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Targeted Deletion of the Murine apobec-1 Complementation Factor (acf) Gene Results in Embryonic Lethality

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RNA editing is an important genetic adaptation that permits distinct gene products to be encoded from a single genomic template. One of the most extensively studied examples of this model of mammalian gene regulation is substitutional C-to-U RNA editing of the apolipoprotein B (apoB) transcript. This editing is essential for the synthesis of a functional lipoprotein while also being a key posttranscriptional modulator of apoB mRNA stability and transport. Recent work has identified the major functional domains (including three canonical RNA recognition motifs) within ACF that mediate both protein-protein and protein-RNA interactions and that both facilitate and constrain C-to-U RNA editing in vitro, thereby establishing the role of ACF in apoB RNA editing in vivo. Small interfering RNA knockdown of ACF in either rat (apoB-expressing) or human (apoB-deficient) cell lines decreased ACF protein expression and induced a commensurate increase in cell survival, particularly during early embryonic development.

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XmnI, the 3’ arm was inserted into the NotI/Apal sites of pL339 (accession number AF335420; a gift from Tim Ley, Washington University) using restriction sites introduced by PCR. The 5’ arm-GFP cassette was added to the KpnI/BamHI sites of the pL339-3’ arm vector upstream of a PGK-Neo cassette. The targeting construct was linearized with Apal and electroporated into 129/Sv embryonic stem (ES) cells. Genomic DNA was isolated from G418-resistant colonies, and correctly targeted clones were identified by Southern blot analysis.

Genotyping analysis. The genotype of ES cells was determined by Southern blotting. Genomic DNA was digested with XbaI, resolved on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a 32P-labeled 793-nucleotide (nt) probe corresponding to the sequence downstream of the 3’ arm. Over 140 clones were analyzed for the homologous recombination event. A single positive clone was identified in which a 6.2-kb band was detected in addition to the 8-kb wild-type band. Targeted clones were expanded in culture, and 200 blastocysts were injected into pseudopregnant recipient C57BL/6 mice. A total of three chimeras were obtained, from which two mice were selected for breeding to establish germ line transmission of the targeted allele. The acf genotypes were determined by PCR with two different sets of three primers (see Fig. 2A). Set 1 included the following: a sense primer (Sce1), FSpmBAC (5’-CTGAACTGGG CATTGGGATGAGTG-3’), and an antisense primer (ASce1), acf/mCOS 625-3’-CTTTCAAGAATCCGCAGCTATGTTG-3’. A second primer (ASce1b), GAPDH (5’-GGCGCGAAGGGGCCACCAAAGAACGG-3’), corresponding to the GFP sequence, will amplify the targeted allele, producing a 388-bp band. Set 2 included the following: the sense primer (Sce2a), F4dEx2 (5’-ATGGAATCAAATCACAATCCGG-3’), and antisense primer (ASce2b), PGK (5’-AGGTGGCGCGAAGGGGCCACCAAAGAACGG-3’), corresponding to the PGK sequence of the Neo cassette, will amplify the targeted allele generating a 230-bp product. acf+/- mice were mated and monitored daily for plugs. Embryos at the indicated timed intervals (days postcoitum [dpc]) were recovered by microdissection for histological analysis or genomic DNA extraction with a Puregene DNA purification kit (Gentra Systems) and genotyping as described above.

**Protein and RNA extraction and analysis.** Tissues were flash frozen in liquid nitrogen and stored at -80°C until used. Protein concentrations were determined (Bio-Rad protein assay) on tissue extracts prior to Western blotting. RNA was isolated using TRIzol (Invitrogen) and 2 μg of DNase-treated RNA used for primer extension analysis of apoB RNA editing and quantitative and semiquantitative reverse transcription (RT)-PCR analysis (7). Real-time quantitative PCRs were performed with an ABI Prism7000 instrument (Applied Biosystems) by use of SYBR Green Master Mix according to the manufacturer’s instructions, and mRNA abundance was determined by normalization to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level in each sample. PCR primers were as follows: ACF (5’-GCCGAACTTGCAAAATCCA-3’ and 5’-AGCATACCTCCCTTTTTGA-3’), and GAPDH (5’-GGCGCGAAGGGGCCACCAAAGAACGG-3’ and 5’-CTTTCAAGAATCCGCAGCTATGTTG-3’). The distribution of ACF mRNA in mouse tissues was assessed by semiquantitative RT-PCR amplification using the following primers: ACF FwdEx2 (5’-ATGGAATCAAATCACAATCCGG-3’), and antisense primer (ASce2b), GAPDH (5’-AGGTGGCGCGAAGGGGCCACCAAAGAACGG-3’). A total of 12 days mouse embryonic development (Fig. 1B and C). ACF immunostaining was readily detected in the embryonic day 12 (E12) developing heart, spinal cord, and lung tissues (Fig. 1D), in which there is virtually no detectable ACF mRNA or protein in adults (data not shown).

**acf gene targeting and phenotype characterization following heterozygous deletion.** A replacement-type targeting construct (see Materials and Methods) (Fig. 2A) was electroporated, and ES cells were screened by Southern blotting (Fig. 2B). acf+/− ES cells were injected into wild-type blastocysts to create germ line acf+/+ mice. PCR genotyping (Fig. 2C) was performed to identify mutant mice. acf−/− mice appeared healthy, grew at a rate similar to wild-type mice, and were fertile. ACF protein content was reduced in the kidney, liver, and small intestine of acf−/+ mice relative to that found in wild-type littermates (Fig. 3A).

**acf and apobec-1 interaction yields an enzyme complex whose activity is critically dependent on the stoichiometry of its minimal component subunits (2, 7). To determine the functional consequences of the aforementioned alterations in ACF protein content, we examined the C-to-U editing of endogenous apoB mRNA; the findings demonstrate a consistent and statistically significant increase in the proportions of U606 containing transcripts in both liver and kidney (Fig. 3B). apoB mRNA editing was unchanged in the small intestines of these animals, exceeding 95% in both wild-type and acf−/+ mice (Fig. 3B). The demonstration of increased apoB mRNA editing in liver and kidney, by contrast, was somewhat unexpected and suggests that heterozygous deletion of ACF alters the stoichiometry of the core components of the holoenzyme.** Among the possible considerations in this scenario are alterations in a metabolically active nuclear ACF pool, changes in

**RESULTS AND DISCUSSION**

**Murine acf gene structure.** The murine acf gene contains 15 exons with the known functional domains illustrated in Fig. 1A. These findings are generally in agreement with the recent findings of Dur and colleagues (10), with the exception that there are three noncoding exons (exons 5, 6, and 11) reported in the current report (Fig. 1A). The two major protein products (ACF64 and ACF65) differ, as in humans, by the differential splicing of a single exon (Fig. 1A), which results in an eight-amino-acid insertion in the larger protein. A similar pattern of alternative splicing was demonstrated in the human ACF gene (14). ACF mRNA expression, as determined by real-time quantitative PCR, was distributed predominantly in the adult liver, small intestine, and kidney (data not shown). This pattern confirms that reported by Dur and colleagues (10). Recombinant human ACF64 and ACF65 reveal indistinguishable biochemical activities (5), but the possibility of an alternative biological function for these isoforms cannot be excluded. ACF43, a splice variant recently described in the rat (26), appears to be a minor transcript (<1%) in murine tissues (data not shown). ACF mRNA and protein were detected at 12 days of mouse embryonic development (Fig. 1B and C). ACF immunostaining was readily detected in the embryonic day 12 (E12) developing heart, spinal cord, and lung tissues (Fig. 1D), in which there is virtually no detectable ACF mRNA or protein in adults (data not shown).

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**siRNA silencing of ACF.** Small interfering (siRNA) oligonucleotides were selected following established parameters (11), and several candidate primers, both murine and human specific, were purchased from Dharmaco Research Inc. Rat hepatoma cells (McArdle7777, American Type Culture Collection), a rodent cell line known to express ACF (7, 32), and human hepatocarcinoma cells (HepG2) were grown to 40% confluence on coverslips and transfected with 100 nM ACF or control-scrambled siRNA oligonucleotides according to the manufacturer’s instructions (Ambion). Forty-eight hours posttransfection, cells were fixed in 10% formalin (Sigma) and probed with a rabbit anti-caspase 3 (active form) antibody (Cell Signaling) followed by a probe with Cy3 secondary antibody (Jackson ImmunoResearch). The nuclei were identified using 4’,6-diamidino-2-phenylindole (DAPI) (1 mg/ml in phosphate-buffered saline). Silencing of ACF protein expression was also assessed by Western blotting of cell lysates from siRNA or scrambled oligonucleotide-transfected cells grown under identical conditions with a rabbit polyclonal ACF antibody (7).
nuclear cytoplasmic shuttling of apobec-1 and/or ACF (9), or even changes in ACF interaction with other protein partners. These possibilities will of course require formal evaluation in future studies.

In order to determine whether the increased C-to-U editing activity in acf<sup>+/−</sup> liver was confined to the canonical site at nt 6666, 10 clones were randomly selected from RT-PCR products, and a total of over 3,100 nt sequences was obtained from a region containing eight C residues, including the canonical site (Fig. 3C). The results reveal only one clone (Fig. 3C) in which there was an additional edited C, suggesting that promiscuous editing (25) or hyperediting (31) was not taking place in the setting of increased C-to-U editing at nt 6666. The C-to-U editing of other RNA targets was not examined. Taken together, the functional outcome of the heterozygous targeting of murine acf points to a tissue-specific increase in endogenous apoB RNA editing activity, possibly reflecting a subtle but distinct alteration in the stoichiometry of the core editing complex.

Phenotype associated with homozygous deletion of murine acf. Three separate lines of acf<sup>+/−</sup> mice were established from independent founders, two of which were littermates and one of which was from a separate breeding. The progeny were genotyped by PCR (Table 1). We detected no acf<sup>−/−</sup> mice from any of these lines after genotyping 42 litters and 332 offspring, indicating that disruption of the acf locus resulted in embryonic lethality. The litter sizes from these heterozygous matings were similar to those from wild-type littermates (range, 7 to 10), and there was no apparent gender bias. In addition to the absence of homozygous acf<sup>−/−</sup> offspring, we...
FIG. 1—Continued.
observed a significant increase in heterozygous offspring among all three lines, with the ratio of heterozygous to wild-type mice averaging 10:1 (range, 8.3 to 12.6) (Table 2), instead of the 2:1 ratio predicted from Mendelian inheritance. These genotypes were confirmed by two independent primer sets, as detailed in Fig. 2C, suggesting that trivial explanations, such as genotyping errors, are unlikely to account for the apparent increase in the number of heterozygous offspring. In

FIG. 2. Targeted disruption of acf gene. (A) Mouse acf gene and targeting vector. Upon homologous recombination of the 5' and 3' arms of the targeting vector with the acf locus (top), the exon 2 is replaced by the coding sequence of GFP and a LoxP-flanked Neo cassette. (B) Southern blot analysis of genomic DNA isolated from recombinant ES cells. Upon digestion with XbaI, the wild-type (+/+) allele gives rise to a 8-kb fragment, while the digestion of the mutant allele (+/-) generates 8- and 6.2-kb fragments, detected with a 3' probe (hashed box). (C) PCR genotyping of wild-type and heterozygous mutant mice. Primers Sse1, ASse1a, and Asse1b, indicated in blue, were mixed in a single PCR and used to amplify a 338-bp product corresponding to the wild-type (+/+) and a 408-bp product associated with the targeted allele (+/-), lanes 1 and 2. Similarly, primers Sse2a, Sse2b, and ASse2, indicated in green, amplified a 610-bp wild-type band (lane 3) and a 230-bp band corresponding to the targeted allele (lane 4).

FIG. 3. Endogenous apoB mRNA editing in wild-type and acf+/− mice. (A) Immunodetection of ACF protein in liver, small intestine (SI), and kidney isolated from wild-type or heterozygous mice. Equal amounts of protein were resolved by sodium dodecyl sulfate-PAGE and probed with antisera to ACF, Hsp40, and GFP. (B) apoB mRNA editing of acf+/− and wild-type mice. Upper panel, apoB mRNA editing was determined by primer extension. The relative mobility of the unedited (C) and edited (U) products is indicated on the left. Lower panel, bar graph representing the data (mean ± standard deviation; n = 4) for each group. Statistically significant differences between groups are indicated by asterisks. (C) Promiscuous editing of hepatic apoB mRNA in acf+− mice. A schematic representation of cytidine residues promiscuously targeted (31) is shown. A 350-bp fragment of apoB mRNA was amplified by RT-PCR, subcloned into pPCR-Script vector, and sequenced. Ten clones from two acf+− mice were analyzed. Nine clones exhibit a uridine residue at the canonical site 6666; only one clone (clone 8) shows an additional C-to-U change at position 6762. Note that a reduction of the ACF protein level does not make the apoB mRNA more susceptible to promiscuous editing.
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% apoB-RNA Editing

Liver | SI | Kidney
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| 100 | 80 | 50 |

* indicates significant difference

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addition, further crosses of the heterozygous acf+/− (mixed background 129/SvJ × C57BL/6) into an acf wild-type C57BL/6 apobec1−/− line resulted in two litters containing a total of 12 mice, all of which were acf+/− (data not shown). The karyotype of the targeted ES cells revealed a normal 40 X,Y distribution, suggesting that there is no chromosomal duplication or major rearrangement occurring in the ES cells used to generate the lines. To our knowledge, there are no other examples of an embryonic lethal phenotype where the distribution of genotypes favors the heterozygosity of the candidate gene, but further dissection of the underlying mechanisms will clearly require additional investigation.

To determine the stage at which homozygous mutant mice die, we analyzed embryos at different times of gestation. Genotyping of embryos dissected at E7.5 and E12.5 revealed no acf−/− mice (Table 1). Furthermore, histological examination of the implantation sites (n = 6) in uterine sections obtained at E6.5 and E7.5 failed to reveal evidence of thrombi at the positions of putative resorbed deciduae (data not shown). In addition and as expected, no empty deciduae were noted under positions of putative resorbed deciduae (data not shown). In E6.5 and E7.5 failed to reveal evidence of thrombi at the positions of putative resorbed deciduae (data not shown). In E6.5 and E7.5 failed to reveal evidence of thrombi at the positions of putative resorbed deciduae (data not shown).

Among the mechanisms we considered for the failure of acf−/− embryos to implant was an inability to develop properly to the stage where implantation can occur. To assess this possibility, blastocysts (E3.5) were harvested from heterozygous acf+/− mice, cultured for 4 to 7 days, and examined for their ability to develop blastocyst outgrowths (27). The outgrowths were then genotyped by PCR. From 62 cultures, 40 blastocysts remained attached and revealed histological evidence of proliferation of both trophoblasts and inner cell masses (8, 28) (data not shown). Genotype analysis of these 40 outgrowths revealed that 22 were wild type and 18 were homozygous acf−/−. However, no acf−/− outgrowths were detected, consistent with the suggestion that acf−/− blastocysts degenerate and undergo cell death within the first 4 days of culture. These findings collectively point to the possibility that ACF is required for preimplantation development.

**ACF knockdown in somatic cells results in apoptotic death.**

To investigate the possibility that ACF is required for cell survival, we undertook ACF knockdown using siRNA-mediated gene silencing. Rat hepatoma cells were transfected with candidate siRNAs targeting different regions of ACF mRNA (Fig. 4A). ACF protein abundance was reduced by >70%, with siRNAs targeting regions located 316 and 823 nucleotides downstream of the initiator ATG, whereas no effect was observed when cells were transfected with scrambled siRNAs (Fig. 4A). In the setting of ACF knockdown, we observed an increased number of apoptotic cells, as indicated by the number of caspase 3-positive nuclei detected in siRNA4-treated cells (Fig. 4B and D). The initiation of apoptosis was supported by the demonstration of cleavage of caspase 3 into its active form (Fig. 4C). The induction of apoptosis was further analyzed by staining with terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling. The delivery of siRNA4 to rat hepatoma cells resulted in a significant, approx-
RNA-editing complementation activity and of ACF expression but does not express apobec-1 (12). siRNA-mediated knockdown of ACF expression in HepG2 cells resulted in an ~76% (four independent assays) decrease in ACF protein expression (Fig. 5A) and a ~30-fold increase in caspase 3-positive-staining nuclei (Fig. 5B). These results reinforce the conclusion that ACF knockdown in somatic cells results in apoptosis. In addition, the findings in HepG2 cells strongly imply that this effect is independent of apobec-1 expression.

To further resolve the observed embryonic lethality associated with homozygous acf deletion in vivo, we endeavored to determine the exact timing and cell type in which apoptosis occurs in E3.5 blastocysts. However, as inferred from immunofluorescent staining, ACF expression was below the level of detection in blastocysts isolated from heterozygous acf matings, precluding a formal conclusion to the question of whether apoptosis occurs exclusively in acf null blastocysts (data not shown). In addition, the transfection of siRNA oligonucleotides into cultured acf+/− ES cells produced inconsistent effects concerning the number of dead and apoptotic cells, which were possibly the result of uncontrolled variations in transfection efficiency (data not shown). We suspect that the resolution of the mechanisms and pathways leading to apoptosis following targeted acf deletion will require the development of a conditional deleter line.

Summary conclusions. The central finding of this report is that the homozygous deletion of murine acf is associated with embryonic lethality, most likely the result of a developmental defect in blastocyst growth, leading to preimplantation failure. What functional properties of ACF could account for this phenotype? Crucial to its role in catalyzing C-to-U RNA editing is the fact that ACF is an RNA binding protein with high affinity (low \( k_d \)) for AU-rich RNAs, including apoB RNA, its presumed target (7, 17, 18). In this regard, it is relevant that several AU-rich RNAs, including those for interleukin-6, interleukin-1β, Cox2, and gamma interferon, are all expressed in the early stages of embryonic development, including the blastocyst stage (24). Although the binding of ACF to these targets has yet to be demonstrated, it is not unreasonable to speculate that altered binding of ACF to any of these or other AU-rich targets might interrupt the development of acf+/− embryos. Probing these underlying mechanisms will require alternative approaches to bypass the early lethality associated with germ line deletion. A potentially important clue to the mechanisms involved in the early embryonic lethality has emerged from recent findings by Kinnaird and colleagues (15). These workers have demonstrated that RNA interference-mediated silencing of the Caenorhabditis elegans ACF homolog, HRP-2, leads to embryonic arrest, with 48 h of exposure resulting in a failure to progress beyond the 64-cell stage (15). These findings, considered together with the current data, suggest that ACF is an hnRNP family member with a conserved and nonredundant role in early embryonic development.

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