracellular trafficking of editing factors in the regulation of editing activity.

APOBEC1 contains signals for both nuclear localization (NLS) and cytoplasmic retention (CRS).[171] The CRS of APOBEC1 is a dominant determinant that must be masked or inhibited before APOBEC1 can enter the nucleus. Although it has not been completely resolved, the NLS within ACF may determine trafficking of both proteins to the nucleus.[172,173] Metabolic regulation of hepatic ACF and APOBEC1 (e.g., through ethanol or insulin signaling pathways) promotes nuclear retention of these proteins through phosphorylation of key serine residues in ACF by protein kinase C.[174] Hyperphosphorylated ACF is retained in the nucleus but ACF nuclear import and ACF binding to APOBEC1 do not require phosphorylation. Biochemical studies have shown that the interaction of hyperphosphorylated ACF with APOBEC1 is improved and is more efficient in complementing editing activity. Consistent with this is the finding that in vitro editing

activity in hepatocyte nuclear extracts was reduced by treating them with phosphatase.[160] In this regard, reduction of serum insulin concentration in fasting animals or the removal of insulin from primary hepatocyte cultures resulted in dephosphorylation of ACF, accumulation of ACF in the cytoplasm and a reduction of *apoB* mRNA editing activity in situ.[155,175,176]

Regulation of activity through protein trafficking is also seen for AID.[46,177,178] In this instance, CSR in activated B-cells is dependent on an evolutionarily conserved, nuclear export signal (NES) within the C-terminus of AID.[29,46,177,178] In addition to regulating AID trafficking to the nucleus, interactions through the NES are proposed to target AID editing activity to select ssDNA sequences within the genome and thereby induce nonhomologous recombination for CSR and GC. Protein kinase A phosphorylation of serine within the N-terminus of AID enhances binding to replication protein A (RPA) and promotes both CSR and SHM.[41,179-181] Although recombinant AID can bind to and deaminate ssDNA in vitro,[141,182-184] RPA is likely to serve in vivo as a molecular chaperone for trafficking of AID and its targeting of appropriate ssDNA within chromatin.[40]

Protein phosphatase I-dependent dephosphorylation of ACF results in ACF nuclear export and reduced binding to APOBEC1.[174] Given that phosphorylated and dephosphorylated ACF appear to bind equally well to *apoB* mRNA,[185,186] it has been proposed that ACF remains bound to *apoB* mRNA during nuclear export to the cytoplasm.[19,174] ACF phosphorylation (and nuclear retention) therefore may regulate not only editing activity but also the amount of *apoB* mRNA transported to the cytoplasm and available for translation. Evidence suggesting that ACF is bound to *apoB* mRNA during translation was first presented by Edward Fisher's lab who showed that *apoB* mRNA translation complexes (polysomes) were atypically buoyant in sedimentation gradients and that this characteristic was mooring sequence dependent.[187] ACF had not been discovered at that time but by inference, the data suggest that the buoyancy of these polysomes was due to a 'parachute effect' from high molecular mass complexes containing ACF bound to the mooring sequence. The next line of evidence came from immunoelectron microscopy of rat liver thin sections demonstrating that ACF is concentrated along the exterior surface of the endoplasmic reticulum[159] (the site of *apoB* mRNA translation). Finally, edited *apoB* mRNA is stabilized in the cytoplasm even though the presence of the premature UAA stop codon would otherwise subject the mRNA to rapid degradation by the nonsense codon mediated decay (NMD) mechanism.[173] The block to NMD on edited *apoB* mRNA is dependent on the mooring sequence at the editing site and the expression of ACF. Active stabilization of edited *apoB* mRNA relative to unedited *apoB* mRNA may be a contributing factor to a long standing observation that in species with hepatic *apoB* mRNA editing, VLDL containing ApoB48 are produced and secreted in greater abundance than those that assembled on ApoB100.[188]

Stringent Control of APOBEC Proteins

APOBEC1 fidelity for editing sites is coupled to the level of its expression. Constitutive high levels of APOBEC1 ectopic expression in cell lines[136,189,190] or transgenic animals[191-193] leads to aberrant site editing and neoplastic transformation. High levels of site-specific editing such as that observed in the small intestine in vivo are thought to be due to the interaction of APOBEC1 with ACF and their constitutive activation.[148] However APOBEC1 abundance in liver and intestine is extremely low (not readily detectable by western blotting) whereas ACF is a moderately abundant protein (estimated to be 100- to 500-fold less abundant than β actin in rat liver based on 2D PAGE, Smith unpublished data). Moreover, the bulk of both proteins are sequestered in the cytoplasm as complexes that are not active in editing (see discussion below). The underlying basis for neoplastic transformation may have been due to excessive amounts of APOBEC1 that aberrantly edited mRNA(s) that otherwise were not substrates leading to the expression of a dysfunctional proteome.[192]

Protein overexpression leading to a cancer phenotype has also been observed with other APOBEC members such as AID[182,194-198] and members of the APOBEC3 subgroup.[79,199] In these situations genotoxicity due to ssDNA editing has been proposed as the underlying transforming

mechanism. It was in fact in the course of studies on AID and APOBEC3 that APOBEC1 was shown to be a very effective ssDNA editing cytidine deaminase.[77,79,80,116] This finding suggested an alternative hypothesis that excessive expression of APOBEC1 can become genotoxic when its abundance exceeds a threshold that cellular factors can regulate. In this hypothesis, APOBEC1 is free to diffuse to the nucleus and once there, binds to and mutates ssDNA within actively transcribed regions of the genome.

Regulation of protein expression and restricted access to the cell nucleus is in fact a characteristic found for many APOBEC family members (Fig. 2). Although the abundance of AID can become higher than that of APOBEC1 (AID is readily detected by western blotting and immunocytochemical staining of B-cells (http://www.lsbio.com/Products/GeneDetail.aspx?LSID = 170008), it is acutely expressed during B-cell activation[40,200,201] and rapidly eliminated by ubiquitination-dependent degradation.[202] AID deaminase activity on ssDNA can be inactivated through its interaction with RNA.[71] By analogy to other family members, it is likely that the ZDD of AID binds to RNA and this inhibits or displaces ssDNA from the active site.[203] AID also can be regulated by restricting its access to the cell nucleus[46,177] through interactions with auxiliary proteins[181,204] and phosphorylation.[179,180]

APOBEC3G and APOBEC3F are more abundant than APOBEC1 and AID. APOBEC3G is estimated to be 200- to 700-fold less abundant than β actin in human peripheral blood mononuclear cells and APOBEC3F is estimated to be 5- to 10-fold less abundant that APOBEC3G (Leonard and Smith, unpublished ELISA data). APOBEC3G is restricted to the cytoplasm by its own CRS located immediately C-terminal to the N-terminal ZDD[205,206] The CRS is likely to restrict APOBEC3G to the cytoplasm through protein-protein interactions although APOBEC3G interactions with several cytoplasmic RNAs through its N-terminal ZDD[86,207-216] also would contribute to cytoplasmic retention. APOBEC3G is expressed at different basal levels in the various white blood cell types.[210,217-220] APOBEC3G expression can be transcriptionally activated by various mitogens and cytokines[210,218,221] however this does not necessarily lead to increased abundance of catalytically active enzyme. APOBEC3G ssDNA deaminase activity and function as a host defense factor can be suppressed through the formation of high molecular mass (HMM) ribonucleoprotein complexes with a variety of cytoplasmic RNAs.[208,218,219] Cells that are most resistant to HIV infection maintain cytoplasmic APOBEC3G in low molecular mass (LMM) complexes that have little or no bound RNA (reviewed in ref. 49).

Regulation of APOBEC3 abundance is also important for viral infectivity. Upon HIV infection APOBEC3G (and APOBEC3F) is rapidly polyubiquitinated and degraded through the proteosomal protein degradation pathway (reviewed in ref. 49). It is not certain whether ubiquitination-dependent degradation of APOBEC3G/3F is a normal cellular mechanism for turnover, however polyubiquitination of the HIV Vif is required for rapid degradation of APOBEC3G.[63,222-227] There are several residues within the N-terminus of Vif that are essential for binding to APOBEC3G and/or APOBEC3F[228-232] and the C-terminus contains residues that bind to Cullin 5 and Elongin C of the cellular ubiquitination machinery[230,233-237] (Fig. 1). APOBEC3G interacts with human Vif through key residues within its N-terminal half, one of which (D128) determines species-specific Vif-APOBEC3G interactions[69,231,238-243] (Fig. 1). Through these interactions Vif chaperones APOBEC3F and 3G to the proteosome for degradation, thereby eliminating these proteins and in the process is itself degraded[222] (reviewed in ref. 49).

In the absence of a Vif viral defense mechanism, newly synthesized APOBEC3 proteins[219] assemble with HIV virions through interactions with HIV RNA genomes, viral Gag protein and cellular RNAs.[86,92,208,214,239,244-248] Following infection, APOBEC3F/3G in the viral core interferes with viral replication and hypermutates nascent proviral ssDNA (reviewed in ref. 49). This is possible because Vif is not expressed until late stages of infection and therefore cannot block APOBEC3 coming in with virions. This is why HIV virions that do not contain APOBEC3F/G can still be arrested if APOBEC3F/3G is maintained in cells as LMM complexes (such as is the case in resting T-lymphocytes) but are fully infectious in cells when APOBEC3F/3G is inactivated in HMM complexes (as is the case in activated T-lymphocytes).[210,219]

Regulation Through Macromolecular Complex Formation

The current hypothesis is that a dimer of APOBEC1[4,116,249,250] binds to ACF as the minimal in vitro C to U editosome (118 kDa) and this complex binds to the mooring sequence for site specific editing.[116] The composition of C to U editosomes in situ remains an open question and there is evidence from yeast two hybrid analysis that ACF can homodimerize.[116] Glycerol gradient sedimentation of functional C to U editosomes isolated from rat liver nuclear extracts[155,160] or assembled on an *apoB* RNA reporter (490 nt long) in vitro[116,144,145] suggested these complexes were 27S (>500 kDa). The kinetics of in vitro C to U editosome assembly suggested that protein complexes with *apoB* reporter RNA proceeded through an 11S intermediate complex (~250 kDa).[116,144,145] Atomic force microscopy of affinity purified catalytically active C to U editosomes assembled in vitro in McArdle hepatoma cell extracts with recombinant 6His tagged APOBEC1[251] suggested complexes equivalent to 650 kDa, consistent with glycerol gradient sedimentation studies (http://dbb.urmc.rochester.edu/labs/smith/photo_gallery.htm). Taken together the data suggested that the C to U editosome in cells has a higher-order state that is more complex than the minimally functional editosome.

Atomic force microscopy,[168] size exclusion chromatography[139,217,218] and small angle X-ray scattering[139] also have suggested higher order complexes of APOBEC3G as homo dimers, tetramers and hexamers. The oligomeric state of APOBEC3G has been suggested to be essential for 3′ to 5′ processivity of deaminase activity along ssDNA and the orientation of the APOBEC3G catalytic domain relative to the cytidines in the ssDNA.[168] However the catalytic domain of APOBEC3G can be expressed as a soluble, monomeric C-terminal fragment following selective mutagenesis and this construct retained catalytic activity despite being unable to dimerize.[252] NMR analysis showed that the fragment largely conformed to the structure of known cytidine deaminases (see Chapter 19) and chemical shifts indicated select residues in the catalytic pocket that interacted with ssDNA oligonucleotides.[253] These findings have fueled a controversy over whether monomers or multimers of APOBEC3G are catalytically active despite the knowledge that all known cytosine/cytidine deaminase function as homo or heteromultimers (see Chapter 19).

The higher order organization of AID is also controversial. Co-immunoprecipitation of mutant and wild type AID coupled with activity analyses suggested that AID dimers form through its N-terminal 60 amino acids and that dimerization is required for activity.[254] The crystal structure of an N-terminal truncated form of APOBEC2 (which is the approximate size of AID) has been determined as an elongated N-terminal dimer.[255] Modeling of AID upon this structure suggested a good fit with an N-terminal dimeric interface. Conflicting with these conclusions are data from atomic force microscopy coupled with functional analyses suggesting that AID is active as a monomer.[256]

Although the controversy has centered on whether APOBEC proteins can be active as monomers or must form homomultimers for activity, it is important to not lose track of the consistent finding that APOBEC family members reside in higher-order complexes within cells and that their association with cellular proteins (such as ACF for APOBEC1) are likely to have important regulatory roles in the cell.[87,101,139,168,181,209,255] Among the largest of these complexes mentioned earlier in this chapter are the HMM ribonucleoprotein particles (RNP) containing APOBEC3F and 3G that range from 5 to 15 megadaltons. These complexes are held together through RNA-bridged interactions with proteins associated with cytoplasmic stress granules and RNA-processing bodies (p-bodies).[208,209,211] Not only are these complexes instrumental in dynamically regulating active and inactive APOBEC host-defense factors (described above), but their assembly with various retroviral/retroelement RNA, micro RNAs[207] and cellular RNAs[50,87,208,209,211] also are proving to be important in regulating translation and other RNA functions in the cell (reviewed in refs. 49, 257). The composition of macromolecular complexes regulating the function of other APOBEC family members is likely to be an important focus of future research in this field.

Conclusions and Prospects

Research on apolipoprotein B (*apoB*) mRNA editing over the past twenty years has led to the discovery of APOBEC1, its complementing factors and the physiological and cellular dynamics that regulate editosomal complexes. Although these discoveries occurred in the context of research on cardiovascular disease, the identification of the APOBEC family comprising twelve structural homologs within the past ten years has led to new discoveries demonstrating the diverse functions these proteins have and their broad impact on human health and disease (Fig. 3). Examples of systems affected by APOBEC proteins include: the control of retroelements, DNA recombination, cell signaling, genome mutation, intracellular trafficking of proteins, cytoplasmic ribonucleoprotein function, lipoprotein metabolism, neoplastic transformation, proteome diversification, proteosomal function, regulation of siRNA in the control of translation, RNA turnover and viral infectivity.

The field needs to continue to progress in the area of structural analysis of APOBEC proteins and their interactions with nucleic acids and other cellular or viral proteins. High-resolution structures of APOBEC proteins in complex with RNA and ssDNA will further our understanding of not only the catalytic mechanism but also address the key issue of regulation such as substrate specificity and processivity. Knowledge of the amino acid residues necessary for nucleic acid binding and deaminase activity will also facilitate experiments to determine why RNA binding to the deaminase domain of AID, for example, or to the N-terminal noncatalytic ZDD of enzymes such as APOBEC3G inhibits ssDNA deaminase activity. High resolution structure-function analyses of interacting proteins such as ACF, RPA and Vif will be important for understanding how these

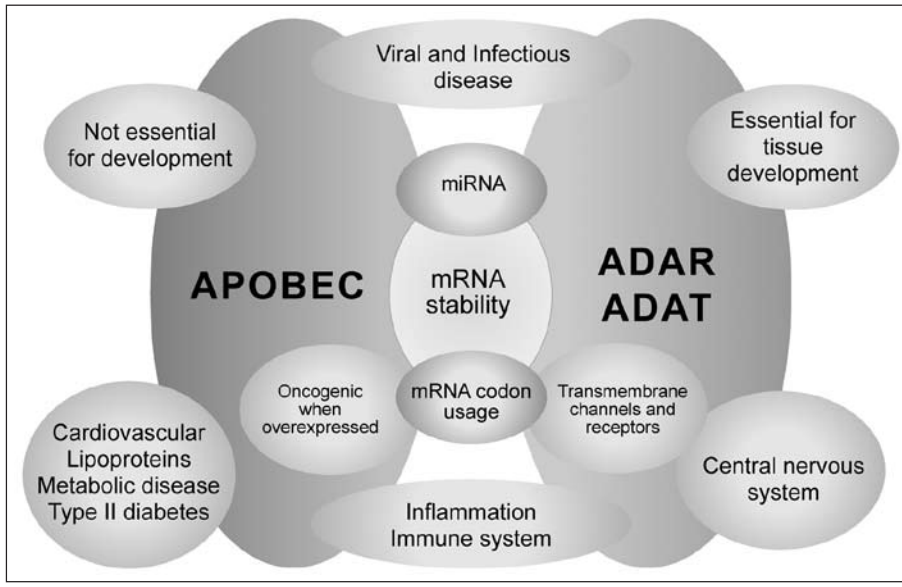

Figure 3. Biological systems impacted by the function of editing enzymes. The APOBEC family of C to U editing enzymes (12 proteins) are structurally related to the ADAR family of A to I editing enzymes (3 proteins) active on dsRNA and the ADAT family of A to I editing enzymes (3 proteins) active on tRNA. Research over the past 20 years has revealed that the expression of these enzymes is essential for the function, or in some cases dysfunction, of a broad array of mammalian physiology (discussed throughout this chapter). Shown in Venn diagram format are the APOBEC and ADAR/ADAT families of enzymes. Members in each family play critical roles in various physiological systems or disease states as represent through overlapping spheres and ovals (the size of which are arbitrary). For more information see Chapters 19, 30 and 40.

proteins regulate APOBEC and target binding to RNA or DNA. The open question of whether APOBEC proteins are functional in biological systems as subunits or multimers must be addressed through structure-guided functional assays (see Chapter 19).

Future experiments also need to focus on understanding regulation of APOBEC proteins in the cell. Cell signal transduction, cell cycle progression, the differentiated phenotype of cells, embryogenesis, neoplastic transformation and viral life cycle have now all have been linked to the expression of APOBEC proteins and the macromolecular interactions that regulate deaminase activity. We currently do not understand the molecular basis for these linkages. Future studies need to address transcriptional and translational regulation of APOBEC protein expression and determine how posttranslational modifications regulate APOBEC protein abundance, activity and intracellular trafficking.

The unifying theme in the APOBEC family of activity regulation through the formation of higher order complexes tells us that there are dynamic protein-protein and protein-RNA interactions that cells use in the acute and long-term control of APOBEC functions. These areas of research are likely to become the major focus for the next two decades as they address the central question of the mechanisms that cells and viruses use to manage the activities of potentially genotoxic proteins.

A major translation research problem that lies before this field is whether we can use the knowledge of APOBEC protein structure, function and cell/viral regulation to understand human health and disease. Beyond this, the next generation of research will have new gene delivery systems and stems cells that will enable biotechnology and the development of therapeutics that targeting APOBEC proteins to improved healthcare.

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