Phosphorylation is a regulatory mechanism in apolipoprotein B mRNA editing

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The editing of apolipoprotein B (apoB) mRNA is under tissue-specific, developmental and metabolic regulation. We found that multiple protein kinase inhibitors or activators increased apoB mRNA editing up to 2.5-fold in Caco-2 cells and 3–8-fold in McA7777 and FAO rat cells respectively. The phosphorylation-agent-induced modulation is independent of the apolipoprotein B editing catalytic subunit 1 (APOBEC-1) and of apoB mRNA expression levels, indicating the involvement of a protein modification, such as phosphorylation, regulating the cellular editing of apoB mRNA. Transient expression of protein kinase C- θ more than doubled apoB mRNA editing in FAO cells. Chronic exposure to ethanol, a treatment known to increase the expression of protein kinases and to change protein phosphorylation status, increased apoB mRNA editing in FAO cells up to 2.5-fold without increasing the mRNA abundance of APOBEC-1. The

elimination of potential phosphorylation sites 47 and 72 of human APOBEC-1 decreased its activity to approx. one-eighth of control levels by a Ser⁴⁷ → Ala mutation, but more than doubled the activity by a Ser⁷² → Ala mutation. The activity modulation was reversed by a Ser → Asp mutation at sites 47 and 72, which introduced a phosphorylation-like carbonic acid group. Both human APOBEC-1 dephosphorylated by alkaline phosphatase and the Ser^{47,72}-to-alanine double mutant protein demonstrated a shifted isoelectric focusing pattern compared with the wild type, indicating phosphorylation at these sites. Taken together, these results suggest that phosphorylation might be an important mechanism in the regulation of apoB mRNA editing.

Key words: APOBEC-1, kinase, mutation, quantification.

INTRODUCTION

A large hydrophobic protein synthesized in mammalian liver and small intestine, apolipoprotein B (apoB) has a central role in the assembly, secretion and metabolism of both triglyceride-rich lipoproteins (chylomicrons and very-low-density lipoproteins) and low-density lipoprotein [1]. ApoB mRNA editing is a site-specific deamination reaction that converts a single C base into U in the glutamine codon (CAA) at amino acid position 2153 to form an in-frame stop codon (UAA), resulting in the translation of a truncated protein, apoB-48 [2,3]. ApoB-48 is collinear with the N-terminal half of apoB-100, but lacks the domains that mediate interaction with the low-density lipoprotein receptor. As a result, lipoprotein particles containing apoB-48 are directed to a different metabolic pathway and undergo more rapid catabolic clearance than particles containing the full-length protein, apoB-100. The amount of edited apoB mRNA is an important determinant of the proportion of apoB-100 and apoB-48 in the metabolism of total apoB [4], low-density lipoprotein and very-low-density lipoprotein [5].

ApoB mRNA editing is an exquisitely precise process under physiological conditions. The site-specific deamination reaction is catalysed by an enzyme complex that recognizes the defined sequence elements flanking the edited base [6]. The enzyme complex consists of a catalytic subunit designated APOBEC-1 (for apolipoprotein B editing catalytic subunit 1) and other incompletely identified auxiliary proteins [7–9]. APOBEC-1 alone cannot catalyse the editing of apoB mRNA *in vitro* in the absence of auxiliary proteins [10]. The putative auxiliary proteins have a widespread distribution and can be detected in extracts from cells that do not synthesize apoB mRNA [7,11]. APOBEC-1 com-

plementation factor ('ACF') has been cloned recently [12,13]. ACF and APOBEC-1 comprise the minimal protein requirements for the specific and efficient editing of apoB mRNA *in vitro*. Other candidate auxiliary proteins such as the heterogenous nuclear ribonucleoprotein A/B analogue ABBP-1 [14] and heterogenous nuclear ribonucleoprotein C [15], mooring-sequence selective RNA binding proteins of 100 and 55 kDa and a general RNA-binding protein of 40–44 kDa [8,16] have been identified through their affinity for APOBEC-1 or apoB RNA. A protein complex referred to as AUX240, identified by using monoclonal antibodies raised against a 27 S editing complex assembled *in vitro*, has also been proposed to contain auxiliary proteins [17]. The specific functional role that each candidate auxiliary protein might have in apoB mRNA editing remains to be clarified further.

ApoB mRNA editing *in vivo* is under tissue-specific, developmental and metabolic regulation [18–20]. ApoB mRNA editing occurs predominantly in the human intestine. Although the human liver is a major site of apoB expression, apoB mRNA editing does not occur in human liver owing to the absence of APOBEC-1 expression [21]. In contrast, editing is present in both rat liver and small intestine [18]. ApoB mRNA editing in rat hepatocytes can be significantly modulated by a variety of dietary and hormonal interventions, including treatment with ethanol [22,23], treatment with thyroid hormone [24], fasting and carbohydrate refeeding [25], treatment with a high dose of oestrogen [26], treatment with growth hormone [27] and chronic treatment with insulin [28]. A close correlation between APOBEC-1 expression levels and the proportion of edited apoB mRNA during fasting and carbohydrate refeeding suggests that the regulation of apoB mRNA editing in these settings might be

Abbreviations used: apoB, apolipoprotein B; APOBEC-1, apolipoprotein B editing catalytic subunit 1; β_2 M, β_2 -microglobulin; PKC, protein kinase C; RT-PCR, reverse-transcriptase-mediated PCR.

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largely due to the abundance of APOBEC-1 [18]. However, several studies have indicated that apoB-mRNA-editing activity can be modulated independently of APOBEC-1 expression. For example, the expression of APOBEC-1 mRNA in rat liver is unaltered despite significantly increased apoB-mRNA-editing activity induced by chronic treatment with ethanol or thyroid hormone [22,24]. The rat liver protein extracts prepared from adult animals treated with thyroid hormone demonstrate an increased apoB-mRNA-editing activity *in vitro*; the editing activity is not increased by additional APOBEC-1 protein [24]. In addition, apoB mRNA editing in both human and rat small intestine is significantly developmentally regulated and also does not correlate well with the expression level of APOBEC-1 [18,19].

The molecular basis of the hormonal, metabolic and developmental regulation of apoB mRNA editing remains largely unknown. As apoB mRNA editing is achieved through a deamination reaction catalysed by a multiprotein complex, editing regulation can be achieved through altering the levels of requisite protein(s) and/or altering protein enzyme activity by protein modification without a protein level change. The most common mechanism of protein modification is phosphorylation, which can potentially alter protein enzyme activity and protein-protein interactions [29]. An involvement of a process such as phosphorylation is suggested by the following observations: (1) rat hepatocytes cultured in hyperosmotic medium increase apoB mRNA editing independently of any transcriptional or translational mechanism [30]; (2) transient treatment of McA7777 rat hepatoma cells with ethanol increases nuclear apoB mRNA editing in as little as 15 min without affecting APOBEC-1 expression [31]; and (3) Ca^{2+} , a potential activator of protein kinases, also increases apoB mRNA editing in cultured cells [32].

Here we show that cellular apoB mRNA editing can be modulated by the administration of protein kinase inhibitors or activators, by the expression of protein kinase C (PKC) isoenzymes and by chronic treatment with ethanol, which is known to alter protein kinase activity. The editing activity of APOBEC-1, the catalytic component of the editing enzyme complex, can also be significantly altered by eliminating or mimicking potential phosphorylation sites. These results suggest that phosphorylation might be an important mechanism in the regulation of apoB mRNA editing.

MATERIALS AND METHODS

Cell culture and treatment

The human colon adenocarcinoma cell line Caco-2, the human liver hepatoblastoma cell line Hep G2 and the rat hepatoma cell lines FAO and McA7777 were obtained from A.T.C.C. (Manassas, VA, U.S.A.). Caco-2 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) fetal bovine serum. Hep G2 cells were maintained in Eagle's minimum essential medium containing 10% (v/v) fetal bovine serum and 2 mM glutamine. McA7777 cells were maintained in DMEM containing 20% (v/v) horse serum and 5% (v/v) fetal bovine serum on a plate precoated with collagen (Boehringer Mannheim). FAO cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal bovine serum. The cell treatments by protein kinase inhibitors or activators or phosphatase inhibitor were conducted by changing the cells to a fresh medium containing the phosphorylation agents and incubating them at 37 °C for 6 h or for the durations indicated. Caco-2 cells were cultured for 14 days on plastic tissue-culture plates (Corning) before the treatment. The agents were dissolved in either water or DMSO, and the final concentration of DMSO in the medium was

no more than 0.1% (v/v). The control tests for the solvent were performed with 0.1% (v/v) DMSO; no effect was observed on apoB mRNA editing.

For chronic treatment with ethanol, FAO cells were cultured in six-well plates unsealed [33] or sealed with Parafilm [34] in media containing the indicated amount of ethanol; the media were changed every day. Similar effect on apoB mRNA editing was observed with the unsealed or sealed plates. After treatment, the cells were washed and total cellular RNA was isolated by Trizol reagent (Gibco BRL) in accordance with the manufacturer's instructions.

ApoB mRNA editing assay

Endogenous editing of apoB mRNA in cells was determined by primer extension after amplification by reverse-transcriptase-mediated PCR (RT-PCR) as described previously [20]. Total cellular RNA samples were pretreated with DNase RQ1 (Worthington) and Sau 3AI (Gibco BRL). The first-strand cDNA was generated with a random primer by using reverse transcriptase from Moloney-murine-leukaemia virus. The PCR was performed for 35 cycles of 40 s at 94 °C, 1 min at 58 °C, 2 min at 72 °C (for human apoB) and 5 cycles of 1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C followed by 32 cycles of 1 min at 94 °C, 1 min at 56 °C, 2 min at 72 °C (for rat apoB). The resultant PCR amplicons (415 and 297 bp for human and rat apoB respectively) were then purified with a GeneClean II kit (Bio 101) and annealed to an anti-sense ^{32}P -labelled oligonucleotide (apoB-extension primer) at 42 °C for 1 h. Primer extension was performed at 42 °C for 10 min. The primer extension products were then resolved on an 8% (w/v) polyacrylamide/urea gel, and the ratio of edited to unedited apoB mRNA was determined with a PhosphorImager.

Construction of PKC isoenzymes and transfection in FAO cells

Full-length human PKC- α , β II and δ were amplified by PCR from cDNA of K562 cells (human erythroleukaemia cell line) with sense and anti-sense primers containing *Kpn*I and *Xba*I restriction enzyme sites respectively, then cloned into a pHOOK-3 expression vector (Invitrogen). Human PKC- ϵ , θ and ζ and PKC protein inhibitor were purchased from Invitrogen and subcloned into pHOOK-3 after re-amplification by PCR with primers containing *Kpn*I and *Xba*I restriction enzyme sites. The gene constructs were confirmed by sequencing analysis and transfected into FAO cells with lipofectin (Gibco BRL) in accordance with the manufacturer's instructions. At 2 days after transfection, the cells expressing the genes were selected with Capture-Tec Beads (Invitrogen) on the basis of the co-expression of a single-chain antibody (sFv) on the surface of transfected cells in accordance with the manufacturer's instructions. The gene-expressed cells were bound to magnetic beads coated with antigen for sFv. Total cellular RNA was extracted from the selected cells by Trizol reagent (Gibco BRL) and the amount of apoB mRNA editing was determined.

Site-directed mutagenesis and expression in Hep G2 cells

Full-length APOBEC-1 cDNA was generated from total RNA extract of human small intestine by RT-PCR and cloned into the eukaryotic expression vector pCR3.1-Uni and pcDNA3.1(-)/Myc-His C (Invitrogen). Site-directed mutants of APOBEC-1 were constructed with a two-step PCR method [35] and were cloned into the same expression vectors as above. All constructs were confirmed by sequencing both strands. APOBEC-1 and mutant DNA constructs were transfected into Hep G2 cells by

Table 1 Primers used in this studyAbbreviations: subcloning primer, primer for PCR amplification of PKC- ϵ , θ and ξ and inhibitor (Invitrogen) for subcloning; S, sense; AS, anti-sense.

Designation	Sequence
APOBEC-1-outside 5' primer	5'-GAATTCGTGGGACAGAGCACCATGACTTCTGAGAA-3'
APOBEC-1-outside 3' primer	5'-GCCAGAGTAATACATATTTATTGTTGGTTAAGCTACAGAG-3'
3–13 deletion-S	5'-TGAATTCGTGGGACAGAGCACCATGACTCTGAGGAGAAGAATCGAAC-3'
Ser ^{3,8} → Ala-S	5'-TGAATTCGTGGGACAGAGCACCATGACTGCTGAGAAAGGCTCTGCAACCG-3'
Ser ⁴⁷ → Ala-S	5'-GAAATCAAGTGGGGCATGGCCCGGAAGATCTG-3'
Ser ⁴⁷ → Ala-AS	5'-AGATCTTCGGGCCATGCCCACTTGATTTCG-3'
Ser ⁵⁴ → Ala-S	5'-AAGATCTGGCGAACGGCAGGCAAAACACC-3'
Ser ⁵⁴ → Ala-AS	5'-GGTGTTCCTGCCGTCGCCAGATCTTC-3'
Thr ⁷¹ → Val-S	5'-TTTATAAAAAATTTGTGTGAGAAAGAGATTTTCAC-3'
Thr ⁷¹ → Val-AS	5'-ATCTCTTCTGACACAAATTTTTTATAAAATTAACCTC-3'
Ser ⁷² → Ala-S	5'-TTTATAAAAAATTTACGGCAGAAAGAGATTTTCAC-3'
Ser ⁷² → Ala-AS	5'-ATCTCTTCTGCCGTAATTTTTTATAAAATTAACCTC-3'
Ser ¹⁹⁶ → Ala-S	5'-CTGTTTAAAGATTGCAAGAGATGGCAAAATCATC-3'
Ser ¹⁹⁶ → Ala-AS	5'-TTGCCATCTCTTCTGCAATCTTAAACAGG-3'
Human β_2 M-S	5'-CATCCATCCGACATTGAAGTTGAC-3'
Human β_2 M-AS	5'-ATCCAATCCAAATGCGGCATCTTC-3'
β_2 M-competitor-AS	5'-ATCCAATCCAAATGCGGCATCTTCTATCTTGGGCTGGACAAAGTC-3'
Human APOBEC-1-S (nt 152–174)	5'-TGGGGCATGAGCCGGAAGATCTG-3'
Human APOBEC-1-AS (nt 512–534)	5'-GGGTATTGTGGCCAGTGAGCTTC-3'
APOBEC-1-competitor-AS	5'-GGGTATTGTGGCCAGTGAGCTTCTGATGCTCTCATAATCTG-3'
Human apoB-S	5'-CTGGGAAACTCCACAGCAAG-3'
Human apoB-AS	5'-CCACATTTTGAATCCAGGATGCAG-3'
ApoB-extension-primer	5'-TATCTTAATATACTGATC-3'
ApoB-competitor-AS	5'-CCACATTTTGAATCCAGGATGCAGGTAGATCATGGATTG-3'
PKC- α -S	5'-CGGGGTACCCCGGGGACCATGGCTGACGTTTCCCGGGCAACGAC-3'
PKC- α -AS	5'-GCTCTAGAGCTGAGTTTCACTAGCACTCTG-3'
PKC- β II-S	5'-CGGGGTACCCCGGGGCAAGATGGCTGAC-3'
PKC- β II-AS	5'-GCTCTAGAGCACATCTACTAGCTCTGAC-3'
PKC- δ -S	5'-CGGGGTACCCACCATGGCGCCGTCCTGCGCATC-3'
PKC- δ -AS	5'-GCTCTAGAGCTGTCCAGGAACCTCAATCTCCAG-3'
Subcloning primer (sense)	5'-CGGGGTACCCCGGAGACCCAAGCTGGCTAG-3'
Subcloning primer (anti-sense)	5'-GCTCTAGAGCGAATGGGTGACCTCGAAG-3'
Rat apoB-S	5'-AGAGGATCCCTGAGCAGGCTTCCTCAGCAG-3'
Rat apoB-AS	5'-TTAAGCTTCAATGATTCTATCAATAATCTG-3'
Rat apoB-extension-primer	5'-TATCTTAATATACTGATC-3'
Rat APOBEC-1-S	5'-CCCCGGGAACCTCGGAAAGAG-3'
Rat APOBEC-1-AS	5'-GGGGGTACCTTGCCCAATG-3'
Rat APOBEC-1-competitor-AS	5'-GGGTACCTTGCCCAATGAGCTTGCTCCGTCATGATCTGG-3'
Rat β_2 M-S	5'-AAGCCCAACTTCTCAACTGCTAC-3'
Rat β_2 M-AS	5'-GATGATTCAGAGCTCCATAGAGCTTG-3'
Rat β_2 M-competitor-AS	5'-GATGATTCAGAGCTCCATAGAGCTTGTTAACTCTGCAAG-3'

using lipofectin (Gibco BRL) by following the manufacturer's instructions. Stable transfectants were isolated by selection with 1 mg/ml Geneticin (Gibco BRL). The expression of the transfected genes was confirmed by the introduction of endogenous apoB mRNA editing in the cells and RT-PCR analysis for APOBEC-1 mRNA.

Protein preparation *in vitro* and analyses

The DNA constructs of mutant and native human APOBEC-1 used for protein expression in Hep G2 cells were transcribed and translated to generate ³⁵S-labelled proteins by TNT Quick coupled transcription and translation system (Promega) by following the manufacturer's instructions. Aliquots of the protein preparations were tested for apoB RNA editing activity *in vitro*, and the amounts of proteins made were quantified by SDS/PAGE [15% (w/v) gel] followed by PhosphorImager exposure. The apoB RNA editing assay was performed *in vitro* by incubating 10 fmol of a synthetic 419 nt apoB RNA (nt 6471–6889) with 5 μ l of sample and 30 μ g of chicken intestine enterocyte extract at 30 °C for 3 h in 20 mM Hepes (pH 7.9)/50 mM

KCl/10 mM EDTA/1 mM dithiothreitol/10% (v/v) glycerol containing 40 i.u. of RNase inhibitor in a final volume of 60 μ l, as described previously [36]. After reaction, the RNA was extracted and subjected to RT-PCR and primer extension as described above to determine the proportion of edited and unedited apoB mRNA species. The apoB RNA editing represented the editing activity of APOBEC-1 and mutants after being normalized to the same protein level.

Other aliquots were dephosphorylated by incubating the protein preparation with 500 units/ml calf intestinal alkaline phosphatase (New England Biolabs) at 30 °C for 30 min. The samples were diluted with 5% (w/v) SDS (3:7) and analysed by two-dimensional electrophoresis as described by O'Farrell [37] (Kendrick Labs). The isoelectric focusing was performed in glass tubes of inner diameter 2.0 mm containing 2% (v/v) Nonidet P40 and 2% (v/v) pH 3.5–10 Ampholines (Pharmacia) for 9600 V h. After equilibration for 10 min in 0.0625 M Tris/HCl (pH 6.8)/2.3% SDS/50 mM dithiothreitol/10% (v/v) glycerol, each tube gel was sealed to the top of a stacking gel and subjected to SDS/PAGE [10% (w/v) gel]. The resolved proteins were detected by exposure to film.

Quantification of mRNA abundance by competitive RT-PCR

Quantitative RT-PCR was conducted with a competitor DNA generated by internal deletion as internal standard, as described previously [38]. Total cellular RNA (3 μ g) was taken for each sample, and first-strand cDNA was generated with a random hexamer primer by using reverse transcriptase from Moloney-murine-leukaemia virus. The cDNA products were diluted 2-fold to 60 μ l with water. cDNA aliquots with the same volume were amplified by PCR for β_2 -microglobulin (β_2 M), APOBEC-1 and apoB in the presence of a selected concentration of competitor and 0.3 μ l of [α - 32 P]dCTP (3000 Ci/mmol, 10 Ci/ml; ICN). PCR amplification was performed in a RoboCycler Gradient-40 (Stratagene) as follows: (1) β_2 M, 2 min at 94 °C, 21 cycles of 30 s at 94 °C, 1 min at 62 °C for human β_2 M or 60 °C for rat β_2 M, 2 min at 72 °C and 10 min at 72 °C; (2) APOBEC-1, 2 min at 94 °C, 28 cycles (for Caco-2) or 22 cycles [for human liver hepatoblastoma Hep G2 cells stably expressing APOBEC-1 (APOBEC-Hep G2 cells)] of 30 s at 94 °C, 1 min at 63 °C, 2 min at 72 °C and 10 min at 72 °C or 2 min at 94 °C, 22 cycles of 30 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C and 10 min at 72 °C for rat APOBEC-1; and (3) apoB, 2 min at 94 °C, 19 cycles of 30 s at 94 °C, 1 min at 58 °C, 2 min at 72 °C and 10 min at 72 °C.

The DNA competitors used above were generated by PCR amplification of target cDNA with the target DNA sense primer and an anti-sense primer located approx. 45 nt upstream of the target DNA anti-sense primer site and tailed with the target DNA anti-sense primer [38]. Thus the resultant competitor amplicons had the same primer-binding sequence as target DNA, but were shorter than the target DNA by approx. 45 bp. To obtain an optimal working concentration of competitor, serial 10-fold dilutions were made from the gel-purified competitor. An aliquot (5 μ l) of the diluted competitor and individual samples were amplified simultaneously. A suitable concentration was chosen for the competitor that resulted in a band of similar intensity to the target band.

The resultant PCR products (10 μ l aliquot) were resolved by 4% (w/v) PAGE and quantified with a PhosphorImager. The ratio of target to competitor product represented the relative abundance of target mRNA in multiple samples after normalization to the same β_2 M level. For normalization of the apoB-mRNA-editing activity in Hep G2 cells induced by APOBEC-1 and mutants relative to the expression levels of both β_2 M and APOBEC-1 mRNA, the editing activity value was divided by the corresponding ratio of β_2 M, multiplied by the ratio mean value of β_2 M in wild-type APOBEC-1 control group, and then repeated in the same way for APOBEC-1 mRNA levels. The resulting value represents the APOBEC-1 editing activity in Figure 7.

Oligonucleotides

The oligonucleotide primers used in APOBEC-1 site-directed mutagenesis, PKC isoenzyme cloning and RT-PCR quantification are listed in Table 1.

RESULTS

Multiple protein kinase inhibitors and activators increase apoB mRNA editing in cultured cells

The possible role of protein phosphorylation in modulating apoB mRNA editing was first investigated by determining the effect of phosphorylation-regulating agents, i.e. protein kinase

Figure 1 Effect of phosphorylation agents on apoB mRNA editing and mRNA expression in Caco-2 cells

(A) Editing of apoB mRNA in the cells. Caco-2 cells 14 days old were exposed for 6 h at 37 °C to a fresh medium containing H-7 (50 μ M), PMA (50 ng/ml), H-89 (50, 20 or 1 μ M), forskolin (50 μ M), A3 (40 μ M), KN-93 (20 μ M), okadaic acid (75 ng/ml), bisindolylmaleimides (0.5 μ M), Ro-31-8220 (5 μ M) or thymeleatoxin (0.5 μ M). After treatment, total cellular RNA was isolated and analysed for apoB mRNA editing as described in the Materials and methods section. (B, C) Expression levels of APOBEC-1 mRNA (B) and apoB mRNA (C) in the cells. Quantitative RT-PCR was performed for the same samples as in (A) for β_2 M, APOBEC-1 and apoB mRNA. The APOBEC-1 and apoB mRNA expression levels were normalized relative to the β_2 M level. Values are means $\pm$ S.D. and represent triplicate determinations of three different extract preparations. * P < 0.001 compared with control.

inhibitors or activators and a phosphatase inhibitor, on apoB mRNA editing in cultured cells, including the human colon adenocarcinoma cell line Caco-2, rat hepatoma FAO cells and McA7777 cells. As shown in Figure 1(A), multiple phosphorylation agents had significant stimulatory effects on apoB mRNA editing in Caco-2 cells. The Caco-2 apoB mRNA editing was increased 1.8–2.5-fold by the protein kinase inhibitors H-7, H-89, A3 and KN-93 [39]. The editing was also increased 1.3–2-fold by

Figure 2 Time course and dose dependence of apoB mRNA editing after treatment with H-7 in APOBEC-Hep G2 cells

APBEC-Hep G2 cells were incubated in media containing different concentrations of H-7 at 37 °C for the indicated duration; apoB mRNA editing was analysed. The values are means and represent duplicate determinations of two different extract preparations.

the protein kinase activators forskolin [40], PMA [41] and thymeleatoxin [42], whereas okadaic acid [43] and the PKC inhibitors bisindolylmaleimides and Ro-31-8220 [44] had no obvious effect. Similarly to Caco-2, APOBEC-Hep G2 cells also showed an up to 3.5-fold increase of apoB mRNA editing by H-7, H-89, KN-93, PMA and thymeleatoxin (results not shown). These stimulatory effects on apoB mRNA editing were the result of cellular modulation, because no effect was observed for the agents when using an *in vitro* system (results not shown).

H-7 is a kinase inhibitor with inhibition constants (K_i) of 6 μ M, 3 μ M and 5.8 μ M for PKC, cAMP-dependent kinase and cGMP-dependent kinase respectively [39]. The time and dose dependence of apoB mRNA editing for phosphorylation agents was evaluated with H-7 in APOBEC-Hep G2 cells, which, unlike Caco-2 cells, have apoB mRNA editing independently of cell culture age. As shown in Figure 2, the editing was increased after 2 h of treatment and a plateau was reached after 6 h. An effect on apoB mRNA editing was observed at concentrations as low as 10 μ M H-7. The comparability between the inhibition constants and the concentrations that exert a significant effect on apoB mRNA editing suggests that the H-7 effect on editing is achieved predominantly through a cellular phosphorylation alteration.

The study on rat hepatoma cells (Figure 3) showed that apoB mRNA editing increased 6.5-fold and 8-fold in FAO and 2-fold and 3-fold in McA7777 cells after exposure to H-7 and H-89 respectively. H-89 is an inhibitor derived from H-7 that more potently inhibits cAMP- and cGMP-dependent kinases, with K_i values of 0.05 and 0.5 μ M respectively. H-89 can also inhibit PKC at a high concentration with a K_i of 30 μ M [39]. No significant effect was observed with H-89 at concentrations between 0.3 and 15 μ M, concentrations selected for the inhibition of cAMP- and cGMP-dependent kinases in both FAO and McA7777 cells. Multiple protein kinase inhibitors and activators had effects on apoB mRNA editing in the human cell lines Caco-2 and APOBEC-Hep G2. In contrast, only H-7 and H-89 had significant effects in rat FAO and McA7777 cells. Taken together, these results demonstrated that apoB mRNA editing in cultured cells from both human and rat can be modulated by protein kinase inhibitors or activators that alter protein phosphorylation. The selective effect of H-7 and H-89 in rat cells and the overall differential magnitudes of response indicate that there are species-specific and tissue-specific differences in the response to these agents.

Figure 3 Effect of phosphorylation agents on apoB mRNA editing in rat hepatoma FAO and McA7777 cells

FAO (A) and McA7777 (B) cells were treated with protein kinase inhibitors or activators or phosphatase inhibitor as in Figure 1(A). Total RNA was extracted and analysed for apoB mRNA editing by primer extension. Values are means $\pm$ S.D. and represent triplicate determinations of three different extract preparations.

To determine whether changes in mRNA expression level are responsible for the observed effects of the phosphorylation agents, the mRNA levels of APOBEC-1 and apoB were quantified by RT-PCR. As shown in Figure 1, H-7, H-89, A3 and KN-93 significantly increased apoB mRNA editing without altering the APOBEC-1 expression level in Caco-2 cells. PMA and thymeleatoxin increased apoB mRNA editing by approx. 30% and increased APOBEC-1 mRNA expression 2-fold and 3-fold respectively. Forskolin increased apoB mRNA editing 2-fold, with an approx. 2-fold decrease in APOBEC-1 mRNA expression level. Consequently, the results suggest that there is not a good correlation between the editing activity and mRNA level changes in these phosphorylation-altering treatments. No significant changes were observed for apoB mRNA levels, except for those of forskolin and A3, which decreased the total level of apoB mRNA while increasing the apoB mRNA editing in Caco-2 cells (Figure 1C). Taken together, these results demonstrate that multiple protein kinase inhibitors or activators can modulate apoB mRNA editing without a direct relationship to altering APOBEC-1 or apoB mRNA levels, and suggest that a mechanism such as altered phosphorylation status is responsible for the increased apoB mRNA editing.

Figure 4 Effect of transiently transfected PKC isoenzymes on apoB mRNA editing in FAO cells

PKC- α , β II, δ , ϵ , θ and ζ and PKC inhibitor were transiently transfected into FAO cells with lipofectin. The transfected cells were selected with co-expressed sFV on cell surface and analysed for apoB mRNA editing as described in the Materials and methods section. (A) Representative primer extension assay for apoB mRNA editing. (B) ApoB mRNA editing in cells with transfected PKC isoenzymes. Values are means $\pm$ S.D. and represent triplicate determinations of three different extract preparations.

Transient expression of PKC- θ increases apoB mRNA editing in FAO cells

The 6.5-fold and 8-fold stimulation effect of apoB mRNA editing by the protein kinase inhibitors H-7 and H-89 in FAO cells (Figure 4) suggest that phosphorylation might be important in regulating rat apoB mRNA editing. The stimulatory effects by H-89 were observed only with a concentration higher than the inhibition constant against PKC, suggesting that one or more PKC family members act as regulatory factors. In contrast, no effect was observed with other PKC inhibitors, bisindolylmaleimides and Ro-31-8220, and the PKC activators PMA and thymeleatoxin, indicating that the molecular basis for the stimulation by H-7 and H-89 of apoB mRNA editing might not be a simple inhibition of PKC. To verify the possible involvement of protein phosphorylation in modulating rat apoB mRNA editing, members of the human PKC family expression genes, including α , β II, δ , ϵ , θ and ζ and p53-regulated inhibitor, were transiently transfected in FAO cells. The cells expressing these genes were selected with co-expressed sFV on the cell surface and the editing of apoB mRNA in the cells expressing the gene was analysed. As shown in Figure 4, the transient expression of PKC- θ resulted in a greater than 2-fold increase in apoB mRNA editing in FAO

cells, whereas the other PKC isoenzymes had no effect. These results provide additional evidence that protein phosphorylation can modulate apoB mRNA editing in FAO cells.

Chronic treatment with ethanol increases apoB mRNA editing in FAO and McA7777 cells without corresponding changes in APOBEC-1 mRNA levels

Chronic treatment of rats with ethanol increases apoB mRNA editing in liver to 100% [22]. Chronic treatment of cultured cells with ethanol has also been reported to increase the expression of different PKC isoforms [33], to cause the translocation of PKC [45] and the catalytic subunit of cAMP-dependent protein kinase to the nucleus [46,47], and to enhance the activation of mitogen-activated protein kinases by growth factors [34]. The effect of chronic treatment with ethanol on apoB mRNA editing was examined in FAO cells to determine whether there was any regulatory effect on apoB mRNA editing under conditions known to alter cellular phosphorylation. FAO cells were incubated with increasing concentrations of ethanol (0–200 mM) for 2 or 4 days. The apoB mRNA editing increased in a dose-dependent manner up to 2.5-fold (Figure 5). An increase in apoB mRNA editing was observed at an ethanol concentration as low as 25 mM. Quantification of APOBEC-1 and apoB mRNA expression levels showed that there was no significant change in the normalized APOBEC-1 and apoB mRNA abundances for either samples treated for 2 or 4 days except for that with 200 mM ethanol for 4 days, which produced an approx. 35% increase in APOBEC-1 mRNA (Figure 5B). However, the increase in APOBEC-1 mRNA accounted for only a small portion of the change in apoB mRNA editing in the cells, because the apoB mRNA editing increased 2.5-fold under these conditions. A similar chronic effect of ethanol was also observed in McA7777 cells (results not shown). Taken together, these results demonstrate that apoB mRNA editing in FAO cells increased without a corresponding APOBEC-1 and apoB mRNA change after chronic treatment with ethanol. Because treatment with ethanol is known to alter protein phosphorylation, these results provide additional support to the contention that phosphorylation is involved in regulating apoB mRNA editing.

APOBEC-1 editing activity is altered by the elimination of potential phosphorylation sites

APOBEC-1 is the best characterized component of the apoB mRNA editing complex and performs the catalytic function [10]. To examine further the possible involvement of phosphorylation in regulating editing, we investigated whether APOBEC-1 protein activity could be changed by eliminating predicted phosphorylation sites. Human APOBEC-1 contains consensus phosphorylation sites for cAMP- and cGMP-dependent kinases (Thr⁷¹), PKC (Ser³, Thr¹³, Ser⁴⁷, Ser⁵⁴, Ser⁷² and Ser¹⁹⁶) and casein kinase II (Ser⁸) on the basis of analysis with the PC/GENE program (IntelliGenetics) and the papers of Hadjiagapiou et al. [21] and Lau et al. [48]. A two-step PCR method was employed to generate site-directed mutations of Ser⁴⁷ to Ala, Ser⁵⁴ to Ala, Thr⁷¹ to Val, Ser⁷² to Ala and Ser¹⁹⁶ to Ala. The Ser^{3,8} $\rightarrow$ Ala mutant was generated by direct PCR with the mutant primer. The Thr¹³ site was eliminated, by deletion of residues 3–13. The editing enzyme activity of wild-type APOBEC-1 and different mutants was evaluated in Hep G2 cells.

The expression of an exogenous gene encoding APOBEC-1 induces apoB mRNA editing in Hep G2 cells [21], and the editing level can be used to evaluate APOBEC-1 editing activity. To evaluate the activity of the different APOBEC-1 mutants, we

Figure 5 Effect of chronic treatment with ethanol on FAO cells

FAO cells were exposed to a medium containing the indicated concentrations of ethanol (EtOH) in unsealed plates at 37 °C for 2 or 4 days with a medium change every day. Total RNA was extracted after treatment. ApoB mRNA editing and mRNA quantification of β_2M , APOBEC-1 and apoB were determined. (A) ApoB mRNA editing results. (B) APOBEC-1 mRNA levels normalized to β_2M . (C) ApoB mRNA levels normalized to β_2M . Values are means $\pm$ S.D. and represent triplicate determinations of three different extract preparations.

initially quantified the correlation between the level of induced apoB mRNA editing and the level of APOBEC-1 transgene expression. The expressed APOBEC-1 protein levels were too low to be quantified in the stably transfected Hep G2 cells (results not shown), but the expressed APOBEC-1 mRNA was easily detected by RT-PCR. Different ratios of Hep G2 cells transfected with either wild-type APOBEC-1 or empty vector were mixed and cultured to generate mixed populations of Hep G2 cells with different expression levels of APOBEC-1 mRNA. The mixed cell populations prepared in this way were similar to a Hep G2 cell line stably transfected with a mixture of wild-type APOBEC-1 and empty vector DNA constructs (results not shown). Total RNA was extracted from each cell preparation cultured to a cell density of approx. 90% confluence and was analysed for the mRNA levels of a housekeeping gene (β_2M) (Figure 6A) and APOBEC-1 (Figure 6B) by RT-PCR as well as for apoB mRNA editing (Figure 6C). As shown in Figure 6(D), the expression level of APOBEC-1 mRNA represented by the

Figure 6 Correlation between expression level of APOBEC-1 mRNA and apoB mRNA editing in Hep G2 cells expressing APOBEC-1

APOBEC-Hep G2 and vector-Hep G2 cells were mixed in the indicated ratios and cultured to 90% cell confluence. Total RNA was analysed for mRNA level and apoB mRNA editing. (A) Quantification of β_2M mRNA on a polyacrylamide gel. (B) Quantification of APOBEC-1 mRNA on a polyacrylamide gel. (C) Analysis of apoB mRNA editing on a polyacrylamide gel. (D) Graphical representation of the correlation between apoB mRNA editing levels and APOBEC-1 mRNA expression levels, normalized to β_2M levels. The ratio of APOBEC-1 to competitor (T/C) represents the relative mRNA expression level of APOBEC-1.

ratio of APOBEC-1 to competitor normalized to β_2M was closely correlated with the induced endogenous apoB-mRNA-editing activity ($R^2 = 0.988$). These results indicate that the induction of apoB mRNA editing in Hep G2 cells can be altered linearly depending on the APOBEC-1 expression level and that the enzyme activity of different APOBEC-1 mutants can be compared by normalizing their apoB-mRNA-editing activity in Hep G2 cells relative to their level of expression of APOBEC-1 mRNA.

Wild-type APOBEC-1 and mutants were transfected into Hep G2 cells and stably transfected cells were selected. As shown in Figure 7(A), the expression of empty vector in Hep G2 cells resulted in a background of only 0.2% apoB mRNA editing, an activity intrinsic to the assay system itself [49]. Expression of wild-type APOBEC-1 and site-directed mutants of APOBEC-1 resulted in variable levels of apoB mRNA editing. However, the mRNA abundance of β_2M and APOBEC-1 was also variable, as shown by RT-PCR quantification (Figures 7B and 7C). The

Figure 7 Effect of elimination of potential phosphorylation sites on APOBEC-1 editing activity

The potential phosphorylation sites of human APOBEC-1 were eliminated by site-directed mutagenesis. Stable Hep G2 cell lines expressing wild-type APOBEC-1 and mutants were prepared. Total RNA was analysed for apoB mRNA editing and mRNA levels. (A) A representative example of the endogenous apoB mRNA editing assay of the stably transfected cells on a sequencing gel. (B) Quantification of β_2M mRNA on a polyacrylamide gel. (C) Quantification of competitive RT-PCR of APOBEC-1 mRNA on a polyacrylamide gel. The ratio of APOBEC-1 to competitor (T/C) represents the relative mRNA expression levels of APOBEC-1. (D) Graphical representation of apoB mRNA editing level of cell lines expressing different mutants, normalized to both β_2M and APOBEC-1 mRNA levels. Values are means $\pm$ S.D. and represent triplicate determinations on three different extract preparations. * $P < 0.001$ compared with control.

APOBEC-1 mRNA expression level varied more than 3-fold between different DNA preparations (Figure 7C), and significant variability existed even after normalization to housekeeping gene mRNA levels. The apoB-mRNA-editing activity induced by APOBEC-1 and mutants was then normalized relative to the expression levels of both β_2M and APOBEC-1 mRNA (see quantification in the Materials and methods section). As shown in Figure 7(D), the editing activities of Ser^{3,8} → Ala and Ser¹⁹⁶ → Ala mutants were comparable with that of the wild type. The

Figure 8 Comparison studies of mutating Ser to Ala and Ser to Asp on APOBEC-1 editing activity in cells and *in vitro*

(A) Effect of a partial phosphorylation mimic on APOBEC-1 activity in Hep G2 cells. The Ser⁴⁷ → Asp and Ser⁷² → Asp mutants of APOBEC-1 were expressed in Hep G2 cells and their editing activity was evaluated as described in the legend to Figure 7. Values are means $\pm$ S.D. and represent triplicate determinations on three different extract preparations. (B) Editing activity evaluation of wild-type and mutant APOBEC-1 *in vitro*. The wild-type and mutant APOBEC-1 proteins were transcribed and translated *in vitro* in the presence of [³⁵S]methionine. Aliquots were quantified for protein generation by SDS/PAGE [15% (w/v) gel] followed by counting with a PhosphorImager and analysed for their apoB-RNA-editing activity *in vitro*. The editing activity was normalized to the protein level. Filled bars, Ser → Ala mutation; open bars, Ser → Asp mutation.

3–13 deletion and the Ser⁵⁴ → Ala and Thr⁷¹ → Val mutants had approx. 50 %, 68 % and 75 % of wild-type APOBEC-1 activity respectively. The Ser⁴⁷ → Ala mutation had only approx. 15 % of wild-type APOBEC-1 activity. However, the Ser⁷² → Ala mutation yielded a 2-fold increase above the wild-type control.

The human APOBEC-1 editing activity changes induced by the Ser⁴⁷ → Ala and Ser⁷² → Ala mutations were also identically observed by an evaluation of the APOBEC-1 editing activity of APOBEC-1 and mutants translated *in vitro* with a rabbit reticulocyte lysate (Figure 8). These results indicate that the APOBEC-1 editing activity can be regulated by phosphorylation at the predicted phosphorylation sites, Ser⁴⁷ and Ser⁷². The potential phosphorylation at Ser⁴⁷ and Ser⁷² residues up-regulated and down-regulated the APOBEC-1 editing activity respectively.

To determine whether Ser⁴⁷ and Ser⁷² had opposite regulatory effects with potential phosphorylation, a double mutant of Ser⁴⁷ and Ser⁷² to Ala was generated and tested for its editing activity. As shown in Figure 8, the double mutation of Ser⁴⁷ and Ser⁷² to Ala had 79 % of wild-type APOBEC-1 editing activity in the Hep G2 cell detection system and 49 % in the *in vitro* system, values intermediate between the editing activity of the Ser⁴⁷ → Ala and

Ser⁷² → Ala mutants. These results indicate that residues 47 and 72 have opposite regulatory effects.

Ser⁴⁷ and Ser⁷² are phosphorylation sites

Serine residues 47 and 72 were predicted to be potential human APOBEC-1 phosphorylation sites on the basis of their consensus kinase phosphorylation sequences. The changes in APOBEC-1 editing activity induced by the Ser⁴⁷ → Ala and Ser⁷² → Ala mutations above were probably due to elimination of the protein phosphorylation site. However, they did not rule out the possibility that a protein conformation change was responsible for the alteration in editing activity, although the amino acid replacement at the sites replaced a hydroxy group in serine with a hydrogen in alanine and minimized the change. Direct evidence of APOBEC-1 protein phosphorylation permits an interpretation of the alteration in APOBEC-1 activity caused by phosphorylation site elimination. However, the analysis for the direct evidence of phosphorylation requires a large amount of APOBEC-1 protein purified from cells. The low expression level of APOBEC-1 in cultured cells currently precludes a direct demonstration [50,51].

To determine further whether Ser⁴⁷ and Ser⁷² are potential phosphorylation sites, Ser⁴⁷ and Ser⁷² were mutated to aspartic residues, which replaced the hydroxy group in serine with a carbonic acid group and partly mimicked the site's being phosphorylated [52]. The partial phosphorylation mimic was predicted to result in an activity alteration opposite to that of the elimination of the phosphorylation site above. As shown in Figure 8(A), the Ser⁴⁷ → Asp mutant had an editing activity approx. 30 % of the wild type, a more than 2-fold increase compared with the Ser⁴⁷ → Ala mutant. The editing activity of the Ser⁷² → Asp mutant was about the same as that of the wild type, half the activity of the Ser⁷² → Ala mutant. Similar results were also obtained with mutant proteins translated *in vitro* (Figure 8B). These results are consistent with the proposal that Ser⁴⁷ and Ser⁷² are respectively positive and negative phosphorylation-regulatory sites of human APOBEC-1 editing activity.

To provide further evidence that the APOBEC-1 activity changes induced by Ser⁴⁷ → Ala and Ser⁷² → Ala mutations were due to the elimination of phosphorylation sites, we used two-dimensional gel electrophoresis to investigate phosphorylation of the APOBEC-1 and mutant proteins translated *in vitro*. As shown in Figure 9, the wild-type APOBEC-1 protein was primarily present as two spots in the two-dimensional gel. Both APOBEC-1 after alkaline phosphatase treatment and the mutant with eliminated phosphorylation sites 47 and 72 had two spots in the region of the higher pI spot of the wild-type APOBEC-1. The two spots were more widely separated in alkaline-phosphatase-treated APOBEC-1, although it was not known whether phosphorylation was removed completely by the treatment. These relative shifts were further confirmed by including ¹⁴C-labelled protein markers in the samples as reference for the shift comparison (results not shown). These alterations to the two-dimensional gels demonstrated that the protein charge of human APOBEC-1 was altered by the phosphorylation removal treatment or the mutation of serine to alanine, indicating that the protein was phosphorylated at Ser⁴⁷ and/or Ser⁷² residues.

DISCUSSION

ApoB mRNA editing is an enzymic deamination reaction performed by a complex of multiple protein components and undergoes tissue-specific, developmental and metabolic regulation. The activity of the editing enzyme determines the degree

Figure 9 Verification of potential phosphorylation sites

³⁵S-labelled APOBEC-1 and mutant proteins prepared as described in the legend to Figure 8(B) were incubated with or without alkaline phosphatase. The resultant preparations were analysed for the presence of the proteins by SDS/PAGE [15% (w/v) gel] and the protein isoelectric forms by two-dimensional electrophoresis. The proteins were detected by direct exposure to film. (A) Protein analysis results with SDS/PAGE; (B) protein analysis results using two-dimensional electrophoresis. Abbreviations: Wt, wild-type APOBEC-1; Wt-CIP, alkaline-phosphatase-treated wild-type APOBEC-1. The positions of molecular mass markers are indicated (in kDa) at the left of (A).

of apoB mRNA editing, and its modulation should be the molecular basis for the regulation of apoB mRNA editing. Phosphorylation is the most common and important mechanism of rapidly and reversibly regulating protein enzyme activity [29]. Here we have characterized for the first time the potential role of phosphorylation in the regulation of apoB mRNA editing.

ApoB mRNA editing is modulated by altering cellular phosphorylation

The initial evaluation investigated the effect of protein kinase and phosphatase-modulating agents on apoB mRNA editing in cultured cells. Our results demonstrate that apoB mRNA editing in Caco-2, FAO and McA7777 cells, but not with an *in vitro* system, are significantly modulated by multiple protein kinase inhibitors or activators. The stimulation of apoB mRNA editing is phosphorylation-specific, as indicated by the comparability between the effective H-7 concentration and H-7 inhibition constants (Figure 2), and is not correlated with the expression levels of APOBEC-1 and apoB (Figure 1). Of the tested cells, FAO cells are the most responsive to the phosphorylation-modulating agents (see Figure 3). The apoB mRNA editing was stimulated more than 2-fold by the transient transfection of PKC- θ . Taken together, these results suggest that apoB mRNA editing is regulated by cellular modulation of phosphorylation and that phosphorylation might have an important role in regulating apoB-mRNA-editing activity.

ApoB mRNA editing is a nuclear event that occurs before apoB mRNA is transported from the nucleus [53].

Phosphorylation altering agents increase apoB mRNA editing up to 2.5-fold in Caco-2 cells and up to 8-fold in FAO and McA7777 cells. These alterations in apoB mRNA editing reflect the relative proportions of apoB-48 and apoB-100 in total apoB mRNA. This indirect measurement of apoB-mRNA-editing activity represents a downstream effect, because the entire pool of apoB mRNA is evaluated rather than the pool that has just been newly transcribed [29]. The actual phosphorylation effect on apoB mRNA editing of nascent transcripts would be expected to occur much more rapidly than on the entire apoB mRNA pool [31]. Most phosphorylation agents exert their effect on cultured cells within 15–30 min [41,43]. Consequently, apoB-mRNA-editing activity might be rapidly altered by phosphorylation agents, but changes in the degree of editing in the total apoB mRNA pool might not be observed for hours (Figure 2), owing to the prolonged stability of apoB mRNA [54] and the limitation of the assay method in determining the edited mRNA changes in total apoB mRNA rather than a delay in alterations in protein activity.

For a single protein it is sometimes possible to predict which protein kinase is responsible for an effect on the protein on the basis of the effect of different phosphorylation agents. However, predicting apoB mRNA editing effects is complicated because the editing enzyme is an incompletely characterized multiprotein complex. In addition, the kinase or phosphatase target(s) for each kinase inhibitor/activator or phosphatase inhibitor are unknown, because many subfamily members exist for each kind of protein kinase [29] and there is the potential for non-selective effects for these various agents [55]. Phosphorylation-related changes in apoB mRNA editing could reflect the alteration by phosphorylation of one or more of the components in the editing enzyme complex. Additionally, if more than one protein is phosphorylated by a particular kinase, divergent effects could be observed, making it difficult to predict which protein kinase is responsible for the regulation. Consequently, the changes in apoB mRNA editing induced by protein kinase inhibitors/activators or phosphatase inhibitor are not conclusive for a prediction of responsible kinases, but the significant modulation does suggest that the editing can be regulated by phosphorylation.

H-7 functions as an inhibitor of PKC, which induced an 8-fold increase in apoB mRNA editing in FAO cells (Figure 3). In contrast, the transient expression of PKC- θ , which elevated cellular PKC- θ , also increased apoB mRNA editing in FAO cells 2-fold (Figure 4). These apparently discordant results could reflect complexities related to the many different members of the PKC family, including at least 11 isoenzymes that are classified by their cofactor requirements [56]. In addition to differences in their requirements for an activating cofactor, these isoforms also differ in tissue expression, subcellular localization and substrate specificity, which seem to account for the wide variety of processes mediated by PKC [57]. The protein component(s) of apoB mRNA editing enzyme complex targeted by H-7 might be different from those of highly expressed PKC- θ . Thus the net effect on apoB mRNA editing could be very complex and difficult to compare. Even an observation of no change by the other PKC inhibitors or activators in FAO cells (Figure 3) might be misleading because there could be simultaneous inhibition(s) and stimulation(s) resulting in no net change in editing.

ApoB mRNA editing is modulated by chronic treatment with ethanol in FAO cells, a process involving an alteration in protein phosphorylation

Chronic treatment of rats with ethanol *in vivo* increases apoB mRNA editing in liver by up to 100% [22]. In this study we

reproduced the chronic stimulatory effect of ethanol in the whole animal with the rat hepatoma cell line FAO. ApoB mRNA editing increases in a dose-dependent manner by chronic treatment with ethanol in FAO cells without a corresponding change in APOBEC-1 mRNA level. It is well documented that PKA and PKC isoenzymes can act as the molecular targets for ethanol, particularly during chronic exposure to alcohol [33,34,45–47], and that phosphorylation regulates protein enzyme activity without a change in protein expression level [29]. The increased apoB mRNA editing induced by chronic treatment with ethanol suggests that phosphorylation might regulate editing.

Ethanol can increase apoB mRNA editing in the nucleus within 15 min in McA7777 cells [31]. This observation suggests that phosphorylation is probably involved in the rapid onset of apoB mRNA editing, because an acute treatment with ethanol induces changes in the translocation and expression levels of protein kinases in rat liver Golgi apparatus and rat hepatocytes [58,59], and phosphorylation can induce a change in a very short time [29]. Chronic treatment of McA7777 cells with ethanol (results not shown) increases apoB mRNA editing to a similar degree (1.7-fold) to that reported for transient treatment with ethanol (1.8-fold), suggesting that a similar mechanism, namely phosphorylation, might be involved in their regulatory effect on apoB mRNA editing. The stimulation of apoB mRNA editing by the transient treatment with ethanol remains constant over 18 h even though 90% of the initial added ethanol is evaporated [23]. Consistent with this result is the observation of a similar chronic effect of ethanol regardless of the prevention of ethanol evaporation (results not shown).

APOBEC-1 activity is modulated by a phosphorylation mimic and site elimination

APOBEC-1 is the most characterized protein in the apoB mRNA editing complex and functions as the catalytic component of the deamination reaction [60]. The use of site-directed mutagenesis of APOBEC-1's potential phosphorylation sites and expression in Hep G2 cells permits comparative studies of native and mutant APOBEC-1 editing activity. The results demonstrate that human APOBEC-1 editing activity is significantly decreased by a Ser⁴⁷ → Ala mutation but increased by a Ser⁷² → Ala; an opposite change is observed for the Ser⁴⁷ → Asp and Ser⁷² → Asp mutations, which partly mimic phosphorylation at these sites (Figure 8). Ser⁴⁷ and Ser⁷² are near the putative zinc-co-ordinating residues His⁶¹, Cys⁹³ and Cys⁹⁶, an important region for activity that could potentially be affected by phosphorylation. These results suggest that human APOBEC-1 editing activity is regulated by phosphorylations at Ser⁴⁷ and Ser⁷².

To determine whether Ser⁴⁷ and Ser⁷² are actually phosphorylated, [³²P]phosphate labelling or isoelectric-point shifts can be performed [61]. However, the native APOBEC-1 expression level in Caco-2 cells is very low. The transfected APOBEC-1 expression level in Hep G2 cells is also very low [50,51]. The transfected APOBEC-1 expression level was improved by using the rat hepatoma cell line McA 7777 cells, but was still not high enough to provide a sufficient amount of material for protein analysis (results not shown). In addition, overexpressing APOBEC-1 in McA7777 cells results in a selective increase in cytoplasmic promiscuous apoB RNA editing [31], indicating that the overexpressed APOBEC-1 in McA7777 cells might not closely represent the native APOBEC-1 in cell distribution and post-translational modification. In contrast, APOBEC-1 and mutants translated *in vitro* can be generated in large quantities and show editing properties identical with those expressed in Hep G2 cells, including the changes in activity

caused by eliminating phosphorylation sites and mimicking phosphorylation of the sites (Figure 8), suggesting that the phosphorylation of APOBEC-1 translated *in vitro* should closely represent that found in Hep G2 cells.

With the use of native and mutant APOBEC-1 translated *in vitro*, site-specific phosphorylation was analysed by two-dimensional electrophoresis. High-resolution two-dimensional gel analyses of APOBEC-1 proteins were difficult to obtain and the protein isoforms were located close to the top of the isoelectric-focusing gel because of a high isoelectric point (a pI of approx. 8.8 as calculated by the GCG program) and complex formation with the protein components from the rabbit reticulocyte lysate. By mixing samples with SDS and using a large gel system, this problem was partly solved. SDS broke the complex and was stripped off the proteins in the isoelectric focusing tube gel containing Nonidet P40 [37]. The inclusion of SDS in loading samples permitted a better focusing of the protein without observable adverse effects. In addition, the assay was a parallel comparison, and identical results were obtained when including marker proteins that migrated appropriately (results not shown). Compared with the wild type, a similar alteration of the APOBEC-1 isoelectrofocusing pattern by the dephosphorylation treatment with alkaline phosphatase and site-directed mutations at Ser⁴⁷ and Ser⁷² demonstrates that APOBEC-1 translated *in vitro* is phosphorylated at sites 47 and/or 72 (Figure 9). This result is consistent with previous ones showing that a protein can be appropriately phosphorylated in an *in vitro* translation system [62,63].

Various factors have been found to modulate apoB mRNA editing *in vivo*, including thyroid hormone [24], growth hormone [64], fasting and refeeding [25], developmental stage [18–20], oestrogen [26], ethanol [22,23] and insulin [28]. Changes in enzyme activity of the apoB mRNA editing complex could reflect either alterations in the relative level of one or more of the components of the apoB mRNA editing enzyme caused by translation or transcription changes or protein modifications such as phosphorylation, which could alter the activity of the apoB mRNA editing enzyme without a change in protein or mRNA level. Of these factors, growth hormone and ethanol are the most likely candidates to exert their effects through phosphorylation. Growth hormone often exerts its effects on cell function through receptor-mediated phosphorylation signal transduction [29]. Chronic treatment with ethanol has been reported to induce the translocation and expression of protein kinases in cultured cells [33]. Consistent with a potential phosphorylation mechanism is the observation that the APOBEC-1 mRNA level is not altered with chronic treatment with ethanol [22].

In summary, multiple experiments were performed evaluating the potential role of phosphorylation in regulating apoB mRNA editing. Our results demonstrate that: (1) apoB mRNA editing can be significantly modulated by protein kinase inhibitors or activators altering cellular phosphorylation in cultured cells without correlation with changes in APOBEC-1 and apoB mRNA expression; (2) transient expression of PKC- θ results in increased apoB mRNA editing in FAO cells; (3) chronic treatment with ethanol, which is known to have phosphorylation modulation effects, increases apoB mRNA editing dose-dependently in FAO cells; (4) human APOBEC-1 editing activity can be significantly altered by eliminating potential phosphorylation sites at serine residues 47 and 72; (5) the use of partial phosphorylation mimics at sites 47 and 72 alters human APOBEC-1 editing activity in the opposite direction and to a smaller degree than elimination of the phosphorylation sites, suggesting phosphorylation at these sites; and (6) a similarly altered two-dimensional electrophoresis pattern is seen with the

double mutant lacking phosphorylation sites at serine residues 47 and 72 and with alkaline-phosphatase-treated human APOBEC-1, suggesting that serine residues 47 and 72 are phosphorylated. Taken together, these results suggest that phosphorylation regulates apoB mRNA editing; considering the magnitude of the H-7 and H-89 stimulation in rat liver cell lines, this might be an important regulatory mechanism.

This research was supported in part by an appointment to the Postgraduate Research Program at the Center for Biologics Evaluation and Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

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Received 28 September 2000/30 April 2001; accepted 31 May 2001