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

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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 traffick 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 an increase in nuclear localization of ACF (24,29,32).

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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.

McArdle 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 ^{32}P labeling was performed by intraperitoneal injection of rats with 12.5 mCi of orthophosphoric acid (10 mCi/ml $^{32}PO_4$; 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 $MgCl_2$ 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 ^{32}P-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* IC_{50} 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 cantharadin 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.

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 $MgCl_2$ 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 $MgCl_2$, 0.1 mM $ZnCl_2$, 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 μg nuclear or 340 μg cytoplasmic extract in two-dimensional (2D) loading buffer (7.5 M urea, 1.0 M thiourea, 1% CHAPS, 58 mM DTT and 0.2% biolytes) and electrophoresed to equilibrium.

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.

Two-dimensional phosphoamino acid analysis

ACF was immunopurified using the ACF CT antibody from 27S enriched nuclear extracts of ^{32}P-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). Lyophilized hydrolysates were spiked with unlabeled phosphoserine, phosphothreonine and phosphotyrosine (Sigma) and resolved on thin layer chromatography plates (Merck, Germany) by 2D electrophoresis using an HTLE 7000 peptide mapping system (CBS Scientific Co. Del Mar, CA) (39). The migration of the unlabeled standards and ACF radiolabeled amino acid(s) were visualized by ninhydrin staining and PhosphorImager Scanning densitometry, respectively.

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.

Given the selective recovery of APOBEC-1 with nuclear ACF (Figure 1), we investigated whether the interaction between ACF and APOBEC-1 in nuclear extracts was mediated by post-translational modifications such as protein phosphorylation. Treatment of nuclear extracts with alkaline phosphatase (10 U CIAP) resulted in a 3-fold reduction in HA-tagged APOBEC-1 recovered with immunoprecipitated ACF (Figure 2A).

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

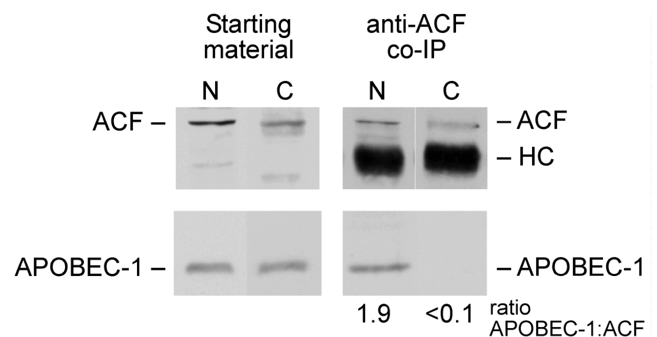

Figure 1. Hepatic APOBEC-1 co-immunopurifies with nuclear ACF. A McArdle cell line overexpressing HA-tagged APOBEC-1 was treated with 0.9% ethanol for 4 h and fractionated into cytoplasm and nuclear extracts. Nuclear (N) and cytoplasmic (C) extracts were western blotted with the ACF NT (top left panel) 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 re-probed 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.

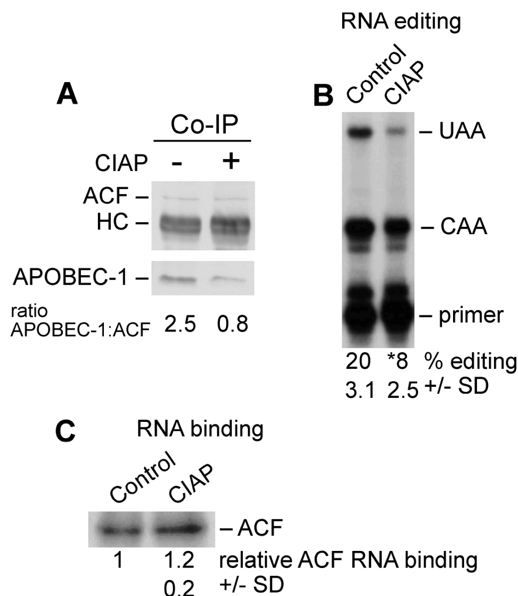

Figure 2. Phosphatase treatment inhibits *apoB* mRNA editing. (**A**) Co-immunoprecipitation: The HA-tagged APOBEC-1 overexpressing McArdle cell line was treated with ethanol for 4 h and fractionated. Nuclear extracts were immunoprecipitated with ACF CT antibody and processed as described in Figure 1. For CIAP treatment extracts were adjusted to 5 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1 mM $ZnCl_2$ and 25% glycerol and then incubated with 5 U CIAP for 1 h at 30°C. Control extracts were treated similarly but lacked phosphatase. The ratios of APOBEC-1:ACF were determined by scanning densitometry and quantitation using ImageJ Software. HC, position of Ig heavy chain. ($n = 4$, SD ±0.2). (**B**) *In vitro* editing activity: Liver nuclear extract was treated with CIAP as described above. *In vitro* editing activity was determined using the poisoned-primer extension assay. The percent editing was quantified by PhoshorImager (Molecular Dynamics) scanning densitometry. ($n = 4$, SD ± 0.2, $p \leqslant 0.01$). (**C**) RNA binding: ACF RNA binding activity was determined by ultraviolet light induced cross-linking of liver nuclear extracts. Quantitation of relative amounts of ACF bound was performed using PhoshorImager scanning densitometry. ($n = 4$, SD ± 0.2, $p \leqslant 0.01$).

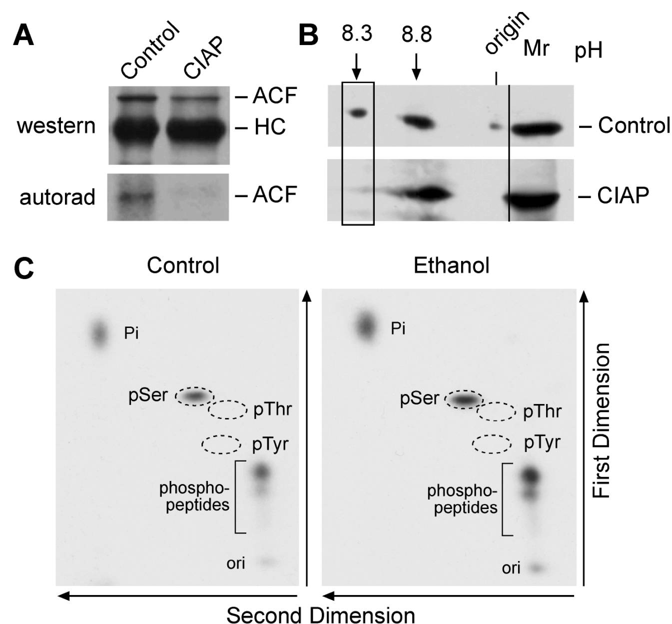

Figure 3. Hepatic ACF is a phosphoprotein. (**A**) Hepatic ACF is phosphorylated *in vivo:* ACF was immunopurified from radiolabeled 27S containing glycerol gradient fractions (i.e. editosomes) and incubated with 100 U CIAP or buffer alone (control). Membranes were exposed to X-ray film (autorad) and subsequently reacted with ACF NT antibody (western). HC, position of Ig heavy chain. Data shown are representative of three independent experiments. (**B**) Nuclear ACF contains more than one phosphorylated 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 immunoprecipitated 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.

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 C_θ 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*, rats were radiolabeled for 4 h via an intraperitoneal injection of orthophosphoric acid in HEPES-buffered saline. Following extensive digestion of hepatic nuclear extracts with DNase I and RNase T1 to remove radiolabeled nucleic acids, ACF was immunoprecipitated with the CT antibody, resolved by SDS–PAGE, transferred to nitrocellulose and analyzed by autoradiography as well as immunoblotting with ACF NT antibody (Figure 4A). A single band was detected by autoradiography that super-imposed with ACF-specific immunoblot reactivity. Greater than 90% of the ^{32}P-label was removed by CIAP treatment (Figure 4B), consistent with the post-translational phosphorylation of protein. Cytoplasmic

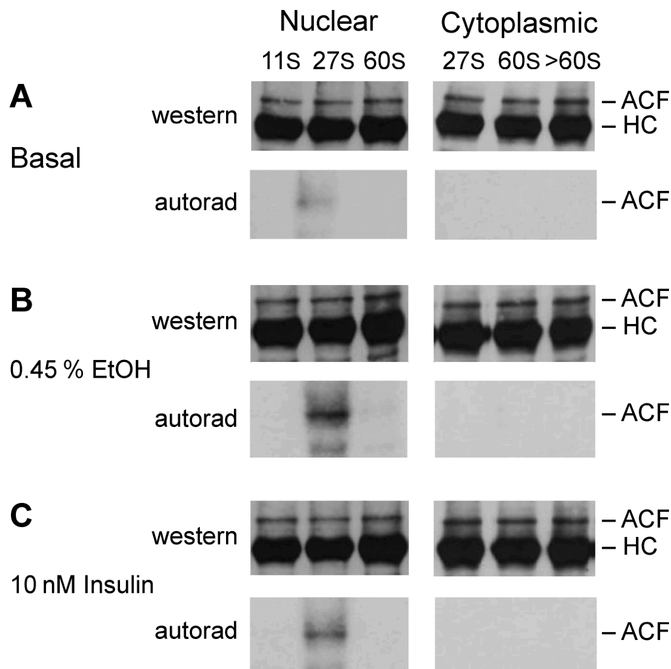

Figure 4. Phosphorylation of ACF is metabolically regulated and is localized to nuclear active 27S editosomes: Cytoplasmic and nuclear extracts were prepared from rat primary hepatocytes that had been labeled with 0.5 mCi $^{32}PO_4$ for 4 h in the presence of (**A**) basal 0.1 nM insulin, (**B**) 0.45% 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.

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 (pI) 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 pI (43). Nuclear ACF was detected as two predominant charge isoforms (Figure 3B). The major isoform migrated at pI 8.8 and is likely unmodified ACF or ACF containing both acidic and basic modifications. A second, less abundant, isoform migrated with a pI 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 (pI 8.3) and concomitant increase in the pI 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 pI 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 phospho-ACF 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

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 traffick to the cytoplasm, we reasoned that inhibition of the appropriate protein phosphatase would result in a nuclear accumulation of phosporylated 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 IC_{50} 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% ($P \leqslant 0.01$) in hepatocytes treated with okadaic acid at concentrations 10-times the IC_{50} for PP1 (Table 1). Similarly, treatment with endothall at a concentration 10-times the IC_{50} for PP1 stimulated editing to statistically significant levels ($P \leqslant 0.01$) (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.

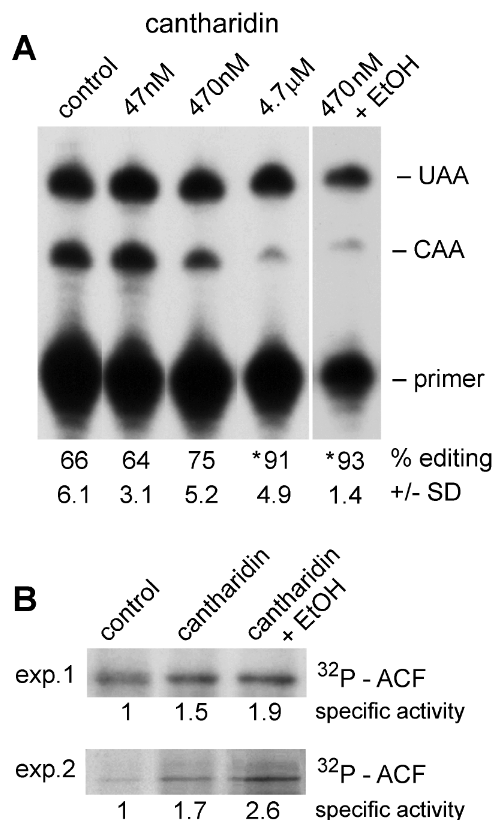

Figure 5. Protein phosphatase I inhibitors modulate *apoB* mRNA editing and ACF phosphorylation. (**A**) RNA Editing: rat primary hepatocytes were incubated with concentrations of cantharidin ranging from 47 nM (IC_{50} of PP2A) to 4.7 μM (10 times IC_{50} PP1) and 0.45% ethanol where indicated. *In 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 $P \leqslant 0.01$ by unpaired *t*-test relative to DMSO control $n > 5$. (**B**) ACF phosphorylation and specific activity: *in vivo* ACF ^{32}P 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 ^{32}P 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. *Statistical significance was determined to be $P \leqslant 0.01$ by unpaired t-test relative to DMSO control when $n \geqslant 3$.

	Mean	STD	n
Okadaic acid[a]			
Primary hepatocytes	66.3	2.8	5
DMSO	68.5	4.2	5
1 nM	71.2	2.0	3
10 nM	73.0	3.1	3
100 nM	91.4*	3.4	4
Endothall[b]			
Primary hepatocytes	65.2	1.9	4
DMSO	69.8	3.0	4
100 nM	66.5	4.1	4
500 nM	65.2	6.0	4
5 μM	63.7	2.8	3
50 μM	83*	3.0	4
Cyclosporin A[c]			
Primary hepatocytes	66.2	1.3	6
DMSO	66.4	4.2	6
5 nM	66.2	4.2	4
50 nM	66.8	6.6	5
500 nM	71.8	3.8	4
Cypermethrin[d]			
Primary hepatocytes	64.9	–	2
DMSO	63.1	–	2
50 pM	69.4	–	2
5 nM	65.9	–	2

[a]IC_{50}; PP1 = 10–15 nM, PP2A = 0.1 nM
[b]IC_{50}; PP1 = 5 μM, PP2A = 90 nM
[c]IC_{50}; PP2B = nM, range
[d]IC_{50}; PP2B = 40 pM

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 dephosphorylation prevents its nuclear export.

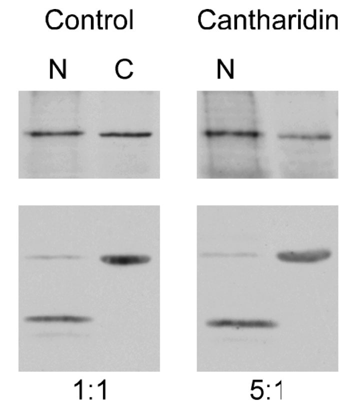

Figure 6. Inhibition of PP1 results in nuclear accumulation of ACF: Rat primary hepatocytes were incubated with 470 nM cantharidin for 4 h and fractionated. Nuclear (N) and cytoplasmic (C) extracts were western blotted for ACF and subsequently for actin and H1 histone. N/C ratio were determined by normalizing N ACF to the corresponding recovery of H1 histone and C ACF was normalized to corresponding recovery of actin for each sample using scanning densitometry of non-saturated immunoblots. Data shown are representative of four independent experiments (SD ±2.1-fold for cantharidin).

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. Our data suggest that nuclear retention/import of ACF is increased in ethanol or insulin treated hepatocytes through modulation of ACF phosphorylation state. We propose a model in which phosphorylation of ACF results in its nuclear accumulation and enhances or stabilizes APOBEC-1 nuclear retention and ACF binding, leading to increased editing activity. In support of this model, phosphatase treatment of cell extracts was associated with reduced co-immunoprecipitation of APOBEC-1 with ACF and reduced editing activity.

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) of which 90% was associated with chromatin (53). Nuclear ACF is also associated with chromatin (24) placing it theoretically within the general domain of nuclear PP1. The ability of phosphoACF to serve as substrate for PP1 remains to be formally addressed.

The lack of labeled ACF in the cytoplasm also suggests that ACF is dephosphorylated prior to or during nuclear export. These data suggest an interesting hypothesis: if phosphorylation of ACF is restricted to the nucleus and associated with enhanced editing activity, then dephosphorylation of ACF might regulate its nuclear export. Given that ACF binds to both unedited and edited *apoB* mRNA (6) and that dephosphorylated ACF binds to *apoB* RNA. ACF is likely to remain bound to *apoB* mRNA and co-export to the cytoplasm following *apoB* mRNA editing and ACF dephosphorylation. In this scenario, the regulation of ACF dephosphorylation would modulate *apoB* mRNA export to the cytoplasm in addition to protecting edited *apoB* mRNA from nonsense mediated decay (NMD) (31).

Under basal conditions, 2D gel electrophoresis analyses suggested that ACF contained 2–3 phosphates. 2D phosphoamino acid analyses indicated serines were the residues phosphorylated following metabolic stimulation. ACF phosphorylation and whether these sites are the same as the 'basal' phosphorylation sites or are additional sites of phosphorylation remains to be determined. Although parallels were observed when comparing ethanol with insulin editing induction (i.e. ACF accumulation in the nucleus and ACF hyperphosphorylation), we cannot assume that the same sites of ACF phosphorylation are involved or that the phosphorylation state of additional editing factors is not affected. In fact, previous studies involving alanine and aspartic acid site-specific mutagenesis of predicted serine phosphorylation sites suggested that APOBEC-1 may have two sites of phosphorylation which had opposing effects of 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 McArdle 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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