Targeted Disruption of the Mouse apobec-1 Gene Abolishes Apolipoprotein B mRNA Editing and Eliminates Apolipoprotein B48*

(Received for publication, January 31, 1996, and in revised form, February 27, 1996)

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A site-specific C to U editing reaction modifies nuclear apolipoprotein B100 (apoB100) mRNA, producing apolipoprotein B48 in the mammalian small intestine. This reaction is mediated by a multicomponent enzyme complex, which contains a catalytic subunit, Apobec-1. We have used gene targeting to disrupt mouse apobec-1 in order to establish its requisite importance in apoB mRNA editing and also, in view of its widespread tissue distribution in rodents, as a preliminary indication of other potential roles. Both heterozygous (apoB-1/−) and homozygous (apoB-1/−/−) gene-targeted mice appear healthy and fertile with no alterations in serum cholesterol or triglyceride concentrations. The liver, on the other hand, contains only unedited apoB mRNA and secretes the full-length form, apoB100 (1, 9). ApoB100 contains the functional domains required for binding to the low density lipoprotein receptor as well as the cysteine residue required for its association with apolipoprotein(a) (1, 10, 11). By corollary, the absence of these domains from apoB48 has major functional consequences for lipoprotein catabolism and may influence the atherogenic potential of the apoB-containing lipoproteins (reviewed in Ref. 1).

ApoB mRNA editing is mediated by a multicomponent enzyme complex that includes a catalytic subunit, Apobec-1, as well as other as yet uncharacterized factors that are required for the functional integrity of the complex (12–14). The primary structure of Apobec-1 reveals homology to other cytidine and deoxycytidine deaminases, particularly within a conserved motif His-(X)24–30-Cys-X-X-Cys (15, 16). Furthermore, Apobec-1 demonstrates zinc-dependent cytidine deaminase activity (15) against both the cytidine at position 6666 of mammalian apoB mRNA as well as a simple monomeric cytidine substrate (17). The biological significance of this latter activity and its role, if any, in the regulation of cellular nucleoside metabolism are currently unknown.

In attempting to ascribe an integrated, biological role for Apobec-1, it was difficult to make a priori predictions, since recent reports suggest a range of potential functions. For instance, in addition to its activity as an apoB RNA-directed cytidine deaminase, Apobec-1 is an RNA binding protein, with broad specificity for AU-rich templates (16, 18). Other features suggest the possibility of a biological role for Apobec-1 distant from its function in apoB mRNA editing. These include the observation that Apobec-1 is widely expressed and developmentally regulated in both rat and mouse tissues, including many sites such as the kidney, lung, and spleen, which contain trivial or undetectable amounts of apoB mRNA (13, 19, 20).

Thus, although restricted to the gastrointestinal tract in humans and rabbits (21–24), the broad tissue distribution of Apobec-1 in rats and mice, taken together with the RNA binding and cytidine deaminase activity alluded to above, implies the possibility that potential functions exist for this protein beyond its role in apoB mRNA editing. Consistent with this suggestion is the recent report that transgenic mice and rabbits that overexpress Apobec-1 in the liver tend to develop hepatic dysplasia and hepatocellular carcinoma (25). This study suggested that it is possible that other mRNA substrates, in addition to apoB, might undergo editing (25).

In order to determine the potential biological functions of Apobec-1 in vivo, we have used gene targeting to create mice that are homozygous for an apobec-1 gene knockout. We report that homozygous apobec-1 knockout mice (apoB-1/−/−) appear healthy and fertile and have no obvious abnormalities other than the absence of plasma apoB48 and undetectable apoB.
mRNA editing. Heterozygous (apobec-1+/−) mice manifested reduced levels of apoB mRNA editing in the liver; however, apoB mRNA editing was indistinguishable in the small intestine of apoB-1+/− and wild-type animals. These findings suggest that Apobec-1 is normally present in excess in the mouse small intestine but at limiting concentrations in the liver.

MATERIALS AND METHODS

Construction of Targeting Vector—Two genomic fragments of the apoB gene were obtained from a mouse ES C129 P1 clone (Genome Systems, St. Louis, MO). A sequence replacement vector was constructed using a 4.5-kb BamHI fragment and a 2.5-kb HindIII fragment, which were ligated into pNTK (see "Materials and Methods"). The disrupted allele lacks exon 6 and contains new PvuI and Ncol sites. B, Southern blot of DNA from representative G418-resistant and (1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5'-iodouracil-resistant ES clones, digested with either Ncol (N) or PvuI (P) and probed with a 0.8-kb HindIII/SalI fragment (hatched bar, panel A). Targeted clones were verified by Southern blotting using a neo probe (data not shown). C, Southern blot of tail DNA from three wild-type (+/+), one apobec-1+/− (+/−), and two apobec-1−/− animals (−/−), digested with PvuI and probed as in panel B. B, BamHII; H, HindIII; P, PvuI; N, Ncol; TK, thymidine kinase cassette; Neo, neomycin resistance cassette.

ES Cell Culture and Generation of Chimeric Mice—Culture and elecroportion of RFe ES cells were conducted as described (27). RF8 cells were generated from 129/SvTer mouse blastocysts.3 DNA was isolated from G418 (150 μg/ml) and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5'-iodouracil-resistant clones. Aliquots of 10 μg of genomic DNA were digested with Ncol or PvuI and used for Southern analysis as detailed in Fig. 1. The blots were probed with an 800-base pair HindIII-SalI fragment encoding a portion of exon 8 and the contiguous 3'-untranslated region of the apoB-1 gene. ES cells from three independently targeted clones were microinjected into C57BL/6J blastocysts in order to obtain high percentage chimeras. The male chimeric mice generated from all three targeted clones transmitted the ES cell genome to their offspring. Genotyping was undertaken by polymerase chain reaction (PCR) analysis of DNA using the primer pairs indicated in Fig. 2 (sequence below) and confirmed by Southern blotting of PvuI-digested genomic DNA (10–20 μg).

Miscellaneous Assays—Animals were allowed unrestricted access to regular mouse chow and were sacrificed without fasting. Reverse transcription-PCR amplification of apoB mRNA was undertaken using mouse-specific primers and −1 μg of DNase I-digested RNA (22). RNase

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2 K. I. Hirano and N. O. Davidson, unpublished observations.
3 R. V. Farese, Jr., unpublished observations.

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Fig. 1. Targeting strategy for apoB-1 gene knockout. A, schematic map of the wild-type (WT) allele showing B exons. A replacement vector was constructed using a 4.5-kb BamHI fragment and a 2.5-kb HindIII fragment, which were ligated into pNTK (see "Materials and Methods"). The disrupted allele lacks exon 6 and contains new PvuI and Ncol sites. B, Southern blot of DNA from representative G418-resistant and (1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5'-iodouracil-resistant ES clones, digested with either Ncol (N) or PvuI (P) and probed with a 0.8-kb HindIII/SalI fragment (hatched bar, panel A). Targeted clones were verified by Southern blotting using a neo probe (data not shown). C, Southern blot of tail DNA from three wild-type (+/+), one apobec-1+/− (+/−), and two apobec-1−/− animals (−/−), digested with PvuI and probed as in panel B. B, BamHII; H, HindIII; P, PvuI; N, Ncol; TK, thymidine kinase cassette; Neo, neomycin resistance cassette.

Fig. 2. Screening strategy for apoB-1 gene knockout. Two independent PCR reactions were used. One reaction was used to determine the absence of exon 6 of apoB-1, using primers P1 and P2. A second reaction was used to demonstrate the presence of the neo cassette, using primers P3 and P4. A, absence of exon 6 from genomic DNA of apoB-1−/− mouse, M, 40X markers. WT, wild type. B, presence of neo cassette in both apoB-1−/− and apoB-1+/− mice, M, A HindIII marker. C, RNase protection assay on total hepatic and small intestinal RNA (50 μg each), using a 446-nt SalI/Kpnl probe to demonstrate the reduction in Apobec-1 mRNA abundance in apoB-1−/− (−/−) mice (protected fragment, 422 nt) and its absence in apoB-1−/− (−/−) mice. The abundance of the Apobec-1 mRNA was normalized using a 333-nt mouse β-actin probe, which yielded a 247-nt protected fragment.
RESULTS AND DISCUSSION

Gene Targeting of apobec-1—A sequence replacement vector (Fig. 1) was constructed as detailed (see “Materials and Methods”) using two regions of homology. One region spans intron 4 to intron 5, and a second region spans exon 7 to exon 8 of the endogenous apobec-1 gene. Homologous recombination of the targeting vector with the cognate gene in the chromosome results in the elimination of exon 6 from the targeted allele (Fig. 1A). Elimination of exon 6 is predicted to inactivate the gene, because this region encodes the critical zinc-coordinating residues and the catalytic glutamate. These residues have been shown to be essential for both cytidine deaminase and apoB RNA editing activity of Apobec-1 (16, 17, 23). Southern blot analysis of genomic DNA demonstrated that 6/189 ES cell clones were injected into blastocysts, and each resulted in germline transmission. Mice were genotyped by two separate commercial kits (Wako and Sigma, respectively).

ApoB mRNA Editing—ApoB mRNA was amplified from the liver, small intestine, and kidney of homozygous, heterozygous, and wild-type mice and subjected to primer extension analysis. As illustrated in Fig. 4, there was no detectable apoB mRNA editing in any tissue of the apobec-1−/− mice, implying that Apobec-1 is the sole catalytic subunit of the apolipoprotein B mRNA editing enzyme. The apobec-1−/− mice demonstrated reduced levels of apoB mRNA editing in the liver (47 ± 2% versus 71 ± 7% TAA in wild-type, n = 5 in each group, p = 0.0019), consistent with the reduction in Apobec-1 mRNA abundance noted above. By contrast, the extent of apoB mRNA editing was indistinguishable in the small intestine of apobec-1−/− and wild-type mice (>90% TAA in both groups), despite the reduction in Apobec-1 mRNA abundance. Taken together,
these data suggest that Apobec-1 abundance in the liver of mice may be limiting, whereas the small intestine normally contains an excess of this component. ApoB mRNA editing was also abolished in the kidney of apobec-1−/− mice, an observation suggesting that there is no duplication of this RNA editing subunit, even in tissues where Apobec-1 expression occurs independent of any major role in lipoprotein biogenesis.

The availability of apobec-1−/− mice will allow us to examine issues relating to the organizational state and function of the complementation factors of the apoB mRNA editing enzyme, specifically in tissue extracts that now express no detectable quantities of the catalytic subunit. It will be informative, for example, to examine intestinal and hepatic extracts prepared from these apobec-1−/− mice for the presence of the RNA binding activities, which correspond to p44 and p60 (28, 29), in order to determine whether these functional attributes are retained in the absence of Apobec-1.

In addition, it will be of importance to determine whether apobec-1−/− mice are susceptible to atherosclerosis particularly in view of the fact that their serum contains exclusively apoB100. In this regard, their phenotype is similar to that of mice which have been recently generated through targeting of the edited codon of the endogenous apoB100. In this regard, their phenotype is similar to that of mice which have been recently generated through targeting of the edited codon of the endogenous apoB100.4 The outcome of such studies, particularly with respect to the need for a high fat, cholesterol-enriched diet in order to induce atherosclerosis (30), will be of intense interest. These and other issues will be the focus of future reports.

Acknowledgments—We acknowledge the invaluable assistance of Teddy Colbert (Genentech) and Annalise Hausman (University of Chicago).

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J. Biol. Chem. 1996, 271:9887-9890.
doi: 10.1074/jbc.271.17.9887

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