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# Functional characterization of APOBEC-1 complementation factor phosphorylation sites

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**Abstract**

ApoB mRNA editing involves site-specific deamination of cytidine 6666 producing an in-frame translation stop codon. Editing minimally requires APOBEC-1 and APOBEC-1 complementation factor (ACF). Metabolic stimulation of apoB mRNA editing in hepatocytes is associated with serine phosphorylation of ACF localized to editing competent, nuclear 27S editosomes. We demonstrate that activation of protein kinase C (PKC) stimulated editing and enhanced ACF phosphorylation in rat primary hepatocytes. Conversely, activation of protein kinase A (PKA) had no effect on editing. Recombinant PKC efficiently phosphorylated purified ACF64 protein in vitro, whereas PKA did not. Mutagenesis of predicted PKC phosphorylation sites S154 and S368 to alanine inhibited ethanol-stimulated induction of editing suggesting that these sites function in the metabolic regulation of editing. Consistent with this interpretation, substitution of S154 and S368 with aspartic acid stimulated editing to levels comparable to ethanol treatment in control McArdle RH7777 cells. These data suggest that phosphorylation of ACF by PKC may be a key regulatory mechanism of apoB mRNA editing in rat hepatocytes.


*Keywords:* apoB; RNA editing; APOBEC-1 Complementation factor; Phosphorylation; Regulation

## 1. Introduction

ApoB mRNA editing occurs post-transcriptionally and causes the site-specific deamination of cytidine 6666 to uridine thereby creating an in-frame translation stop codon [1,2]. Consequently, two different apoB proteins are expressed, full-length apoB100 and the truncated protein apoB48, each with distinct physiological functions [3]. Minimally, apoB mRNA editing requires the cytidine deaminase APOBEC-1 as a homodimer [4,5], APOBEC-1 complementation factor (ACF) [6], and the RNA substrate. Limited tissue expression of APOBEC-1 restricts editing in humans to the small intestine (≥85% editing), whereas in some rodents apoB mRNA editing also occurs in the liver where it is subject to metabolic regulation [2,3,7,8].

Under normal, physiological conditions, apoB mRNA editing is a nuclear event occurring on spliced and polyadenylated RNA [9,10]. However, in vitro editing activity could be detected in both cytoplasmic and nuclear S100 extracts [11,12]. In vitro and in vivo data demonstrate that the proteins involved in editing exist in two distinct complexes; active nuclear 27S editosomes and inactive, 60S cytoplasmic complexes that can be re-organized into active 27S complexes in vitro [11,13,14]. Proteins that form these complexes traffic between the

*Abbreviations:* APOBEC-1, apolipoprotein B editing catalytic subunit 1; ACF, APOBEC-1 complementation factor; apoB, apolipoprotein B; RRM, RNA recognition motif; PP1, protein phosphatase 1; PKC, protein kinase C; PKA, protein kinase A; NT, N-terminal; CT, C-terminal

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cytoplasm and nucleus and their subcellular compartmentalization is regulated to control editing activity [14–17].

ApoB mRNA editing is regulated by developmental, hormonal, and dietary factors as well as being under tissue-specific control [7,18–23]. Editing can be stimulated primarily through two mechanisms: increased APOBEC-1 expression [24] and/or through modulation of pre-existing editing factors [25]. The fasting/refeeding and insulin induced models of editing are associated with increased APOBEC-1 abundance [24]. Conversely, induction of editing with ethanol was not dependent on de novo protein or RNA synthesis [25], suggesting modification or redistribution of pre-existing auxiliary factors were sufficient to induce editing. In both cases, the mechanism of induction involved the redistribution of ACF from the cytoplasm to the nucleus [14].

Recently, ACF was shown to be a phosphoprotein in rat primary hepatocytes [26]. The phosphorylated form was strictly localized to nuclear 27S editing complexes, the biological site of editing [26]. Dephosphorylation of ACF diminished in vitro editing activity and the interaction between ACF and APOBEC-1, but did not significantly affect RNA-binding activity. Induction of editing by ethanol and insulin resulted in nuclear accumulation of phosphorylated ACF [14,26] suggesting that phosphorylation is a common mechanism to modulate editing in response to metabolic stimuli. Inhibition of protein phosphatase 1 (PP1) activity was associated with nuclear accumulation of ACF, increased recovery of phosphorylated ACF and robust editing levels [26]. However, the protein kinase(s) responsible for phosphorylating ACF remain an outstanding question.

We identified serine 154 (S154) and serine 368 (S368) as two candidate phosphorylation sites. McArdle RH7777 cell lines expressing S154 and S368 mutated to alanine maintained normal basal editing levels, but these levels were refractory to ethanol induction. Conversely, substitution of aspartic acid at these sites significantly increased editing in the absence of ethanol to levels comparable with those achieved by ethanol treated cells expressing wild type ACF. The data support the possibility that these sites are necessary for ethanol-induced editing. Residues S154 and S368, are predicted PKC phosphorylation sites, and are conserved between rat and human ACF. Studies with PKC and PKA activators demonstrated that modulation of PKC activity enhanced editing levels in rat primary hepatocytes, whereas PKA did not. Similarly, purified ACF protein was efficiently labeled by PKC isozymes, but not by PKA. These findings are discussed in terms of structural and functional predictions of ACF phosphorylation.

## 2. Materials and methods

### 2.1. Isolation of rat primary hepatocytes and protein kinase activator studies

Rat primary hepatocytes were isolated from normal, fed male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) (250–275 g BW) and cultured on BIOCOAT type I collagen coated, 60 mm plastic dishes (Becton Dickinson Labware, Franklin Lakes, NJ) in Waymouth's media (Sigma Chemical Co., St. Louis, MO) containing 0.1 nM porcine insulin (Sigma) as described previously [14]. Primary hepatocytes were treated for 6 h with protein kinase activators indolactam V, 8-cpt cAMP, and forskolin at the concentrations encompassing their respective in vivo $EC_{50}$ values, as described by the manufacturer (Calbiochem, La Jolla, CA). Total cellular RNA was harvested in TriReagent (MRC, Cincinnati, OH) and processed through the poisoned-primer extension assay to quantify the effect on apoB mRNA editing [14].

To evaluate the effect of protein kinase activation on ACF phosphorylation, cultures were pre-incubated for 2 h with 12 or 120 μM indolactam V in phosphate-free Waymouth's media and subsequently supplemented with 0.5 mCi $^{32}PO_4$ and incubated for an additional 4 h. Cultures were harvested and subcellular extracts prepared. ACF was immunoprecipitated, resolved by SDS-PAGE, and specific activity was quantified as described previously [26].

### 2.2. Recombinant ACF purification and in vitro ACF phosphorylation

Recombinant ACF64 was expressed in E. coli and purified as described [27]. In vitro ACF labeling reactions were carried out using 2 μg of purified recombinant ACF64 in 5 mM $MgCl_2$, 100 μM $CaCl_2$, 1 mM DTT, 5 μCi $^{32}$P-γATP, 25 mM Tris–HCl pH 7.5 and 1X Lipid Activator (Invitrogen, Carlsbad, CA) with 25 mU of each PKC isozyme (Calbiochem) or 40 mM Tris–HCl pH 7.4, 20 mM Mg Acetate, 5 μCi $^{32}$P-γATP and 25 mU of bovine PKA catalytic subunit (Promega, Madison, WI). Reactions were incubated at 30 °C for 10 min, terminated by acetone precipitation, resolved by 10.5% SDS-PAGE, and transferred to nitrocellulose (Bio-Rad, Hercules, CA). Western transfers were probed with anti-HA monoclonal antibody (Covance Research Products, Berkeley, CA) and developed using Western Lightning reagent (NEN, Boston, MA). $^{32}$P incorporation was monitored by phosphorimager scanning densitometry. PKA enzymatic activity was confirmed via labeling of Kemptide peptide (Promega).

### 2.3. ACF site-directed mutagenesis and functional analysis

Rat ACF64 with a C-terminal V5 epitope tag was cloned into a modified *pcDNAIII* vector (Invitrogen) [14]. ACF64 cDNA was mutated at specific bases to convert specific serine or threonine codons to alanine or aspartic acid codons using the QuikChange® Multi System (Stratagene, La Jolla, CA). Mutagenic primers (T49AT50A; CCAGGCTGGGAT**GCTGCA**CCTCCTGAAAGGGGCTGC, T49DT50D; CCAGGCTGGGAT**GACGAT**CCTCCTGAAAGGGGCTGC, S132A; GGGCGTCTGTGCTG**CTG**TGGACAACTGCCGG, S132D; GGGCGTCTGTGCTG**ATG**TGGACAACTGCCGG, S154A; GAGAGAAGAAATCTTGG**CAG**AGATGAAAAAGGTC, S154D; GAGAGAAGAAATCTTGG**ACG**AGATGAAAAAGGTC, S171A; GTCATTGTCTACCCAG**CCG**CTGCCGATAAAACC, S171D; GTCATTGTCTACCCAG**ACG**CTGCCGATAAAACC, S176A; GTCTACCCAAGCGCTGCTGATAAA**GCC**AAAAACCGGGGG, S176D; GTCTACCCAAGCGCTGCTGATAAA**GAC**AAAAACCGGGGG, S188A; GCCTTTGTGGAATATGAAG**CTC**ACCGCGCAGCCG, S188D; GCCTTTGTGGAATATGAAG**ATC**ACCGCGCAGCCG, S368; CTACCAAAGGACATCTC**GCC**AACAGAGCTCTCATCCG, S368D; CTACCAAAGGACATCTC**GAC**AACAGAGCTCTCATCCG) were extended using *PfuTurbo*® DNA polymerase according to the manufacturer's recommendations. The accuracy of the mutations was verified by sequencing the entire cDNA using T7 (Promega, Madison, WI), V5 (Sigma Genosys), and Seq481 (Sigma Genosys, The Woodlands, TX) sequencing primers (T7, TAATACGACTCTATAGGG, V5; CTAGAAGGCACGTCGAGGC, Seq481; GAACGAGTTGTTGATGTCATTG) and using Big Dye sequencing system (Applied Biosystems, Foster City, CA).

McArdle RH 7777 (McArdle) cells maintained in DMEM (Gibco-BRL, Carlsbad, CA; supplemented with 10% FBS, 10% horse serum, 1% Penicillin, Streptomycin, Fungizone (Invitrogen)) were transfected with 2 μg plasmid containing each ACF64 mutant using Fugene 6 (Roche, Mannheim, Germany). Stable cell lines were created by G418 selection. When applicable, stable cell lines were treated with 0.9% ethanol for 4 h. RNA editing of endogenous apoB mRNA was assessed by poisoned-primer extension analysis as previously described [14].

Expression of ACF mutants was assessed by western blotting of whole-cell extracts prepared by lysing cells for 30 min in RIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM