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ACCELERATED COMMUNICATION

Apolipoprotein B mRNA Editing and the Reduction in Synthesis and Secretion of the Atherogenic Risk Factor, Apolipoprotein B100 Can Be Effectively Targeted through TAT-Mediated Protein Transduction

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ABSTRACT

Hepatic very-low-density lipoprotein particles (VLDL) containing full-length apolipoprotein B100 are metabolized in the blood stream to low-density lipoprotein (LDL) particles, whose elevated levels increase the risk of atherosclerosis. Statins and bile-acid sequestrants are effective LDL-lowering therapies for many patients. Development of alternative therapies remains important for patients with adverse reactions to conventional therapy, with defects in the LDL receptor-dependent lipoprotein uptake pathway and for intervention in children. Editing of apoB mRNA by the enzyme APOBEC-1 changes a glutamine codon to a stop codon, leading to the synthesis and secretion of apoB48-containing VLDL, which are rapidly cleared before they can be metabolized to LDL. Human liver does not edit apoB mRNA because it does not express APOBEC-1. Although initially promising, enthusiasm for *apobec-1* gene therapy for hypercholesterolemia was blunted by the finding that uncontrolled transgenic expression of APOBEC-1 led to nonspecific editing of mRNAs and pathology. We demonstrate that APOBEC-1 fused to TAT entered primary hepatocytes, where it induced a transient increase in mRNA editing activity and enhanced synthesis and secretion of VLDL containing apoB48. Protein transduction of APOBEC-1 transiently stimulated high levels of apoB mRNA editing in a dose-dependent manner without loss of fidelity. These results suggested that apoB mRNA editing should be re-evaluated as a LDL-lowering therapeutic target in the new context of protein transduction therapy.

Cholesterol is carried in blood from one tissue to another as lipoprotein particles by specific carrier proteins called apolipoproteins. Apolipoprotein B (apoB) is an integral and nonexchangeable structural component of lipoprotein particles referred to as chylomicrons, very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). ApoB circulates in human plasma as two isoforms, apoB100 and apoB48. ApoB48 is generated by an RNA editing mechanism that changes codon 2153 (CAA) to a translation stop codon (UAA) (Chen et al., 1987; Powell et al., 1987). Editing is a site-specific deamination event catalyzed by *apoB* mRNA editing catalytic subunit 1 (APOBEC-1) (Teng et al., 1993) with the help of auxiliary factors (Teng et al., 1993; Yang et al. 1997a,b; Lellek et al., 2000; Mehta et al., 2000; Yang et al., 2000; Blanc et al., 2001) as a holoenzyme or editosome (Smith et al., 1991, Harris et al., 1993). ApoB100 and apoB48 play different roles in lipid metabolism; most importantly, apoB100-associated lipoproteins (VLDL and LDL) are much more atherogenic than apoB48-associated lipoproteins (chylomicrons and their remnants and VLDL).

Current lipid-lowering therapies include statins and bile-acid-binding resins. Statins are competitive inhibitors of hydroxymethylglutaryl (HMG)-CoA reductase, which catalyzes the committed step in the synthesis of cholesterol (Davignon et al., 1992). Bile-acid-binding resins sequester bile acids in the intestine, thereby interrupting the enterohepatic circulation of

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ABBREVIATIONS: ApoB, apolipoprotein B; LDL, low density lipoproteins; VLDL, very-low-density lipoproteins; APOBEC-1, ApoB editing catalytic subunit; PTD, protein transduction domain; CMPK, chicken muscle pyruvate kinase; IPTG, isopropyl thiogalactoside; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; HA, hemagglutinin; NLS, nuclear localization signal.

bile acids and increasing the elimination of cholesterol from the body. These are effective therapies for patients with hyperlipidemia; however, adverse effects have been observed in up to 30% of the patients (Anonymous, 1993), suggesting the need for alternative therapies. Mutations in the gene encoding the LDL-receptor or apoB can cause a human genetic disease known as familial hypercholesterolemia, characterized by an elevated level of cholesterol and early atherosclerosis caused by the defect in LDL-receptor mediated cholesterol uptake by cells (Goldstein et al., 1995). Therapy for children with this disorder is needed to prevent morbidity or mortality; however, the National Cholesterol Education Program (NCEP) recommends consideration of drug treatment only for children 10 years of age or older due to the risk that prolonged drug therapy may impair growth and pubertal development (Anonymous, 1992). Developing alternative approaches for lowering serum LDL levels is therefore essential for the sectors of the population still at risk.

Stimulating hepatic apoB mRNA editing is a means of reducing serum LDL through the reduction in synthesis and secretion of apoB100 containing VLDL. In most mammals (including humans), apoB mRNA editing is carried out only in the small intestine. The presence of substantial editing in liver (found in four species) is associated with a less atherogenic lipoprotein profile compared with animals that do not have liver editing activity (Greeve et al., 1993). APOBEC-1 is expressed in all tissues that carry out apoB mRNA editing (Teng et al. 1993). Human liver does not express APOBEC-1 but it does express sufficient auxiliary proteins to complement exogenous APOBEC-1 in apoB mRNA editing in transfected cells (Teng et al., 1993; Sowden et al., 1998).

Transgenic experiments aiming to enhance hepatic editing through *apobec-1* gene transfer have shown a marked lowering of plasma apoB100 and significant reduction of serum LDL (Teng et al., 1994; Hughs et al., 1996; Farese et al., 1996; Kozarsky et al., 1996; Nakamuta et al., 1996; Qian et al., 1998; Wu et al., 1999). ApoB100 is not essential for life, because mice that synthesize exclusively apoB48 (apoB48-only mice) generated through targeted mutagenesis developed normally and were healthy and fertile. Compared with wild-type mice fed on a chow diet, the level of LDL-cholesterol was lower in apoB48-only mice (Farese et al., 1996). The induction of apoB mRNA editing activity through *apobec-1* gene transfer and tissue-specific overexpression faced a significant challenge in that it induced hepatocellular dysplasia and carcinoma in transgenic mice and rabbits (Yamanaka et al., 1995, 1996, 1997). This was proposed to be caused by persistent high levels of APOBEC-1 expression, resulting in unregulated and nonspecific mRNA editing (Sowden et al., 1996a; Yamanaka et al., 1997; Sowden et al., 1998). Adverse effects were not observed in transgenic animals with low to moderate levels of APOBEC-1 expression (Teng et al., 1994; Qian et al., 1998; Wu et al., 1999). The risk of adverse effects stemming from persistent elevated levels of APOBEC-1 expression and the use of adenoviral vectors have diminished enthusiasm for *apobec-1* gene therapy for the treatment and prevention of atherogenic disease. We hypothesized that apoB mRNA editing could be an effective therapeutic target if its induction could be maintained at low levels and, importantly, achieved in a transient manner. To this end, the possibility of using APOBEC-1 directly as a therapeutic agent through the protein transduction domain (PTD) technology was evaluated.

A modified version of APOBEC-1 has been designed that was fused with the protein transducing domain of HIV-1 TAT protein. The TAT domain facilitates the entry of the fusion protein into cells in a receptor or transporter independent fashion (Nagahara et al., 1998; Schwarze et al., 1999; Vocero-Akbani et al., 1999). The construct also contained a C-terminal fusion of chicken muscle pyruvate kinase (CMPK) as means of blunting catalytic activity (Yang et al., 2000; Smith et al., 2001). We report that once inside hepatocytes, TAT-APOBEC-CMPK re-folded and catalyzed an increase in site-specific editing of apoB mRNA in a dose-dependant manner and, importantly, with fidelity. Metabolic labeling of treated rat primary hepatocytes revealed an increase in secreted apoB48 and reduction of apoB100. These results open new possibilities for the treatment of hyperlipidemia through the induction of precisely controlled hepatic editing activity.

Materials and Methods

Generation of TAT fusion protein. A double-stranded oligomeric nucleotide encoding the 11-amino-acid TAT domain flanked by glycine residues and a PCR product encoding HA-APOBEC-CMPK and HA-CMPK (Yang et al., 2000) were inserted into *Nde*I/*Xho*I digested p*PROEX* vector (Life, Gaithersburg, MD). The entire constructs (TAT-HA-APOBEC-CMPK or TAT-HA-CMPK) were inserted into p*ET*-24b (Novagen, Madison, WI) vector to take advantage of the C-terminal His_6 tag. TAT fusion proteins (referred to as TAT-CMPK and TAT-APOBEC-CMPK) were purified from BL-21(DE3) codon plus cells (Stratagene, La Jolla, CA). Two to four 1-liter cultures were inoculated with a 10-ml overnight culture each and induced by 0.1 mM IPTG at 30°C for 1 h. Soluble proteins were obtained by French press in 25 ml of buffer A (8 M urea, 10 mM Tris pH 8, 100 mM NaH_2PO_4). Cellular lysates were cleared by centrifugation, loaded onto a 5-ml Ni-NTA column (QIAGEN, Valencia, CA) in buffer A with 10 to 20 mM imidazole, washed and eluted with imidazole in buffer A 'stepwise' (100, 175, and 250 mM) and loaded onto a HiTrap SP column (Amersham Biosciences, Piscataway, NJ). The column was washed and eluted with 1 M NaCl in buffer A. The urea and high salt were removed from the relevant fractions by rapid dialysis against buffer B (30 mM Tris, pH 8.5, 50 mM NaCl, 10 μM zinc acetate, and 5% glycerol). The elution profile was analyzed by SDS-PAGE. Gels were stained with silver according to manufacture's recommendations (Bio-Rad, Hercules, CA).

Cell Culture. McArdle RH7777 cells were obtained from ATCC (Manassas, VA) and cultured as described previously (Yang et al., 1997a). Rat primary hepatocytes were prepared from unfasted, male Sprague-Dawley rats (250–275 g body weight, Taconic Farm) fed ad libitum normal rat chow as described previously (Van Mater et al., 1998). Recombinant TAT fusion protein was added directly to the cell culture media after dialysis.

RNA Isolation. Total cellular RNA was isolated from cells with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's recommendations. Purified RNAs were digested with RQ-DNase I (Promega, Madison, WI) and with *Rsa*I (Promega) restriction enzyme that has a recognition site between the PCR annealing sites of target substrates to ensure the removal of the contaminating genomic DNA.

Editing Assays. Editing activity was determined by the reverse transcriptase-polymerase chain reaction (RT-PCR) methodology described previously (Smith et al., 1991). First strand cDNA was generated using oligo(dT)-primed total cellular RNA. Specific PCR amplification of rat apoB sequence surrounding the editing site was accomplished using ND1/ND2 primer pairs. PCR products were gel isolated and the editing efficiency was determined by poisoned primer extension assay using [^{32}P]ATP (PerkinElmer Life Sciences, Boston, MA) end-labeled DD3 primer under high concentration of

dideoxy GTP as described previously (Smith et al., 1991; Sowden et al., 1996a). Primer extension products were resolved on a 10% denaturing polyacrylamide gel, autoradiographed, and then quantified by a laser densitometric scanning (Molecular Dynamics, Sunnyvale, CA). Percent editing was calculated as the counts in the UAA (edited) band divided by the sum of the counts in UAA and those in the CAA (unedited) bands and multiplied by 100.

Protein Labeling Methods. Twelve- to eighteen-hour rat primary hepatocytes grown in Waymouth's 752/1 media (Sigma, St. Louis, MO) were treated for 11 h with TAT-APOBEC-CMPK and then incubated for 1 h in DMEM deficient medium (without methionine, cysteine, and L-glutamine) (Sigma, St. Louis, MO) containing 0.2% (w/v) bovine serum albumin, 0.1 nM insulin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. The medium was replaced with fresh labeling medium containing 0.7 μCi/ml L- [^{35}S]methionine and L-[^{35}S]cysteine using EXPRE^{35}S^{35}S protein labeling mix (PerkinElmer Life Sciences). Cells were incubated in the labeling medium for 30 min. One volume of Weymouth's medium with cold cysteine and methionine was added to cells and the labeling was continued for an additional 12 h, after which cell culture medium was collected for the isolation and analysis of secreted apoB protein and RNAs were harvested from the cells for editing assay.

Detecting Secreted apoB in Cell Culture Medium. Immunoprecipitation of apoB from cell culture medium was performed as described previously (Sparks et al., 1996). A rabbit polyclonal antibody raised against rat apoB and reactive with the N terminus of apoB100 and apoB48 (gift from Drs. J. D. Sparks and C. E. Sparks, University of Rochester, Rochester, NY) was used to precipitate apoB. The immunoprecipitants were separated by SDS-PAGE on 5% gel. The gel was dried and exposed to film to reveal the secreted apoB containing lipoprotein profile, which represents the secreted apoB48 and apoB100 during the 12-h labeling period.

Immunofluorescence. McArdle cells, grown on six well cluster plates were treated with either TAT-APOBEC-CMPK or TAT-CMPK for the indicated times. Cells were then washed extensively with PBS and subsequently fixed with 2% paraformaldehyde, permeabilized with 0.4% Triton X-100, blocked with 1% bovine serum albumin, and reacted with affinity purified anti-HA (BABCO, Berkeley, CA) and affinity-purified FITC-conjugated goat anti-mouse secondary antibody (Organon Teknika, West Chester, PA), each at 1:1000 dilution. Fluorescence was observed and electronic images captured on an inverted fluorescence Olympus microscope (Olympus, Tokyo, Japan).

Results

Generation of Full-Length TAT Fusion Protein. We sought to induce hepatic apoB mRNA editing through TAT mediated APOBEC-1 protein transduction into liver cells. It has been shown that linking an 11-amino-acid PTD of HIV-1 TAT protein to heterologous protein conferred the ability to transduce into cells (Nagahara et al., 1998; Schwarze et al., 1999; Vocero-Akbani et al., 1999). PTD-linked protein transduced into ~100% of cells and the transduction process occurred in a rapid and concentration-dependent but receptor- and transporter-independent manner (Schwarze et al., 2000). Liver cells have been shown to be susceptible to transduction (Nagahara et al., 1998). To produce in-frame TAT fusion protein from *Escherichia coli*, we constructed a prokaryotic expression vector that has an N-terminal PTD flanked by glycine residues for free bond rotation of the domain (Schwarze et al., 1999), an hemagglutinin (HA) tag, and a C-terminal 6-histidine tag. Using this vector as a backbone, we constructed a plasmid encoding full-length TAT-APOBEC-CMPK protein (Fig. 1A). APOBEC-CMPK was used in this study because it showed a less robust editing activity in vitro and targeted primarily cytoplasmic mRNAs (Yang et al., 2000). In vitro studies demonstrated that APOBEC-1 retained catalytic activity when conjugated to various lengths of nonspecific proteins (Siddiqui et al., 1999; Yang et al., 2000).

Recombinant proteins were solubilized in 8 M urea buffer from bacterial cells so as to maximize their yield from inclusion bodies. Previous studies have shown that denatured proteins could transduce as well as native proteins (Schwarze et al., 1999). The proteins were purified through metal-chelating affinity chromatography followed by cationic exchange chromatography. The urea was removed using rapid dialysis and the purity of full-length 86 kDa TAT-APOBEC-CMPK was apparent as shown by silver staining (Fig. 1B). The purification of full-length protein was also confirmed by Western blot using anti-His$_6$ antibody (data not shown).

TAT-APOBEC-CMPK Entered McArdle Cells. We first evaluated the uptake of TAT-APOBEC-CMPK into McArdle cell using an antibody reactive with the HA epitope and fluorescence microscopy. Recombinant APOBEC-1 has a tendency to aggregate, a property that persists in TAT-APOBEC-CMPK, apparent as aggregates of HA antibody-reactive material attached to the surface of cells 1 h after the addition of the protein to the media (Fig. 2, A and B). Aggregation was not a property of the TAT motif or CMPK as control protein (TAT-CMPK) at a higher molar concentration appeared as an array of speckles attached to the surface of McArdle cells 1 h after its addition to the media (Fig. 3, A and B).

Within 6 h after treatment both TAT-APOBEC-CMPK (Fig. 2, C and D) and TAT-CMPK (Fig. 3C and D) were apparent inside the cells and the cell surface-attached aggregates seemed to be more disperse. After 24 h of treatment, many of the cells treated with TAT-APOBEC-CMPK demon-

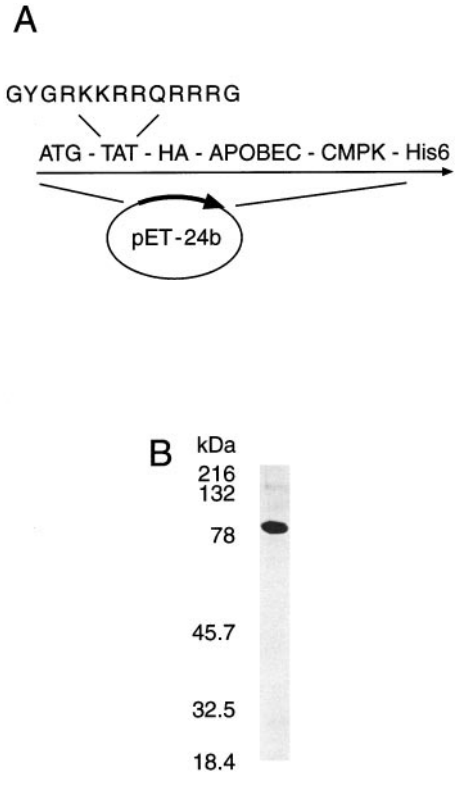

Fig. 1. The purification of full-length TAT-APOBEC-CMPK protein. A, the structure of the prokaryotic expression vector encoding the TAT fusion protein. B, the purity of the full-length protein was apparent after two-column purification as revealed by silver-staining.

strated bright perinuclear fluorescence and a low intensity of fluorescence throughout the nucleus and cytoplasm (Fig. 2, E and F). Cells treated for 24 h with TAT-CMPK demonstrated bright fluorescent speckles in the cytoplasm and fainter homogenous nuclear fluorescence (Fig. 3, E and F). The nuclear distribution of the recombinant protein might have been facilitated by the embedded nuclear localization signal (NLS) in TAT sequence (Schwarze et al., 1999) because APOBEC-1 alone does not have a functional NLS (Yang et al., 1997b; 2001) and 6His-HA-APOBEC-CMPK was excluded from the nucleus (Yang et al., 2000). The data suggested that McArdle cells took up both TAT-APOBEC-CMPK and TAT-CMPK. Comparatively, the efficiency of TAT-APOBEC-CMPK uptake was poorer than that for TAT-CMPK, and the distribution of these proteins within the cells seemed different.

Cells Treated with TAT-APOBEC-CMPK had increased editing activity. Given that TAT-CMPK entered McArdle cells, we evaluated whether this would affect apoB mRNA editing activity (Fig. 4). Cells were treated with the indicated amounts of TAT-CMPK (using the same preparation of protein as in Fig. 3) and total cellular RNA was isolated after 24 h and the proportion of edited apoB mRNA measured. No change in the percentage editing of apoB mRNA relative to untreated cells (see Fig. 5) was observed with TAT-CMPK concentrations ranging from 45 to 1125 nM (5 to 133 μg of protein/ml of media) (Fig. 4).

In contrast, editing activity increased in McArdle cells with 360 nM (62 μg of protein/ml media) TAT-APOBEC-CMPK after 6 h and continued to a peak by 24 h, an increase of more than 3-fold over the level of editing observed in control cells (Fig. 5). The proportion of edited RNA remained elevated up to 48 h after treatment (Fig. 5) and approached baseline by 72 h. It has been reported that the enzymatic activity lagged the appearance of the transduced protein inside the cells, probably because of a slow refolding of the transduced protein (Schwarze et al., 1999). Taken together, the results

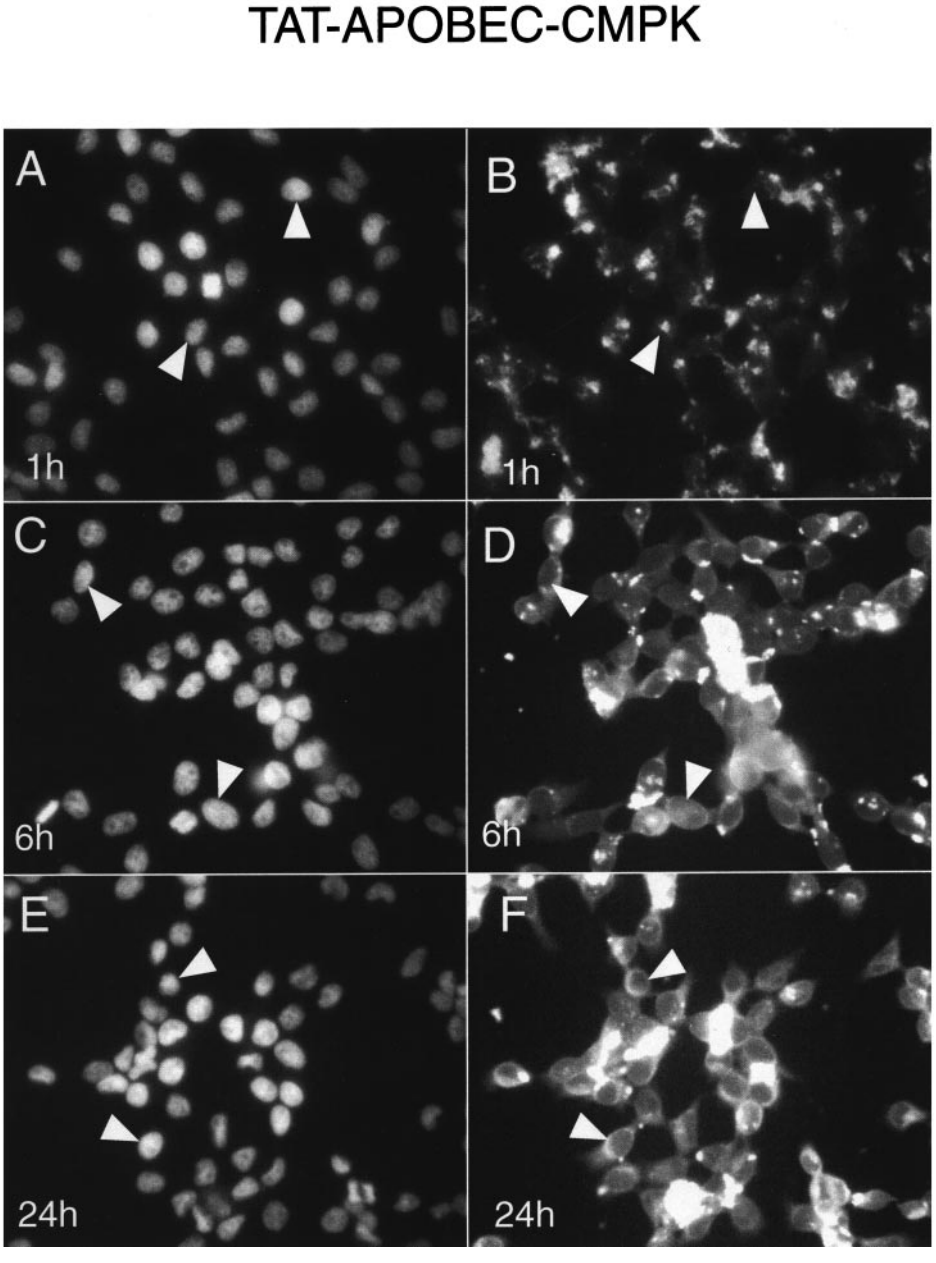

Fig. 2. TAT-APOBEC-CMPK entered McArdle cells. McArdle cells were treated with 650 nM recombinant TAT-APOBEC-CMPK for the indicated times. Cells were fixed, permeabilized, reacted with antibody to the HA epitope and FITC-conjugated anti-mouse secondary antibody and mounted in DAPI containing buffer as described under *Materials and Methods*. Arrowheads indicated the position of select nuclei.

demonstrated that TAT-APOBEC-CMPK transduced into McArdle cells, refolded into an enzymatically active conformation over the first 6 h, and then edited apoB mRNA. The reduction in the proportion of edited apoB mRNA after 48 h was probably due to enzyme inactivation and apoB mRNA turnover. This characteristic was important as it demonstrated the transient and reversible nature of the protein transduction system.

Increased Editing Activity in Primary Rat Hepatocytes. To determine whether this approach would be applicable in primary liver cells, cultured rat primary hepatocytes were prepared and treated with TAT-APOBEC-CMPK. It has been shown that the editing efficiency in primary rat hepatocytes decreased as a result of proliferation after 72 h in culture (Van Mater et al., 1998). Together with the fact that TAT-APOBEC-CMPK maximally increased editing 24 h after treatment in McArdle cells, we elected to evaluate dose response for a fixed time rather than study kinetics. Primary hepatocytes were treated with the indicated amounts of TAT-APOBEC-CMPK and analyzed for edited apoB mRNA 24 h afterward.

The editing activity of hepatocytes increased in proportion to the amount of TAT-APOBEC-CMPK added to the cell culture media relative to cells treated with buffer alone (Fig. 6) or treated with TAT-CMPK (Fig. 4). Given that the primary hepatocytes were seeded at the same cell number as McArdle cells, a comparison of the data in Figs. 5 and 6 suggested that TAT-APOBEC-CMPK was as effective in inducing editing activity in the primary cell culture. This was true for several preparations of recombinant protein and primary cells; therefore, the difference may be because fact that the primary hepatocytes have a higher baseline of editing than McArdle cells (48% versus 7%) and/or may be 'primed' with more auxiliary factors.

Promiscuous editing of additional cytidines in rat apoB mRNA of transfected cells (Sowden et al., 1996a, 1998; Yamanaka et al., 1996) or hyperediting of other mRNAs in trans-

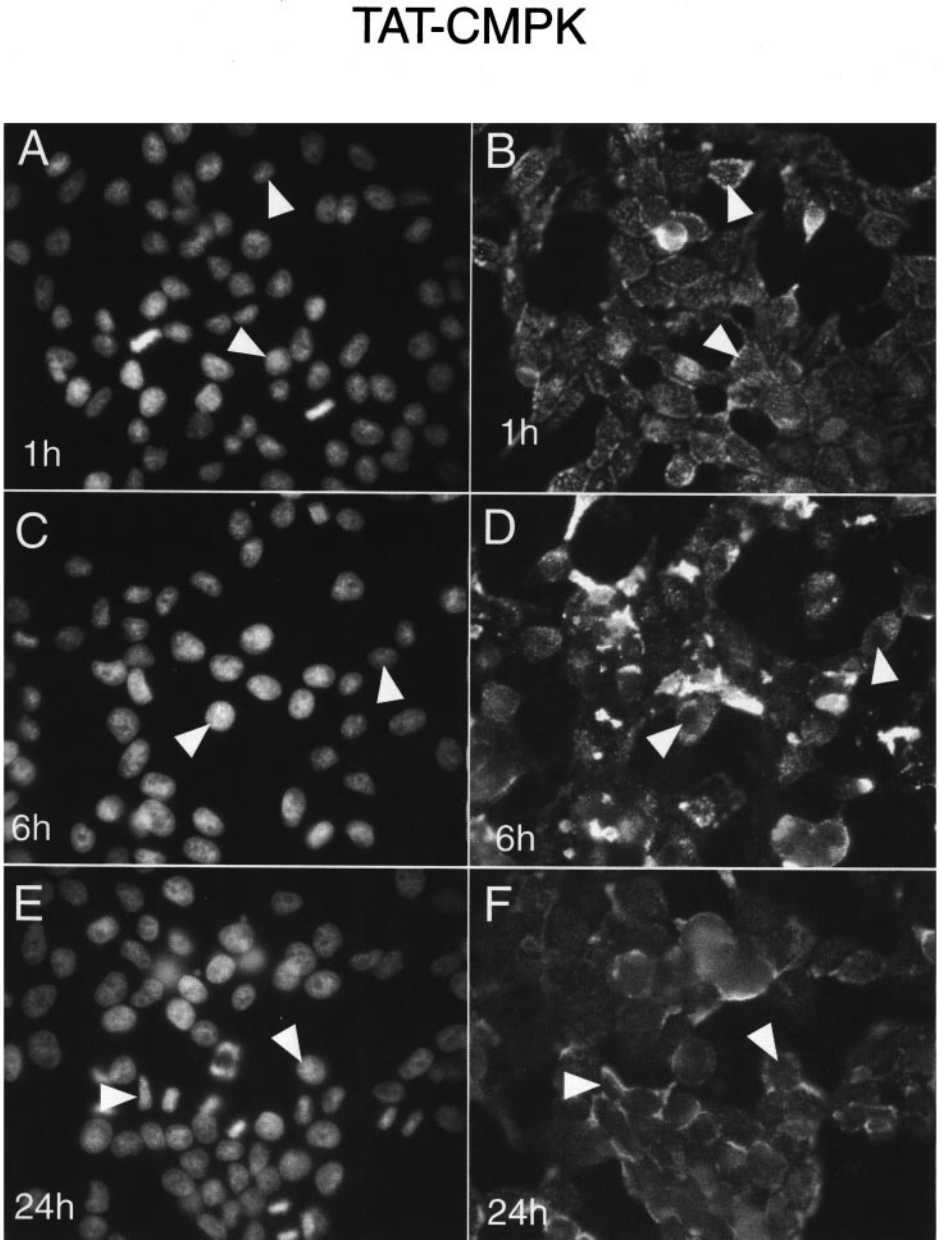

Fig. 3. TAT-CMPK entered McArdle cells. McArdle cells were treated with 1125 nM recombinant TAT-CMPK for the indicated times. Cells were fixed, permeabilized, reacted with antibody to the HA epitope and FITC-conjugated anti-mouse secondary antibody and mounted in DAPI-containing buffer as described under *Materials and Methods*. Arrowheads indicated the position of select nuclei.

genic mice and rabbits (Yamanaka et al., 1996, 1997) has been observed in response to very high levels of APOBEC-1 expression. Editing of cytidines 5′ of the wild type editing site (C6666) was a bellwether for the loss of editing site fidelity in rat cells and could be used to monitor the induction of promiscuous editing in relation to changes in APOBEC-1 expression (Sowden et al., 1998; Siddiqui et al., 1999). Promiscuous editing of cytidine 3′ C6666 in apoB mRNA did not occur to a significant extent in rat cells and hyperediting of mRNAs other than apoB was not a characteristic of APOBEC-1 overexpression in rat cells (Sowden et al., 1998).

Despite the high level of editing activity in treated primary hepatocytes, promiscuous editing (evident as additional primer extension products above UAA (Sowden et al., 1996a, 1998) was not observed (Fig. 6). Given that our detection limit for promiscuous editing was 0.3% (Sowden et al., 1996) the data suggested that TAT-APOBEC-CMPK could be used to substantially increase site-specific editing of apoB mRNA without significant loss of fidelity of the reaction.

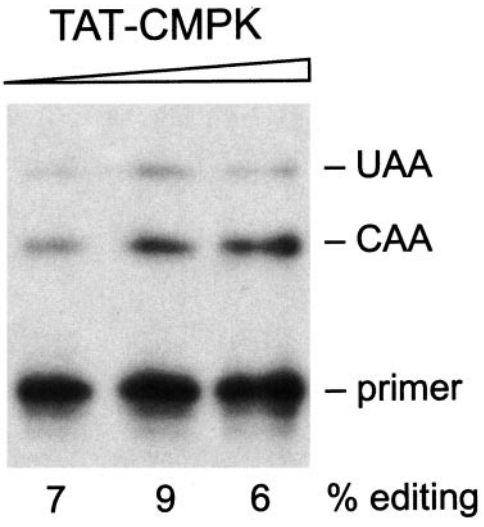

Fig. 4. TAT-CMPK did not stimulate editing. McArdle cells were treated with 45 nM, 225 nM, and 1125 nM recombinant TAT-CMPK for 24 h. Total cellular RNA was isolated and apoB mRNA was selectively amplified by RT-PCR and the proportion of edited apoB RNA determined by poisoned primer extension as described under *Materials and Methods*. CAA, primer extension product corresponding to unedited RNA; UAA, primer extension product corresponding to edited RNA; P, primer.

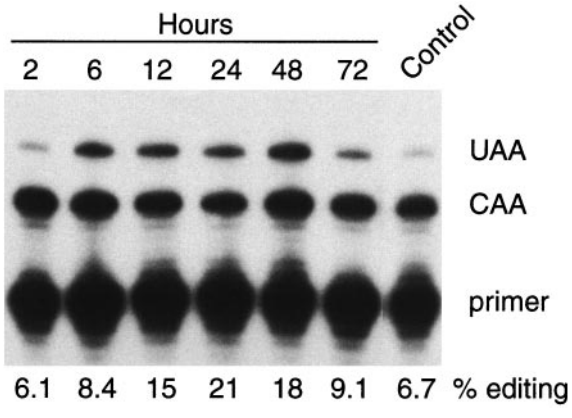

Fig. 5. TAT-APOBEC-CMPK increased editing activity in McArdle cells. TAT fusion protein (360 nM or 62 μg of protein/ml of media) was added into cell culture media and RNAs were isolated subsequent to treatment from wild-type McArdle cells at the indicated time points. Control cells were treated with a corresponding aliquot of buffer B used to dialyze the recombinant protein. The editing efficiency was calculated as described under *Materials and Methods* section. The standard deviations for each of the lanes on the gel, reading left to right, are as follows: 0.9, 2.2, 3.8, 2.1, 1.1, 0.9, 0.2, n =3.

Secreted Lipoprotein Profile Change Due to TAT-APOBEC-CMPK Treatment. To further confirm the efficacy of this method, secreted apoB protein was evaluated in primary rat hepatocytes that were long-term metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine after TAT-APOBEC-CMPK treatment. The secreted ^{35}S-labeled apoB lipoproteins were isolated from the cell culture media exposed to cells for 12 h followed by immunoprecipitation, and analyzed by autoradiography after SDS-PAGE separation. The signal on the gel was in direct proportion to the number of cysteine and methionine residues in apoB100 and apoB48. Because apoB48 was the N-terminal 48% of apoB100, a stronger signal was expected from apoB100 in control cells. However, as the editing efficiency approached 90% due to TAT-APOBEC-CMPK treatment, an increasing amount of apoB48 was secreted, and apoB100 became almost undetectable (Fig. 7). Thus, lowering apoB100-associated atherogenic risk factors through precisely controlled hepatic apoB mRNA editing was achievable by protein transduction with TAT-APOBEC-CMPK.

Discussion

We have designed a novel approach to curtail hepatic output of apoB100 associated atherogenic factors through upregulating apoB mRNA editing by using protein transduction into liver cells. The PTD, amino acid residues 49–57, of HIV-1 TAT protein has been used in other systems to deliver functional full-length protein molecules into cells (Nagahara et al., 1998; Schwarze et al., 1999; Vocero-Akbani et al., 1999). Some of these fusion molecules, when introduced into mice, entered all tissue cells, even crossing the blood brain barrier (Schwarze et al., 1999). Although the detailed mechanism for the cellular uptake of the fusions remains unknown, denaturing of the protein during membrane transduction is thought to be a rapid process, and the rate-limiting event is the renaturing of the transduced protein once inside the cells (Schwarze et al., 2000).

In this regard, the protein transduction method may have

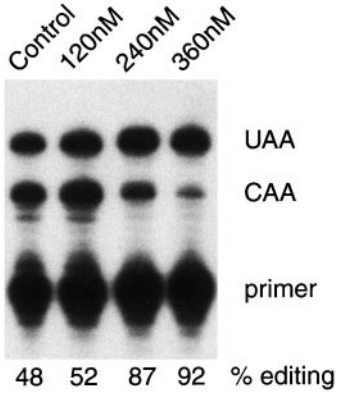

Fig. 6. TAT fusion protein increased editing activity in primary rat hepatocytes. Hepatocytes were prepared and treated with TAT-APOBEC-CMPK as described. Control cells were treated with a corresponding aliquot of buffer B used to dialyze the recombinant protein. The increase in editing activity caused by TAT fusion protein was apparent. The standard deviations for each of the lanes on the gel, reading left to right, are as follows: 2.2, 3.6, 2.5, 1.9, n =3.

limitations in that some proteins may be unable to successfully adopt an active conformation after they have been unfolded. It is significant, therefore, that we are able to show that both TAT-CMPK and TAT-APOBEC-CMPK had the capacity to enter hepatocytes and that TAT-APOBEC-CMPK activated editing within 6 h of its addition to the media. We have observed similar kinetics with TAT-APOBEC-CMPK prepared under native conditions (data not shown).

Importantly, TAT-CMPK could not stimulate editing activity, demonstrating that the observed changes in editing were specific to APOBEC-1 containing recombinant proteins. Considering the tendency for APOBEC-1 containing proteins to aggregate, part of the lag in entering cells could have been due to the inability of these multimeric complexes to cross the plasma membrane and the time it took for TAT-APOBEC-CMPK monomers to dissociated from the aggregates and cross the membrane. This is supported by the finding that TAT-CMPK, which did not seem to form large aggregates, seemed to accumulate within the cells with more rapid kinetics than that observed for TAT-APOBEC-CMPK. The 6-h lag before an increase in editing activity could be measured may have also been because of the time required for the transduced protein to refold and assemble editosomes.

ApoB mRNA editing occurs in the cell nucleus despite the fact that editing factors can also be demonstrated in the cytoplasm (Yang et al., 2000). The mechanism responsible for APOBEC-1's distribution in the nucleus is not understood (Yang et al., 2001), however its mass seemed to be important because the chimeric protein APOBEC-CMPK was excluded from the nucleus (Yang et al., 1997b; 2000; 2001). TAT-APOBEC-CMPK's ability to distribute in both the cytoplasm and the nucleus was consistent with the proposed ability of PTD to act as a nuclear localization signal (Schwarze et al., 1999). Although TAT-APOBEC-CMPK's distribution mimicked that of the wild-type enzyme's distribution (Yang et al., 1997b), we cannot be certain that all of the transduced TAT-APOBEC-CMPK molecules were active in editing, nor can we say with certainty whether cytoplasmic or nuclear transcripts were edited.

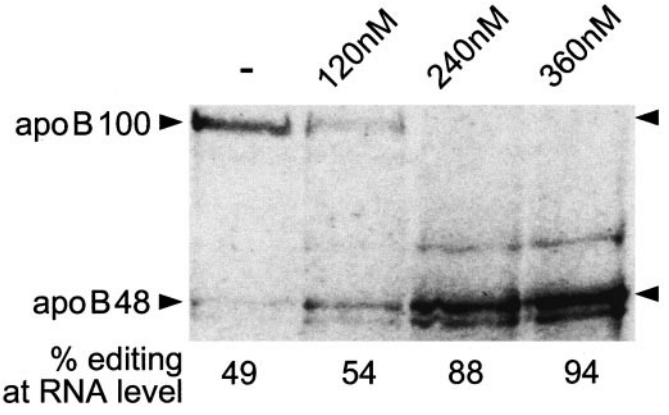

Fig. 7. Secreted lipoprotein profile change due to TAT-APOBEC-CMPK treatment. Primary hepatocytes were treated with TAT fusion protein first, then labeled with [^{35}S]methionine and [^{35}S]cysteine. Control cells (−) were treated with a corresponding aliquot of buffer B used to dialyze the recombinant protein. Cell culture media were collected, apoB48 and apoB100 were precipitated by anti-apoB antibody and separated by SDS-PAGE. The second band below apoB48 might have been due to protein degradation and the band between apoB100 and apoB48 could be C-3 complement (Dr. Janet Sparks, personal communication). The editing efficiency of the same cells is shown at the bottom. The results are from a single experiment representative three experiments with similar results.

Enhancement of editing activity by overexpression of APOBEC-1 through gene transfer has been shown to be associated with promiscuous editing on both nuclear and cytoplasmic transcripts (Sowden et al., 1996a; Yang et al., 2000). Metabolic stimulation of apoB mRNA editing always retained fidelity (Wu et al., 1990; Greeve et al., 1993; Phung et al., 1996; von Wronski et al., 1998). It is therefore highly significant that we observed that the fidelity of the editing activity was retained with TAT-APOBEC-CMPK even when editing was enhanced to >90%. This level of high-fidelity editing could not be achieved without hyperediting in *apobec-1* transgenic animals (Yamanaka et al., 1997). There was no pathology in transgenic animals in which induction of hepatic apoB mRNA editing was achieved at a low level of *apobec-1* expression and these animals had a markedly lower serum apoB-100 and significantly reduced serum LDL compared with controls (Teng et al., 1994; Farese et al., 1996; Hughs et al., 1996; Kozarsky et al., 1996; Qian et al., 1998; Wu et al., 1999). Interestingly, *apobec-1* gene transfer into apobec-1 gene knockout mice restored editing and reduced serum LDL levels (Nakamuta et al., 1996), demonstrating that APOBEC-1 has therapeutic potential in livers with no prior editing activity. The induction of hepatic editing of apoB mRNA in *apobec-1* transgenic rabbits with an LDL receptor deficiency also ameliorated hypercholesterolemia (Kozarsky et al., 1996). Taken together these studies suggested that apoB mRNA editing could be safely targeted as a mechanism for reducing serum LDL and the risk of atherogenic diseases. The advantage of protein transduction therapy is that the dose could be modulated relative to the desired response and that the effect on editing could be terminated by withdrawing therapy.

The PTD should allow protein to enter all cells of the body, even if the protein is delivered intravenously (Schwarze et al., 1999). Ideally, the liver should be specifically targeted with TAT-APOBEC-CMPK and for this, we envision an intraperitoneal injection to accomplish a first pass clearance, transducing most of the protein into hepatocytes. Even though APOBEC-1 is not widely expressed in tissues (Teng et al., 1993), its generalized expression in transgenic animals did not induce pathology (Teng et al., 1994; Hughs et al., 1996; Kozarsky et al., 1996; Farese et al., 1996; Qian et al., 1998; Wu et al., 1999). Studies are being initiated in animals to determine dose response and to determine whetherTAT-APOBEC-CMPK enters other tissues and if so, with what effect. Aside from one study suggesting that overexpression of APOBEC-1 in liver can lead to editing of mRNAs other than apoB (Yamanaka et al., 1997) no other mRNA substrates for APOBEC-1 have been found (Skuse et al., 1996; Sowden et al., 1998). Furthermore, *apobec-1* gene knock out studies have shown that there were no other editing enzymes capable of editing apoB mRNA and that APOBEC-1 was not required for life (Hirano et al., 1996; Nakamuta et al., 1996). Taken together, the data suggest that mRNA editing by APOBEC is self-limited because of its specificity for apoB mRNA; therefore, TAT-APOBEC-CMPK is not likely to have effects in tissues other than those that express apoB mRNA and auxiliary proteins.

Current cholesterol-lowering therapies target circulating cholesterol at the level of enhanced elimination or reduced produc-

tion. A sector of the population remains at risk for atherosclerosis because of side effects from current therapies in some of these patients and the inability of others with defects in apoB and/or the LDL receptor mediated uptake pathway to completely benefit from conventional cholesterol lowering therapies. Hypercholesterolemia is an early onset disease yet the restricted usage of conventional therapies among children due to the potential of interfering with pubertal development has not been resolved. Protein-based therapies such as insulin or growth hormone have been extensively used among children to treat type I diabetes or pituitary dwarfism, respectively. To the patient or the parent of the patient, the reversible nature of protein-based therapy may be more appealing than gene therapy. To this end, our results offer the potential of an alternative to conventional or gene therapy for reducing the risk of atherosclerosis in the sectors of population at risk.

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