Metabolic regulation of ApoB mRNA editing is associated with phosphorylation of APOBEC-1 complementation factor

David M. Lehmann3,4, Chad A. Galloway1, Mark P. Sowden1,2 and Harold C. Smith1,2,3,4,5,*

1Department of Biochemistry and Biophysics, 2Department of Pathology and Laboratory Medicine, 3Department of Toxicology, 4The Environmental Health Sciences Center and 5James P. Wilmot Cancer Center, University of Rochester, Rochester, Rochester, NY 14642, USA

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ABSTRACT

Apolipoprotein B (apoB) mRNA editing is a nuclear event that minimally requires the RNA substrate, APOBEC-1 and APOBEC-1 Complementation Factor (ACF). The co-localization of these macro-molecules within the nucleus and the modulation of hepatic apoB mRNA editing activity have been described following a variety of metabolic perturbations, but the mechanism that regulates editosome assembly is unknown. APOBEC-1 was effectively co-immunoprecipitated with ACF from nuclear, but not cytoplasmic extracts. Moreover, alkaline phosphatase treatment of nuclear extracts reduced the amount of APOBEC-1 co-immunoprecipitated with ACF and inhibited in vitro editing activity. Ethanol stimulated apoB mRNA editing was associated with a 2- to 3-fold increase in ACF phosphorylation relative to that in control primary hepatocytes. Significantly, phosphorylated ACF was restricted to nuclear extracts where it co-sedimented with 27S editing competent complexes. Two-dimensional phosphoamino acid analysis of ACF immunopurified from hepatocyte nuclear extracts demonstrated phosphorylation of serine residues that was increased by ethanol treatment. Inhibition of protein phosphatase I, but not PPIIA or IIB, stimulated apoB mRNA editing activity coincident with enhanced ACF phosphorylation in vivo. These data demonstrate that ACF is a metabolically regulated phosphoprotein and suggest that this post-translational modification increases hepatic apoB mRNA editing activity by enhancing ACF nuclear localization/retention, facilitating the interaction of ACF with APOBEC-1 and thereby increasing the probability of editosome assembly and activity.

INTRODUCTION

ApoB mRNA editing involves the site-specific deamination of cytidine 6666 to uridine within a glutamine codon (CAA) thereby creating an in-frame translation stop codon (1). Consequently, two apoB protein variants are expressed, full-length apoB100 and the truncated protein apoB48, both of which participate in lipid transport, but with markedly different roles as atherogenic risk factors (1). Minimally, apoB mRNA editing requires the cytidine deaminase APOBEC-1 as a homodimer (2–5), APOBEC-1 Complementation Factor (ACF) (6–9) and the tripartite editing motif within the mRNA substrate (10–12). ACF is the mooring sequence-specific RNA binding protein that directs site-specific editing (6–9,13).

Limited tissue expression of APOBEC-1 and apoB mRNA restricts editing in humans to the small intestine (>85% editing), but apoB mRNA editing also occurs in the liver of several species (3,14–16). Hepatic editing is modulated by fasting and refeeding in part due to an insulin-dependent increase in APOBEC-1 expression (17). Hepatic editing is also regulated independently of changes in APOBEC-1 expression levels by developmental, hormonal and nutritional perturbations (17–23). The mechanism for this form of editing activity regulation has not been defined, but involves the nuclear trafficking of editing factors (24–27).

ApoB mRNA editing occurs primarily on spliced and polyadenylated RNA in the nucleus (2,24,25,28–30). Despite this, APOBEC-1 and ACF are distributed in both the cytoplasm and nucleus (24,26,29–31). In nuclear extracts, APOBEC-1 and ACF co-sedimented in 27S, editing-competent complexes, but as inactive 60S complexes in cytoplasmic extracts (6,24). Under in vitro editing conditions, 60S complexes reorganized to active 27S complexes on reporter RNAs (6,24). Furthermore, localization studies demonstrated that ACF and APOBEC-1 trafficking between the cytoplasm and the nucleus (26,27). In support of trafficking as a regulatory mechanism, ethanol, insulin and thyroid hormone stimulation of hepatocyte editing activity were associated with an increase in nuclear localization of ACF (24,29,32).
Induction of editing by ethanol occurred within minutes (21,23,29), and withdrawal of the stimulus both reduced the abundance of ACF in the nucleus and suppressed editing activity (23,24). Ethanol induced editing is not dependent on de novo protein or RNA synthesis (33) suggesting that modulation of pre-existing editing factors is sufficient to support enhanced editing activity. These observations substantiated the possibility that cytoplasmic 60S complexes may serve as a reservoir of editing factors necessary for rapid assembly of nuclear 27S editosomes. Protein phosphorylation is one of the most common protein modifications known and its importance in the regulation of protein activity has been well documented (34). In fact phosphorylation has been implicated as having a role in apoB mRNA editing although its mechanism remains unclear (35).

We show that ACF was phosphorylated on one or more serine residues, and that ethanol and insulin induction of apoB mRNA editing was accompanied by phosphorylation of ACF. PhosphoACF was only detected in the nucleus, and was selectively recovered with active 27S editosomes. Although ACF and APOBEC-1 are both present in the cytoplasm, APOBEC-1 co-immunoprecipitated with ACF only from nuclear extracts. Recovery of ACF/APOBEC-1 complexes and apoB mRNA editing activity were dependent on protein phosphorylation. Protein phosphatase inhibitor studies suggest that protein phosphatase 1 is involved in regulating editing activity, ACF phosphorylation and ACF subcellular distribution. The significance of ACF phosphorylation for ACF trafficking to the nucleus, association with APOBEC-1 and assembly into 27S editosomes and the regulation of editing efficiency is discussed.

**MATERIALS AND METHODS**

**Animal care, primary hepatocyte isolation and hepatoma cell culture**

Male Sprague-Dawley rats (275–325 g BW/Charles River Laboratories, Wilmington, MA) were housed under 12 h light/dark cycles and fed normal rat chow (Purina, St. Louis, MO) ad libitum and euthanized between 9 and 10 a.m. Primary hepatocytes were isolated (23) and plated onto BIOCOAT type I collagen coated dishes (Becton Dickinson Labware, Franklin Lakes, NJ) in Waymouth’s 752/1 media (Sigma Chemical Co., St Louis, MO) containing 0.1 nM porcine insulin (Sigma) for 12–16 h prior to the onset of each experiment.

McArdele RH7777 cells (ATCC Manassas, VA) stably expressing HA epitope-tagged APOBEC-1 (36) were treated for 4 h with 0.9% ethanol and fractionated into nuclear extracts (23).

**In vivo phosphorylation of ACF**

*In vivo* 32P labeling was performed by intraperitoneal injection of rats with 12.5 mCi of orthophosphoric acid (10 mCi/ml 32PO4; NEN, Boston, MA) buffered with 50 mM HEPES, pH 7.0 and 150 mM NaCl. After 4 h, rats were sacrificed and hepatic cytoplasmic and nuclear extracts prepared. Primary hepatocyte cultures in 60 mm dishes were incubated in phosphate-free Minimum Essential Eagle Media (Sigma) containing 0.1 nM porcine insulin for 6 h and subsequently in fresh media containing 10 nM insulin (equivalent to that seen in post-prandial serum) or 0.45–0.9% ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY) (23,29) plus 2.0 mCi orthophosphoric acid. Cultures were labeled for 4 h prior to extract preparation.

**Subcellular extract preparations**

Livers were perfused *in situ* with 0.25 M sucrose, 50 mM Tris, pH 7.0 and 5 mM MgCl2 and protease inhibitors (Roche, Indianapolis, IN) (6,24). Cytoplasmic and nuclear extracts (37) were supplemented with 10 mM NaF and fractionated through glycerol gradients.

Cultured primary hepatocytes and hepatoma cell lines were rinsed with 1× Tris-buffered saline (TBS; 10 mM Tris and 150 mM NaCl, pH 7.5) and scraped into TBS containing proteinase/phosphatase inhibitors (Roche). Nuclear and cytoplasmic extracts were prepared using the NE-PER kit (Pierce, Rockford, IL) supplemented with 10 mM NaF and proteinase inhibitors.

**Glycerol gradient fractionation**

Preparative (35 ml) 10–50% glycerol gradients containing 10 mM NaF were loaded with 20 mg or 2 mg of cytoplasmic or nuclear S100 extract respectively and sedimented at 100 000 *g* for 10 h at 7°C. The distribution of 11S, 27S and 60S complexes (fractions 3/4, 5/6 and 7/8) was determined by the sedimentation of bovine serum albumin (6S), catalase (11S) and 60S spliceosomes (6,24). Gradient fractions were treated with DNase I and RNaseT1 to ensure the removal of DNA or RNA prior to the analysis of ACF 32P-labeling.

**In vitro editing reactions and RNA binding assays**

*In vitro* editing reactions and quantitative poisoned-primer extension assays were carried out using 80 μg of extract proteins as described previously (11,37). ApoB RNA binding assays were performed by subjecting nuclear extract (80 μg) to *in vitro* editosome assembly and ultraviolet cross-linking (37).

**Protein phosphatase inhibitor studies**

Primary hepatocytes were treated for 6 h with cantharidin, endothall or okadaic acid at concentrations encompassing their respective *in vivo* IC50 values as described by the manufacturer (Calbiochem, La Jolla, CA) and their suggested references. The effects of the compounds on apoB mRNA editing were evaluated by RT–PCR and poisoned-primer extension analysis (38). The effect of 470 nM cantharidin on ACF subcellular distribution was investigated by treating primary hepatocyte cultures for 4 h followed by nuclear and cytoplasmic extract preparation.

To evaluate the effect of phosphatase inhibition on ACF phosphorylation, cultures were pre-incubated for 2 h with 470 nM cantharidin in phosphate-free Waymouth’s media and subsequently supplemented with 0.5 mCi 32PO4 and incubated for an additional 4 h. Cultures were harvested and subcellular extracts prepared.
Immunological techniques
Rabbit polyclonal peptide-specific antibodies were raised against ACF N-terminal (NT) sequence (NHKSGDGLS-GTQKE) and C-terminal (CT) sequence (HTLQTL-GIPTEGGD) (24) and affinity purified with the corresponding peptides (Bethyl Laboratories, Inc., TX). For immunoprecipitation analyses all radiolabeled extracts were adjusted to 5 mM MgCl₂ and digested with 100 U of DNase I (Promega, Madison, WI), RNase T1 (Roche) and RNase A (Sigma) for 1 h on ice to remove radiolabeled nucleic acid. Where applicable, extracts were incubated overnight with ACF CT antibody at 4°C and subsequently reacted with Protein A-agarose (Oncogene Research Products, Boston, MA) pre-washed with 1× TBS/10 mM NaF. The immuno-absorbed material was washed three times with 1× TBS/10 mM NaF, three times with 1× TBS/1 M NaCl (36) and finally three times with 1× TBS. Immuno- absorbed material to be treated with alkaline phosphatase was washed with 50 mM Tris, pH 8.4, 1 mM MgCl₂, 0.1 mM ZnCl₂, 25% glycerol and then incubated with 5 U CIAP for 1 h at 30°C and then washed six times with 1× TBS. ACF-antibody complexes were eluted with 3 M sodium thiocyanate, acetone precipitated and analyzed by 10.5% SDS–PAGE followed by autoradiography and/or western blotting with ACF NT antibody. For ACF immunoprecipitation from gradient fractions an equal volume of pooled gradient fractions from sedimentation zones of interest were reacted with sub-saturating amounts of ACF CT antibody to ensure that the recovery of phosphoACF was not simply a reflection of ACF abundance in each zone.

Two-dimensional gel electrophoresis
Extracts isolated from hepatocytes were analyzed for ACF charge isoforms using the Protean isoelectric focusing (IEF) system (Bio-Rad Laboratories, Hercules, CA). Immobilized pH gradient (IPG) strips (Bio-Rad Laboratories, pH range 3–9.3) were hydrated with 150 mM Na₂CO₃ and fractionated. Two-dimensional gel electrophoresis was performed using an HTLE Criterion gel (Bio-Rad Laboratories), transferred to nitrocellulose and reacted with ACF NT antibody. The immuno-absorbed material was washed three times with 1× TBS/10 mM NaF, three times with 1× TBS/1 M NaCl (36) and finally three times with 1× TBS. Immuno-absorbed material to be treated with alkaline phosphatase was washed with 50 mM Tris, pH 8.4, 1 mM MgCl₂, 0.1 mM ZnCl₂, 25% glycerol and then incubated with 5 U CIAP for 1 h at 30°C and then washed six times with 1× TBS. ACF-antibody complexes were eluted with 3 M sodium thiocyanate, acetone precipitated and analyzed by 10.5% SDS–PAGE followed by autoradiography and/or western blotting with ACF NT antibody. For ACF immunoprecipitation from gradient fractions an equal volume of pooled gradient fractions from sedimentation zones of interest were reacted with sub-saturating amounts of ACF CT antibody to ensure that the recovery of phosphoACF was not simply a reflection of ACF abundance in each zone.

Two-dimensional phosphoamino acid analysis
ACF was immunopurified using the ACF CT antibody from 27S enriched nuclear extracts of 3²P-labeled primary hepatocytes cultured in basal media (0.1 nM insulin) or in basal media containing 0.9% ethanol. Immunoprecipitates were resolved by SDS–PAGE, blotted onto PVDF membrane (Bio-Rad, CA) and ACF was identified by autoradiography, excised and acid hydrolyzed in 5.7 N HCl at 110°C for 1 h (39). After completion of IEF the IPG strip was equilibrated in SDS buffer for 30 min and then in iodoacetamide buffer for additional 30 min according to the manufacturer’s recommendations. Proteins were resolved through a 10.5% Criterion gel (Bio-Rad Laboratories), transferred to nitrocellulose and reacted with ACF NT antibody.

RESULTS
The minimal, functional editosome is composed of ACF, a homodimer of APOBEC-1 and the apoB RNA substrate (40,41). ACF and APOBEC-1 are distributed in the cytoplasm and nucleus of editing competent cells where they co-localize in macromolecular complexes of 60S and 27S, respectively (6,24). However, apoB mRNA editing is only associated with nuclear 27S complexes (24,29). Given that endogenous levels of APOBEC-1 are below the detection limits of currently available antibodies (36), the interaction of ACF with APOBEC-1 in the nucleus and cytoplasm was analyzed by co-immunoprecipitation from extracts prepared from McArdle cells that stably express HA-tagged APOBEC-1 (25,36). As anticipated, ACF and APOBEC-1 were abundant in both cytoplasmic and nuclear starting material (Figure 1). However, APOBEC-1 was efficiently co-immunoprecipitated with ACF only from nuclear extracts.

As an interaction between ACF and APOBEC-1 is critical for editing activity (42) we investigated the effect of alkaline phosphatase treatment on in vitro editing activity of hepatocyte extracts. ApoB mRNA editing activity in CIAP-treated McArdle cells stably expressing HA-tagged APOBEC-1 was treated with 0.9% ethanol for 4 h and fractionated into cytoplasm and nuclear extracts. Nuclear (N) and cytoplasmic (C) extracts were western blotted with the ACF NT (top left panel) or anti-HA antibody (bottom left panel). ACF CT antibody immunoprecipitates of nuclear and cytoplasmic extracts were western blotted with the ACF NT antibody (top right panel). The blots were stripped and reprobed with HA antibody (APOBEC-1, bottom left and right panels). ACF, position of ACF, HC, position of Ig heavy chain. The quality of the fractionation was assessed by western blotting for marker proteins as shown in Figure 6. Data shown are representative of three independent experiments. Statistical significance was determined to be P < 0.01 by unpaired t-test relative to nuclear co-IP.

![Figure 1](image-url)
nuclear extract was significantly inhibited by 2.5-fold relative to control extracts (Figure 2B). The observed reduction in editing activity could be attributed to the failure of ACF to interact with APOBEC-1 or with the RNA substrate. In contrast to the interaction with APOBEC-1, CIAP treatment (1, 5 or 10 U) did not significantly increase ultraviolet light cross-linking of ACF to apoB mRNA (Figure 2C). These data suggest that the reduction in editing activity in CIAP-treated extracts was most probably the result of suppression of interactions between ACF and APOBEC-1 rather than an alteration in the binding affinity of ACF to apoB mRNA.

**ACF is a phosphoprotein**

Previous studies using site-directed mutagenesis of APOBEC-1 and overexpression of protein kinase Cα implicated phosphorylation of APOBEC-1 as a mechanism for activating apoB mRNA editing (35). Although our aforementioned data are consistent with this possibility, the low level of endogenous APOBEC-1 expression has prohibited in vivo validation of this finding. Furthermore, in vivo studies carried out in our laboratory using exogenous rat APOBEC-1 were unable to detect APOBEC-1 phosphorylation (data not shown) and the phosphorylation sites suggested by the authors are not conserved between rat and human (35).

To evaluate whether endogenous ACF is phosphorylated in vivo, ACF was immunopurified from radiolabeled 27S containing glycerol gradient fractions (i.e. editosomes) and incubated with 100 U CIAP or buffer alone (control). Membranes were exposed to X-ray film (autorad) and subsequently reacted with ACF NT antibody (western). HC, position of Ig heavy chain. Data shown are representative of three independent experiments. (B) Hepatic ACF contains more than one phosphorlated residue. Rat liver nuclear extracts (150 µg), incubated with or without 100 U CIAP were resolved by equilibrium 2D gel electrophoresis. The applicable range of the gel is shown corresponding to pH 8.3–9.3. The boxed area delineates the acidic isoform of ACF that was not observed following CIAP treatment. Nuclear extract was run on the end of the second dimension gel to verify the relative migration (Mr) of ACF and is delineated by a vertical bar. Data shown are representative of two independent experiments. (C) ACF is phosphorylated on serine residue(s): ACF was immunopurified from control or 0.9% ethanol-treated radiolabeled rat hepatocytes and transferred to PVDF. The radiolabeled band corresponding to ACF western blot reactivity was excised from the blot, acid hydrolyzed and the products from control and ethanol-treated hepatocytes resolved in parallel by 2D thin layer electrophoresis. The migration of phosphoamino acids was determined from ninhydrin staining of known standards spiked into the samples and is indicated and the thin layer plate was autoradiographed. Data shown are representative of three independent experiments.
ACF did not become radiolabeled (Figure 4A). The amino acid sequence of rat ACF is predicted to contain 23 serine/threonine and 7 tyrosine high probability sites of phosphorylation (http://www.cbs.dtu.dk/services/NetPhosK and http://expasy.org/tools/scanprosite). To investigate the complexity of physiologically relevant ACF phosphorylation sites, liver extracts prepared from control rats were resolved by equilibrium 2D gel electrophoresis and immunoblotted with ACF NT antibody (24). The predicted isoelectric point (pl) of ACF is 8.8 (http://www.scripps.edu/~cdputnam/protcalc.html) and the covalent addition of phosphate would be expected to cause an acidic shift of the pl (43). Nuclear ACF was detected as two predominant charge isoforms (Figure 3B). The major isoform migrated at pl 8.8 and is likely unmodified ACF or ACF containing both acidic and basic modifications. A second, less abundant, isoform migrated with a pl of 8.3 and is consistent with phosphorylation of ACF. To verify that the observed charge heterogeneity was due to protein phosphorylation, nuclear extracts were treated with CIAP which resulted in an almost complete loss of the acidic isoform (pl 8.3) and concomitant increase in the pl 8.8 isoform (Figure 3B), which is consistent with the removal of 2–3 phosphates (0.2–0.3 pH units per phosphate) (43). Cytoplasmic ACF migrated as a single isoform at pl 8.8, which did not shift upon phosphatase treatment (data not shown), further confirming that cytoplasmic ACF was not phosphorylated under our assay conditions.

To determine whether serine, threonine and/or tyrosine residues were the target of phosphorylation, primary hepatocytes were labeled to high specific activity with $^{32}$PO$_4$ and ACF was immunoprecipitated from nuclear extracts. 2D thin layer electrophoresis of acid hydrolyzed ACF demonstrated that phosphorylation occurred on serine residues (Figure 3C, left panel). Although this technique is not intended for quantitation, it is interesting that the amount of phosphoserine detected increased (1.5-fold) in ACF immunopurified from an equivalent amount of nuclear extract isolated from primary hepatocytes incubated with 0.9% ethanol during the labeling period (Figure 3C, right panel). The 1.5-fold change in phosphoserine abundance likely represents a minimum as the radioactivity in partially hydrolyzed peptides (migrating along the first dimension) suggest greater $^{32}$P incorporation (see Materials and Methods). These data suggest that threonine and tyrosine residues are not phosphorylated in rat ACF or that their phosphorylation exhibits a slow rate of turnover, preventing them from incorporating $^{32}$P label during the experiment.

Phosphorylated ACF is only recovered with nuclear editosomes

If phosphorylated ACF is relevant to editing activity, it should be associated with nuclear 27S editosomes. To evaluate this, cytoplasmic and nuclear extracts from radiolabeled primary hepatocyte cultures were sedimented through 10–50% glycerol gradients. ACF was immunoprecipitated with sub-saturating quantities of the ACF CT antibody from pooled fractions corresponding to (i) 11S (pre-editosomal ACF and APOBEC-1), (ii) the 27S editosome and (iii) 60S and greater. The ACF immunoprecipitates were resolved by SDS–PAGE, transferred to nitrocellulose, autoradiographed and subjected to PhosphorImager scanning to detect and quantify radiolabeled proteins prior to immunoblotting with the ACF NT antibody. Consistent with prior analyses (24), ACF was recovered by immunopurification from all gradient fractions (Figure 4A). Although ACF was widely distributed in all nuclear fractions, phosphoACF was restricted to fractions containing the 27S editosome. Editing activity, indicative of the assembly of APOBEC-1 with ACF has only been observed in 27S gradient fractions (6). Considering data presented in Figures 1, 2A and B, the selective recovery of phosphorylated ACF in the nuclear 27S editosome fraction is highly suggestive. Thus, a correlation linking phosphoACF to the physiologically relevant 27S editing complexes can be made.

Phosphorylation of ACF is metabolically regulated

*ApoB* mRNA editing is regulated by a variety of hormonal and dietary factors [reviewed in (44)]. To determine if ACF phosphorylation is correlated with metabolic regulation of editing rat primary hepatocytes were labeled with $^{32}$PO$_4$ for 4 h in the presence of either 0.45% ethanol or 10 nM insulin. A 3.5-fold increase of phosphoACF was associated with nuclear 27S editosomes isolated from ethanol-treated hepatocytes (Figure 4B). These data are consistent with the increase in serine phosphorylation observed following ethanol treatment (Figure 3C). Moreover, phosphoACF was

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**Figure 4.** Phosphorylation of ACF is metabolically regulated and is localized to nuclear active 27S editosomes: Cytoplasmic and nuclear extracts were prepared from rat primary hepatocytes that had been labeled with 0.5 mCi $^{32}$PO$_4$ for 4 h in the presence of (A) basal 0.1 nM insulin, (B) 0.45% ethanol or (C) 10 nM insulin and fractionated through glycerol gradients. ACF CT antibody immunoprecipitates were transferred to nitrocellulose and exposed to X-ray film (autorad) and then reacted with ACF NT antibody (western). Autoradiographs from the cytoplasmic fraction immunoprecipitations represent exposures that were twice as long compared to those shown for the nuclear fractions to ensure that phospho-ACF, if present, could be detected. HC, position of Ig heavy chain. Data shown are representative of three independent experiments.
not detected in any cytoplasmic fractions despite a 10-fold higher protein load compared with nuclear extracts onto the gradients and a prolonged autoradiographic exposure (Figure 4B).

To determine if enhanced ACF phosphorylation is a more general mechanism associated with editing induction, we investigated the effect of insulin treatment on primary hepatocytes. Insulin, like ethanol, stimulates apoB mRNA editing (17,24) and the nuclear accumulation of ACF (24). Consistent with data from ethanol-treated primary hepatocytes, addition of 10 nM insulin during the 4 h labeling period resulted in a 2.5-fold increase in the recovery of phosphorylated ACF in nuclear 27S editosomes (Figure 4C). No phosphoACF was detected in any cytoplasmic fractions of insulin stimulated hepatocytes. These data demonstrate that ACF phosphorylation is a general characteristic associated with modulation of apoB mRNA editing activity.

ApoB mRNA editing and ACF phosphorylation can be modulated by protein phosphatase inhibitors

If ACF phosphorylation is integral to the regulation of apoB mRNA editing activity, and ACF must become dephosphorylated in order to traffic to the cytoplasm, we reasoned that inhibition of the appropriate protein phosphatase would result in a nuclear accumulation of phosphorylated ACF and stimulate apoB mRNA editing. To evaluate this, hepatocytes were treated with a series of protein phosphatase inhibitors and editing activity determined. Protein phosphatase inhibitors were selected such that we could identify or rule-out a role for a specific class of protein phosphatases. Cantharidin is a protein phosphatase inhibitor with markedly different inhibitory concentrations for PP2A and PP1 (40 and 473 nM, respectively) (45,46). When tested at 470 nM, the IC₅₀ for PP1, we detected a reproducible, but not statistically significant increase in editing (Figure 5A). Additional experiments using 4.7 μM, a concentration anticipated to inhibit >90% PP1 activity, editing increased from 66 to >90% (Figure 5A). In addition, treatment of hepatocytes with both cantharidin and ethanol simultaneously, conditions proposed to stimulate both ACF nuclear localization and phosphorylation as well as to inhibit dephosphorylation resulted in the largest stimulation of editing activity.

To further support the role of PP1 in editing regulation, two additional protein phosphatase inhibitors, okadaic acid (45) and endothall (47) were tested. Editing was enhanced from 66 to >90% (P ≤ 0.01) in hepatocytes treated with okadaic acid at concentrations 10-times the IC₅₀ for PP1 (Table 1). Similarly, treatment with endothall at a concentration 10-times the IC₅₀ for PP1 stimulated editing to statistically significant levels (P ≤ 0.01) (Table 1).

Since calcium levels have been implicated in the regulation of editing (18), we investigated the effect of inhibition of the calcium-sensitive protein phosphatase 2B (PP2B) (48) on editing. Treatment of hepatocytes with cyclosporin A (49) and cypermethrin (50) did not affect significantly apoB mRNA editing (Table 1). In addition to ruling out PP2B, these data also demonstrate that enhanced editing in the presence of protein phosphatase inhibitors is not a non-specific effect owing to small molecule inhibitors; strengthening our position that PP1 is involved in editing regulation.

**Figure 5.** Protein phosphatase I inhibitors modulate apoB mRNA editing and ACF phosphorylation. (A) RNA Editing: rat primary hepatocytes were incubated with concentrations of cantharidin ranging from 47 nM (IC₅₀ of PP2A) to 4.7 μM (10 times IC₅₀ PP1) and 0.45% ethanol where indicated. In vivo editing activity was determined on endogenous apoB mRNA using apoB specific RT–PCR followed by poisoned-primer extension (38). *Statistical significance was determined to be P < 0.01 by unpaired t-test relative to DMSO control n > 5. (B) ACF phosphorylation and specific activity: in vivo ACF ³²P incorporation was determined by PhosphorImager scanning of ACF immunoprecipitates prepared from rat hepatocytes treated with 470 nM cantharidin. ACF-specific activity (relative to control hepatocytes) was calculated as the ACF ³²P density (PhosphorImager) divided by the recovery of ACF determined from densitometric scanning of ACF western blots (Image J). ACF immunopurified from control hepatocytes was arbitrarily assigned a value of 1 (n = 3), exp. 1 and exp. 2 denote independent experiments 1 and 2.

In order to correlate the observed effects on editing with changes in ACF phosphorylation, ACF was immunoprecipitated from extracts isolated from hepatocytes treated with increasing concentrations of cantharidin (Figure 5B). ACF phosphorylation was increased in hepatocytes treated with 470 nM cantharidin. Maximal ACF phosphorylation was observed when hepatocytes were treated with both ethanol and cantharidin, reflecting the editing activity data described in Figure 5A. Taken together, these data demonstrate that the cellular effects of cantharidin that lead to enhanced editing activity are associated with increased ACF phosphorylation.

Previous reports demonstrated accumulation of ACF in the nucleus of hepatocytes treated with ethanol and insulin and return of ACF to the cytoplasm upon removal of stimuli (24). To evaluate the effect of PP1 inhibition on the nuclear retention of ACF, hepatocytes were incubated with 470 nM cantharidin for 4 h, fractionated into nuclear and cytoplasmic...
Table 1. Protein phosphatase I inhibitors modulate apoB mRNA editing. Rat primary hepatocytes were treated with the indicated concentrations of protein phosphatase inhibitors for 6 h. Editing activity was determined on the endogenous apoB mRNA using apoB specific RT-PCR followed by poisoned-primer extension (38). The mean, standard deviation (STD) and number of replicates (n) are indicated for each determination. aStatistical significance was determined to be P < 0.01 by unpaired t-test relative to DMSO control when n≥3.

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IC50; PP1 = 10–15 nM, PP2A = 0.1 nM
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IC50; PP2B = nM, range
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extracts, resolved by SDS–PAGE and immunoblotted for ACF. Subsequently, the blots were re-probed for actin and Histone H1 to verify cell fractionation quality and to serve as normalization standards for protein loading (Figure 6). The normalized relative abundance of nuclear to cytoplasmic ACF was 1:1 in control cells. Upon incubation with cantharidin a 5-fold increase in nuclear ACF was observed, consistent with our hypothesis that inhibition of ACF diphosphorylation prevents its nuclear export.

**DISCUSSION**

Hepatic apoB mRNA editing is a regulated, nuclear process that requires the assembly of multi-protein editosomes. Reconstitution assays have identified the essential protein factors as the cytidine deaminase APOBEC-1 and the auxiliary factor ACF. Data presented in this report demonstrate that ACF is a phosphoprotein and that phosphoACF is restricted to nuclear 27S editosomes. ACF was phosphorylated on one or more serine residues under basal media conditions and the proportion of total cellular ACF that became phosphorylated increased upon ethanol or insulin treatment, stimuli both known to enhance editing activity. This suggests that ACF phosphorylation is metabolically regulated and a part of the mechanism for activating apoB mRNA editing regardless of whether APOBEC-1 expression is increased [insulin treatment, (17,22)] or not [ethanol treatment, (21)].

Recombinant APOBEC-1 and ACF purified from *Escherichia coli*, baculovirus or prepared from *in vitro* translation extracts can edit apoB RNA *in vitro* suggesting that phosphorylation is not essential for editosome assembly and editing activity. However, we argue that the efficiency of recombinant APOBEC-1 alone with recombinant ACF is entirely dependent on input protein concentration and the reaction has a very poor catalytic turnover, capable of only attomolar RNA substrate editing per hour (3,31,51). This is in sharp contrast to the highly efficient endogenous hepatic or intestinal cell editing activity. In fact, editing activity *in vivo* is regulated in a species- and tissue-specific manner, and inducible during development and in response to metabolic and hormonal perturbations (14,16) [and reviewed in (1)]. In this context, our data suggested that phosphorylation of ACF optimized and/or stabilized the functional interaction with APOBEC-1 in the nucleus leading to efficient editing activity. This mechanism explained how apoB mRNA editing activity can be activated metabolically or during development using pre-existing editing factors.

Under basal conditions, inhibition of PP1 activity resulted in the nuclear retention and increased recovery of phosphoACF and increased apoB mRNA editing activity, suggesting that ACF phosphorylation/dephosphorylation contributes to the modulation of editosome assembly and editing activity. Given that not all nuclear ACF is phosphorylated (Figures 3B and 4) and that not all ACF is assembled in 27S editosomes [Figure 4 and (12,24)] our data suggest that at any given time not all of the cellular ACF is involved in editing. This implies that there is a pool of ACF that can be used to rapidly modulate editosome assembly upon metabolic or hormonal stimuli or that ACF has additional roles in the cell.

In addition to editosome structure and function, ACF plays an important role in the cellular regulation of apoB mRNA editing through its trafficking activity between the cytoplasm...
and the nucleus (24–27,30). Although the site of apoB mRNA editing is within the cell nucleus (2,29) and takes place during or immediately after pre-mRNA splicing (2,28,38), APOBEC-1 and ACF are distributed in both the nucleus and cytoplasm (9,24,26,27,30). The data presented here suggest that nuclear retention/import of ACF was increased in ethanol or insulin treated hepatocytes through ACF phosphorylation. The mechanism for regulating APOBEC-1 and ACF trafficking are unknown, and the dependence of each protein’s trafficking on ACF–APOBEC-1 complex formation is controversial (25–27,30). Data from our laboratory suggest that APOBEC-1 has strong cytoplasmic retention signals, and that its nuclear import is mediated by interactions with ACF (25,30). We report that ACF and APOBEC-1 are present in both the cytoplasm and nucleus of editing competent cells, but that they only co-immunopurify from nuclear extracts. A small proportion of nuclear ACF was phosphorylated in non-stimulated hepatocytes (0.1 nM insulin) (Figures 3B and C) which increased several-fold upon insulin and ethanol stimulation (Figures 3B and C). These data suggested that a low level turnover of ACF phosphorylation maybe required to maintain basal apoB mRNA editing activity. Significantly, PP1 inhibition stimulated editing activity even under basal media conditions. The turnover of editing complexes has been suggested from studies that demonstrated nucleocytoplasmic shuttling of ACF and APOBEC-1 (26,27,30,36) and from in vitro studies of editosome assembly (12). Addition of ethanol (or its catabolite, acetaldehyde) or chemicals affecting protein kinases and phosphatases to nuclear extracts did not affect in vitro editing activity or ACF phosphorylation (data not shown). These data indicate that intact cell signal transduction cascades are required for the regulation of ACF phosphorylation and apoB mRNA editing. The identification of PP1 as a candidate phosphatase involved in regulating phosphate turnover on ACF is relevant, as high levels of PP1 are present in rat hepatocyte nuclei (52) placing it theoretically within the general domain of nuclear PP1. The ability of phosphatase action to affect mRNA editing activity is controversial (25–27,30). Data from our laboratory suggest that APOBEC-1 may have two sites of phosphorylation which had opposing effects on editing activity (35). APOBEC-1 has never been validated as a phosphoprotein through metabolic labeling studies similar to those reported here because the expression of endogenous APOBEC-1 is prohibitively low. We evaluated phosphorylation of APOBEC-1 overexpressed in a stable McArde cell line under basal and ethanol stimulated conditions and found no evidence for radiolabeling during a 4 h incubation period (data not shown). If APOBEC-1 is a phosphoprotein, the sites of phosphorylation may not be subjected to acute regulation and in fact Ser47 and Ser72, which were proposed to be phosphorylated in human APOBEC-1, are not conserved in rat APOBEC-1. The expression level of, and availability of high titre antibodies against, two APOBEC-1 homologs namely activation induced deaminase (AID) and APOBEC-3G have made equivalent studies possible and both proteins were identified as phosphoproteins (54,55). Consequently, phosphorylation of APOBEC-1 and its role in regulating editing activity remains a formal possibility.

In conclusion, regulation of hepatic apoB mRNA editing by ethanol and insulin promotes serine phosphorylation of ACF and its localization to active nuclear 27S editosomes. The data support a role for the metabolic regulation of ACF phosphorylation that promotes its interaction with APOBEC-1, in editosome assembly, as well as ACF nuclear retention/import. Thus, phosphorylation of ACF adds a new level of understanding of the control mechanisms cells use to modulate apoB mRNA editing in the context of current models of editosome composition and assembly.

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