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The expression of *apoB* mRNA editing factors is not the sole determinant for the induction of editing in differentiating Caco-2 cells

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ABSTRACT

Apolipoprotein B mRNA is edited at cytidine 6666 in the enterocytes lining the small intestine of all mammals; converting a CAA codon to a UAA stop codon. The conversion is ~80% efficient in this tissue and leads to the expression of the truncated protein, ApoB48, essential for secretion of dietary lipid as chylomicrons. Caco-2 cell raft cultures have been used as an *in vitro* model for the induction of editing activity during human small intestinal cell differentiation. This induction of *apoB* mRNA editing has been ascribed to the expression of APOBEC-1. In agreement our data demonstrated differentiation-dependent induction of expression of the editing enzyme APOBEC-1 and in addition we show alternative splicing of the essential auxiliary factor ACF. However, transfection of these editing factors in undifferentiated proliferating Caco-2 cells was not sufficient to induce robust *apoB* mRNA editing activity. Only differentiation of Caco-2 cells could induce more physiological like levels of *apoB* mRNA editing. The data suggested that additional regulatory mechanism(s) were induced by differentiation that controlled the functional activity of editing factors.

Introduction

Apolipoprotein B (ApoB) is a non-exchangeable structural component of intestinally derived chylomicrons and of liver derived very low density lipoprotein particles (VLDL). The truncated variant of ApoB lipoprotein (ApoB48) is expressed via post-transcriptional RNA editing [1,2] which involves a site-specific deamination of cytidine (C6666) to form uridine [3], thereby creating an in-frame translation stop codon, UAA, from a glutamine codon, CAA [1,2]. The mammalian small intestine constitutively edits ⩾85% of *apoB* mRNA and stores ApoB48 for the assembly and secretion of chylomicrons containing dietary lipids [1–5].

Site-specific C to U *apoB* mRNA editing requires a macromolecular complex assembly of proteins (editosome) that minimally contains a catalytic subunit, the cytidine deaminase APOBEC-1 (27 kDa) [6], and an essential RNA binding protein known as APOBEC-1 complementation factor (ACF64) [7]. Insulin-stimulated alternatively splicing of *acf* mRNA [8] gives rise to a 65 kDa protein (APOBEC-1 stimulating protein, ASP [9]). ACF64 and ACF65 bind to *apoB* mRNA and APOBEC-1 and have equivalent ability to support *apoB* mRNA editing *in vitro* [8,10,11].

Developmental induction of *apoB* mRNA editing occurs in the fetal intestine beginning with gestation day 17–18 or week 11, in rat or human intestine, respectively, and reaches adult levels in rat by gestation day 21–22 [12–16]. An interesting observation relative to the findings in the current study is that the rate of *apobec-1* mRNA expression lags behind the rate of induction of editing, suggesting that editing activity is activated as soon as APOBEC-1 has been expressed. The findings suggest that *apobec-1* gene expression is an important if not the dominant element determining the onset of intestinal editing activity.

In the present study, we evaluated whether APOBEC-1 expression was the sole determinant for the induction of editing activity during intestinal cell development. We show that *apoB* mRNA editing could not be detected until *apobec-1* mRNA expression was observed in differentiating Caco-2 cells, beginning approximately seven days post plating in differentiating conditions. At this time a shift in expression of alternatively spliced auxiliary protein mRNAs encoding ACF65 and ACF64 was also initiated. The data supported the hypothesis that APOBEC-1 expression and auxiliary protein alternative splicing were regulated during differentiation and responsible for the onset of editing activity. However, transient expression of APOBEC-1 alone or together with ACF64 or ACF65 in undifferentiated and proliferating cells was not sufficient to induce significant changes in *apoB* editing activity. These results suggested that newly expressed APOBEC-1, ACF64 and ACF65 are not fully

Abbreviations: ApoB, apolipoprotein B; APOBEC-1, ApoB editing catalytic subunit 1; ACF, APOBEC-1 complementation factor; PAGE, polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein particles; RT-PCR, reverse transcription-polymerase chain reaction

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functional until other activating regulatory mechanisms have been evoked during the course of Caco-2 differentiation.

Materials and methods

Cell culture. Caco-2 cells were purchased from ATCC (Manassas, VA) and cultured as recommended (EMEM w/2 mM L-glutamine (Gibco, Grand Island, NY), 20% FBS (Gibco), 1 mM sodium pyruvate and 0.1 mM non-essential amino acids (Gibco)). To induce differentiation proliferating Caco-2 cells were plated on 1 μM PET filter inserts (BD Bioscience, San Jose, CA) at 5×10^4 cells per filter with differential media bathing the apical and basal surfaces. Standard media conditions were maintained on the basal surface and serum free media bathed the apical surface, as previously described [17]. Transient transfection of proliferating Caco-2 cells with pcDNA3 encoding HA epitope tagged APOBEC-1 and V5 tagged ACF64 or 65, previously described [8], were conducted using FuGene 6 (Roche, Indianapolis, IN) per manufacture protocol.

Nucleic acid analyses. Total RNA was isolated from Caco-2 cells at one week intervals up to 21 days following plating on filter inserts using TRI-REAGENT (MRC, Cincinnati, OH) per manufacture protocol. *apoB* mRNA editing levels were assessed on 25 ng of cDNA created by oligo dT primed RT-PCR using the primers MS2, CTACTTCCACTTTTGTTAAAATC and MS3, GAAAATACAGAGCAGCCC CTG, in a poisoned primer extension assay as previously described [18]. Transcript levels were observed by RT-PCR. Two micrograms of total RNA isolated during the differentiation time course were primed using oligo dT for reverse transcription then amplified with primers CTGGGAGTTTGACGTCTTC and CCAGCAGTGATAATACTCTG or TCTCTCTTTCTGGCCTGGAG and CTATCTTGGGCTGTGACAAAG to amplify human *apobec-1* or β-*2-microglobulin*, respectively. Products were observed by incorporation of a ^{32}P end-labeled 3′ primer in the PCR at a ratio of 1:10 with unlabeled primer using the cycling conditions; 94 °C, 3 min, 94 °C, 30 s, 53 °C, 60 s, 72 °C, 90 s for 30 cycles. The reactions were resolved on agarose gels, dried and quantified by Phosphorimager scanning densitometry. Transcript levels of *acf64* and *acf65* were assessed in the same manner using the primers CTTTGGGCCAAGTTTATGATCC and CTGTGTATG CCAAATAGCCACG, as previously described [8] and resolved on a 2% agarose Metaphor gel (BMA, Rockland, ME).

Immunological techniques. Caco-2 cells were harvested using the NE-PER kit (PIERCE, Rockford, IL) in the presence of a protease inhibitor cocktail (USB, Cleveland, OH) and then were pooled. Pooled extracts were then placed into two separate immunoprecipitation reactions with a polyclonal anti-ACF antibody recognizing the epitope HTLQTLGIPTEGGD common to the C-terminus of both ACF64 and 65 (ASP) (Bethyl Laboratories, Montgomery, TX). Elutants were resolved by SDS–PAGE and blots were probed with either a polyclonal anti-ACF N-terminus or ACF65 specific antibodies (Bethyl Laboratories) recognizing the epitopes NHKSGDGLSGTQKE or CEIYMNVPVGA, respectively. Equal aliquots of IP material were resolved by SDS–PAGE and probed with anti-β-actin (Sigma, St. Louis, MO) for normalization of loading. Detection of APOBEC-1 and ACF64 or 65 in transient transfections utilized anti-HA (Covance, Princeton, NJ) and anti-V5 (Invitrogen, Carlsbad, CA), respectively.

Results and discussion

Caco-2 cells are a model system for human intestinal cell (enterocyte) differentiation [14–16]. Monolayers of polarized columnar enterocytes form over 14–21 days in culture when grown on filter membranes (raft cultures) exposed to rich media on the basal surface and minimal media on the apical (see Materials and methods). Differentiated Caco-2 cells secrete chylomicron-like lipoprotein particles assembled on apolipoprotein B48 from their basal membranes exposed to the rich media. This differentiation phenotype results from site-specific C to U editing of *apoB* mRNA giving rise to a translation stop codon that limits expression of the ApoB protein to 48% of the length of that encoded by the *apoB* gene [1,2,19]. Induction of this phenotype requires the expression of both APOBEC-1, the cytidine deaminase that catalyzes editing [6] and ACF, the auxiliary protein (APOBEC-1 complementation factor) [9,20] that is required for RNA editing site recognition [12,14,16,21,22].

As anticipated, proliferating Caco-2 cells expressed *apoB* mRNA but did not demonstrate significant levels of editing (Fig. 1A and Table 1). Upon differentiation, *apoB* mRNA editing increased up to 20-fold. Semi-quantitative RT-PCR (see Materials and methods) for *apobec-1* mRNA normalized to *β-2-microglobulin* mRNA (previously demonstrated to be unaffected by differentiation [13] and used to normalize cell input in RT-PCRs) demonstrated very low *apobec-1* mRNA expression until 14 days in culture; after which *apobec-1* mRNA expression was markedly elevated (Fig. 1B). APOBEC-1 protein levels were not determined as its expression level was below the detection limits of available antibodies. However, a comparison of the induction of *apoB* mRNA editing activity (Fig. 1A) with *apobec-1* mRNA expression (Fig. 1B) suggested that the expression of the deaminase was rate limiting and corroborating an early report [13] suggesting that it was a key differentiation-dependent determinant for the induction of editing activity.

In contrast to APOBEC-1, there was significant expression of ACF in proliferating Caco-2 cells and the expression of ACF was maintained throughout differentiation as evident from both the semi-quantitative RT-PCR and western blotting with ACF peptide-specific polyclonal antibodies (see Materials and methods) (Fig. 2A and B, respectively). Several ACF alternatively spliced mRNAs have been identified and the ability of their translation products to complement APOBEC-1 has been determined [8,11,23,24]. Among the variants, ACF64 and ACF65 exhibit the most robust complementation of APOBEC-1 [8,9,11,20]. ACF65 has an additional eight amino acids not found in ACF64 that enable *acf65* mRNA to be differentiated from *acf64* mRNA by radiolabeled RT-PCR (Fig. 2A). The data suggest that the abundance of both mRNAs in proliferating Caco-2 cells was approximately equivalent (Fig. 2A). Following 21 days of differentiation the *acf64* mRNA was the prominent spliced variant (*acf64*:*acf65* ratio of 1.8:1) consistent with the finding that *acf64* mRNA is the predominant spliced variant in fetal and adult human intestine [22] as well as in rat liver, rat intestine and HepG2 human hepatoma cells [11].

Western blotting with anti-ACF65 antibodies (a peptide-specific polyclonal antibody reactive with the eight amino acids unique to ACF65) and with an ACF64/ACF65 common antibody (a peptide-specific polyclonal antibody reactive with the N-terminus common to ACF64 and ACF65) demonstrated that both variants were expressed in Caco-2 cells (Fig. 2B upper and lower panels, respectively). There was reduction in ACF65-specific Western signal relative to the signal from β-actin (used as a cell number loading control). A corresponding loss of ACF64/ACF65 N-terminus Western signal was not observed, which suggested that an increasing amount of this signal came from ACF64 over the time course of differentiation while the signal from ACF65 decreased. Taken together, the data suggested that enhanced APOBEC-1 expression and possibly changes in the relative abundance of ACF65 and ACF64 were integral to the induction of *apoB* mRNA editing activity during Caco-2 cell differentiation.

To directly test this hypothesis under defined *in vitro* conditions, *apoB* mRNA editing activity was quantified in undifferentiated and proliferating Caco-2 cells following transfection and ectopic expression of HA-tagged APOBEC-1. Though APOBEC-1 expression was apparent by western blotting (Fig. 3A), little change occurred in the ability of proliferating cells to support *apoB* mRNA editing activity (Fig. 3B and Table 1). These data suggested editing

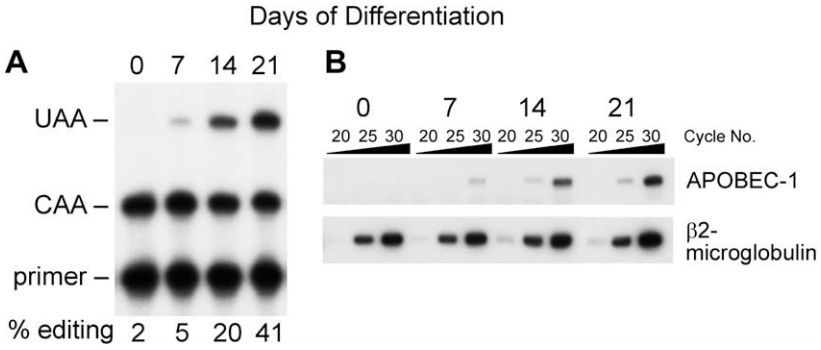

Fig. 1. *apoB* mRNA editing is induced during a 21 day time course of Caco-2 cell differentiation. (A) Total RNA was extracted from undifferentiated and proliferating Caco-2 cells (time 0) and differentiating raft cultures (7, 14 and 21 days post plating shown across the top of the panel) and *apoB* mRNA was amplified and poisoned primer extended to quantify the percentage of editing activity (shown at the bottom of the panel) as described in Materials and methods. (B) *Apobec-1* and *β-2-microglobulin* transcripts were quantified by RT-PCR from total cellular RNA isolated during the differentiation time course (as shown in A). A transcript-specific, ^{32}P end-labeled 3′ primer was incorporated into each PCR in order to quantify radiolabeled RT-PCR products on agarose gels by Phosphorimager scanning densitometry.

Table 1
Summary of apolipoprotein B mRNA editing activity in wild type and transfected Caco-2 cells.

Source of editing factors	% *apoB* mRNA editing[b]
Proliferating Caco-2 cells	2.5 ± 1.1% (n = 5)
14 day differentiated Caco-2 cells	18 ± 3% (n = 3)
Proliferating Caco-2 + [a]APOBEC-1	4.2 ± 1.2% (n = 8)
Proliferating Caco-2 + [a]APOBEC/ACF64	5.7 ± 1.4% (n = 3)
Proliferating Caco-2 + [a]APOBEC/ACF65	4.7 ± 1.1% (n = 3)

[a] Ectopic expression of editing factor was introduced by transfecting cDNAs into proliferating cells.
[b] Values represent the percent of total cellular *apoB* mRNA that was edited ± the standard deviation in the given number of replicate cell cultures.

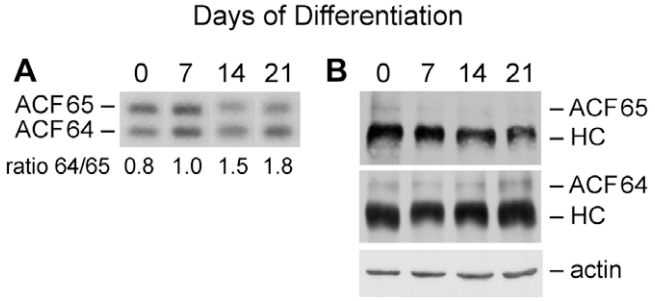

Fig. 2. Expression of alternative spliced variants of APOBEC-1 complementation factor, ACF, during Caco-2 cell differentiation. (A) Alternative splicing of *acf64* and *acf65* mRNA in differentiating Caco-2 cells (days shown across the top of the panel) was evaluated by selectively amplifying *acf64* and *acf65* mRNA using exon-sequence specific primers with radiolabeled 3′ primer as described for Fig. 1. The ratio of *acf64* or *acf65* RT-PCR products was calculated from Phosphorimaging scans. (B) Expression on ACF64 and ACF65 proteins in proliferating and differentiating Caco-2 cells (days shown across the top of the panel) was determined by immunoprecipitating ACF from cell extracts with a C-terminal, peptide-specific polyclonal antibody (peptide sequence common to both proteins) and blotting the IP elutant with either an antibody specific ACF65 or for the N-terminal epitope common to ACF65 and ACF64. Equal loading of protein extracts into the immunoprecipitates was verified by blotting for the recovery of β-actin in all of the cell extracts. HC, heavy chain from the antibodies used for immunoprecipitation.

factors where not assembling as functional editosomes even thought transfected cells had abundant ectopic APOBEC-1 and endogenous ACF.

Co-transfection of undifferentiated and proliferating Caco-2 cells with *apobec-1* and either *acf64* or *acf65* cDNAs lead to the expression of the encoded proteins (Fig. 3A). Despite abundant amounts of APOBEC-1 and ACF64 or ACF65, *apoB* mRNA editing activity was not stimulated (Fig. 3B and Table 1). This surprising result suggested that although there were measurable changes in APOBEC-1 and ACF64/ACF65 abundance over the course of differentiation, the overexpression of these proteins in proliferating cells must not have been sufficient to induce functional interactions of editing factors with *apoB* mRNA and RNA editing activity. We hypothesize that over expression of editing factors was not sufficient to induce editing activity in proliferating cells because: (1) they lacked the expression of yet to be identified differentiation-specific factor(s), (2) they could not carry out the necessary post-translational modifications and/or (3) an inhibitory factor was present. These findings augment the current model for the induction of editing during enterocyte differentiation in which the expression of editing factors is the primary, if not the sole determinant for the onset of activity.

It is of interest in this regard to consider potential parallels in the regulatory mechanisms that have been observed during liver development. Hepatic *apoB* mRNA editing did not demonstrate significant induction until 15 days postpartum and only reached adult levels by 25 days postpartum [12,22,25]. During liver development the induction of *apobec-1* mRNA expression preceded the induction of editing. The absence of a coincident increase in *apoB* mRNA editing upon the induction of rat hepatic *apobec-1* mRNA suggested that the enzyme was not translated or had been maintained in an inactive state by an inhibitory activity and/or that appropriate auxiliary factors needed to be expressed before editosomes could be assembled.

apoB mRNA editing activity is considered to be constitutive in the adult intestine whereas it is known to be highly regulated in adult rodent liver. Hepatic APOBEC-1 expression appeared to account for most of the observed changes in *apoB* mRNA editing in the fasted/refed rat model of hepatic editing (where fasting inhibited editing and refeeding with a low fat, high carbohydrate diet induced editing [12]) and in the hyperinsulinemic state [26,27]. Alternatively alterations in APOBEC-1 expression did not occur when hepatic editing activity was enhanced in hyperthyroid [28] or ethanol-fed rats [29] or in ethanol treated rat primary hepatocytes cultures or McArdle cells [30,31]. Instead, ACF serine phosphorylation was stimulated by insulin or ethanol which resulted in an increased nuclear retention of ACF [10,30,32], an enhanced interaction of ACF with APOBEC-1 promoting editosome assembly and increased editing activity [31]. Phosphorylation of ACF did not affect its interaction with *apoB* mRNA. The data presented in this report suggested that similar regulatory mechanisms controlling the functional interaction of APOBEC-1 with ACF might be operational during enterocyte differentiation. Subsequent to differentiation, intestinal *apoB* mRNA editing has >85% efficiency suggesting a constitutive ability of enterocytes to maintain activated editing

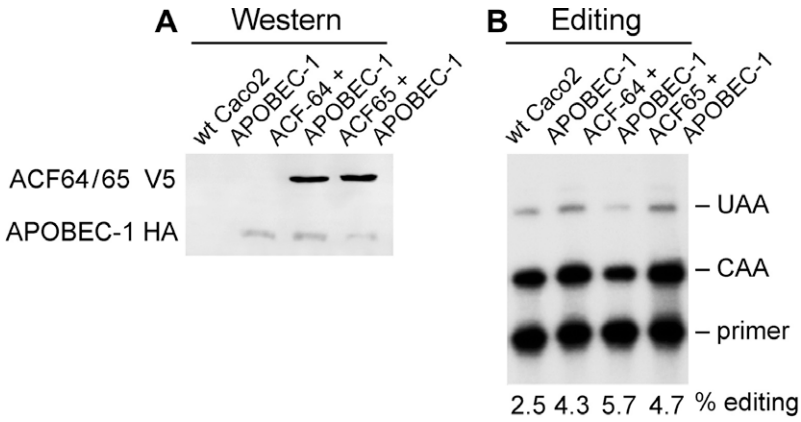

Fig. 3. Expression of APOBEC-1 alone or with either ACF64 or ACF65 was not sufficient to induce *apoB* mRNA editing comparable to that observed in differentiated cells. (A) Total cellular extracts from proliferating Caco-2 cells transfected with pcDNA3 vector expressing HA-APOBEC-1 alone or in combination with either vectors expressing V5 tagged ACF64 or ACF65 were blotted with antibodies specific for the HA or V5 epitope to visualize APOBEC-1 or ACF64/ACF65, respectively. (B) The percent of *apoB* mRNA editing activity (shown at the bottom of the panel) in control Caco-2 cells or cells expressing APOBEC-1 alone or in conjunction with ACF64 or ACF65 was determined by poisoned primer extension following RT-PCR amplification of *apoB* mRNA from total cellular RNA. Reactions were quantified by Phosphorimager scanning of the radioactive signals from PAGE-resolved, primer extension products. Percent editing was calculated as the volumes of UAA band divided by the sum of the volumes UAA + CAA times 100.

factors. The mechanisms have importance for all tissues that edit and will be the subject of our ongoing investigations.

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