Delayed liver regeneration in mice lacking liver serum response factor

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THE LIVER PRESENTS A REMARKABLE capacity to regenerate after injury. Most of our knowledge on liver regeneration derives from studies performed after two-thirds partial hepatectomy (PH) (in rodents). In response to this surgical procedure, the remnant hepatocytes enter the cell cycle in a highly synchronized manner and restore the lost mass in one to two rounds of cell division. The first critical phase, initiated a few minutes after PH, is called the priming phase and shows the induction of immediate early genes (IEGs) at both transcriptional and translational levels (26). It corresponds to the exit of the hepatocytes from the G0 quiescent state, rendering them susceptible to a set of factors leading to their progress through the cell cycle (9). Serum response factor (SRF) is a ubiquitous transcription factor that binds as a homodimer to the CArG box sequence and requires a MCM1, agamous, deficiens, SRF (MADS) box for its transcriptional activity (27). SRF-directed gene activation has been observed at different stages of the cell cycle (11). Moreover, an essential involvement of SRF has been assumed in the control of proliferation and cell cycle progression (24). Total gene invalidation of SRF is embryonic lethal in mouse at the onset of gastrulation (1). SRF is a transcription factor responsible for the induction of IEGs (25), among which some are normally upregulated in the liver during the priming phase after PH, such as c-fos, Egr-1, JunB or pip92 (4, 30), we wondered whether it could play a role in liver regeneration. Using the conditional knockout Cre-Lox strategy, we therefore investigated whether genetic disruption of SRF expression in the liver could affect the normal liver development and the hepatic proliferative response following 70% hepatectomy. We show here that conditionally deleted animals displayed impaired regenerative response after PH. This phenotype was associated with delayed expression of cell cycle regulatory proteins and impaired induction of various SRF target IEGs, such as c-fos, Egr-1, and JunB.

MATERIALS AND METHODS

Animals and surgery. Alfp-Cre mice (14) were bred with SRF exon 2 floxed homozygous mice (SRFfl/+)(23) to obtain double transgenic mice, SRFfl/+Cre+, with a mixed genetic background (129sv; Balb/c, C57Bl/6). Further crosses between SRFfl/+Cre+ and SRFfl/+ mice were used to obtain SRFfl/+Cre+ or SRFfl/+Cre− mice. The SRF allele Δ is constitutively deleted for exon 2. Eight- to twelve-week-old SRFfl/+Cre+ and SRFfl/+Cre− mice were subjected to sham operation or two-thirds PH between 9 AM and 12 PM, as described by Higgins and Anderson (12), under general anesthesia with inhaled isoflurane (n = 4 for each genotype and time point). Animals were killed at different times after surgery. Sham animals were operated on without any liver resection. Livers were either harvested into Formalin for histological evaluation and proliferation studies or snap frozen into liquid nitrogen for mRNA preparations or cellular lysate preparations. All experiments were approved by the institutional committee and were in accordance with European guidelines for the care and use of laboratory animals.

PCR analysis of SRF gene deletion. Cre recombinase-mediated excision of the floxed Srf allele was detected by PCR on DNA from different organs using primers SF1, 5'-CTGTAAGGGAGTGGAGGAGCGA-3'5', SF2, 5'-ATAGGACATGTTAGCCTCA-3'; SF3, 5'-TTTGAGACTCCGCCGGGACTAAA-3'. PCR cycles were as follows: SF1-SF3, 94°C for 4 min, followed by 25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; SF1-SF2, 94°C for 4 min, followed by 25 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. The internal probe SF int, 5'-GCAGATGTAGCTCTCAGAAG-3' and SF3, 5'-TCTGGAATCAGGTGGCCTCAGCA-3', was used for hybridization with PCR products. Positions of primers are indicated in Fig. 1. The TATA box binding protein (TBP) mouse gene was used to normalize the measurements, using oligonucleotides TBP-forward, 5'-TCTGGAATCAGGTGGCCTCAGCA-3' and TBP-reverse, 5'-TACTGAACTGCTGTTGGGT-3'.

Northern blot analysis. Total RNA was extracted from frozen liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). Twenty micrograms of total RNA were size-fractionated by gel electrophoresis on 1% agarose under denaturing conditions, transferred to Hybond N+ (Amer sham, Pharmacia Biotech), and hybridized with 32P-labeled probes.

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RESULTS

SRF gene expression in the liver after PH. To determine whether SRF expression was modulated during liver regeneration, we used the well-characterized model of liver regeneration after two-thirds PH. We investigated SRF transcriptional expression patterns at different times after two-thirds PH in wild-type animals. We demonstrated, using real-time RT-PCR, that SRF is strongly induced after PH. We detected an early upregulation of SRF transcription as soon as 90 min after PH and a peak at around 12 h posthepatectomy (Fig. 1).

Liver-specific inactivation of SRF. To evaluate the role of SRF in the liver development and regeneration process, we created a conditional knockout model of SRF specifically targeted in the liver. Mice homozygous for the floxed SRF allele (SRFfl/fl) were bred with Alfp-Cre transgenic mice that express the Cre recombinase under the control of the albumin promoter and α-fetoprotein enhancer (14). These transgenic mice have been shown to express the recombinase in the hepatic bud before embryonic day 14.5 (E14.5), targeting both hepatocytes and biliary cells but not endothelial cells or mesenchymal cells (7). Following Cre-mediated recombination, the SRF exon 2 that encodes two-thirds of the MADS box is excised, resulting in a nonfunctional truncated protein unable to bind DNA, to homodimerize, or to have a negative transdominant effect (23) (Fig. 2A). Transgenic mice harboring both the Cre transgene and two floxed SRF alleles (SRFfl/Cre+) were born with Mendelian frequencies, and adult animals were healthy and fertile. As expected, recombination occurred specifically in the liver and not in other organs (Fig. 2B) between E10.5 and E12.5 (Fig. 2C). RT-PCR (Fig. 2D) and Northern blot (Fig. 2E) analyses revealed barely detectable full-length SRF transcripts in the liver of adult SRFfl/Cre+ mice, whereas the expected two alternative transcripts (4.5 and 2.5 kb) were detected in Cre− mice. Using PCR on freshly isolated primary hepatocyte DNA, we estimated that the recombinase efficiency reached 90–100% (data not shown). The livers of adult mutant animals show no obvious abnormalities. Altogether, these data indicate that conditional deletion of exon 2 inactivated the SRF gene in the hepatocytes and that this SRF deletion did not impair liver development.

Impaired liver regeneration in SRF mutant mice. We then followed the kinetics of liver regeneration post-PH in invalidated Cre+ mutants and their control Cre− littermates. There was no significant difference in survival after PH between Cre+ and Cre− mice. BrdUrd incorporation was detected by immunohistochemistry to evaluate the proportion of hepatocytes in S phase at different times after PH. As shown in Fig. 3, A and B, BrdUrd incorporation was significantly reduced and delayed 30 and 36 h after PH in Cre+ animals compared with Cre− mice, respectively. To determine whether the progression through mitosis was also delayed or impaired, hematoxylin-eosin-stained liver sections from Cre− and Cre+ animals were analyzed at different times after PH for mitotic figures (Fig. 3, C and D). The results showed that the mitosis frequency at 48 h after PH was significantly greater in SRFfl/Cre− than in SRFfl/Cre+ mice (P < 0.05). Taken together, these data show that, in the liver of mice invalidated for SRF, hepatocellular cell cycle progression was delayed. Seven days after PH, there was no longer a significant difference in the liver weight-to-body...
expression of two IEGs, c-fos, and the recombined allele (allele Δ) of the srf gene. Exons are shown as numbered boxes. Numbered arrows show primer positions for PCR and RT-PCR analyses. A: PCR amplification on liver DNA using SF1 and SF3 primers. A 310-bp fragment corresponding to the deleted allele is amplified specifically in the liver (L) and not in the pancreas (P), the brain (B), the spleen (S), the kidney (K), the muscle (M), or the heart (H) of a representative mutant adult animal. B: PCR amplification of the deleted allele in liver DNA using SF1-SF3 primers in Cre+/+ and Cre−/− animals at embryonic day 10.5 (E10.5), E12.5, and E18.5. D: RT-PCR analysis of liver cDNA from a homozygous SRF+/+Cre− animal showing the normal transcript at 330 bp, a heterozygous SRF+/+Cre−/− animal showing both deleted (192 bp) and normal transcripts, and a homozygous mutant SRF+/+Cre−/− animal showing only the deleted transcript. E: Northern blot analysis revealed 2 expected bands of 4.5 and 2.5 kb in Cre−/− animals. These transcripts are not detected or barely detected in SRF+/+Cre+/+ mice.

weight ratio, indicating that regeneration was finally completed in mutant mice. Moreover, no evidence of enhanced cell death could be seen in mutant livers posthepatectomy by hematoxylin-eosin and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining (data not shown).

To assess whether the observed decrease in DNA synthesis was the consequence of impaired progression of hepatocytes through the G1/S restriction point, we measured the protein levels of cell cycle-associated proteins after PH. Cyclin A and cyclin E were induced in Cre+/+ mutant mice but with a significant delay compared with Cre−/− control mice (Fig. 4, A and B). In Cre+/+ mutant animals, cyclins E and A were induced at 44 h compared with 30 and 36 h in Cre−/− controls, respectively. These results show that SRF deletion causes a delay in the progression through the G1/S transition phase, confirming the results obtained in mutant mice by BrdUrd incorporation.

Altered induction of IEGs. IEGs are involved in the first step of liver regeneration, called the priming phase, which is initiated a few minutes after PH. Because the phenotype observed in mutant liver occurred early during the liver regeneration process, we wondered whether this delay could be a consequence of a blunted induction of IEGs. First, we examined the expression of two IEGs, c-myc and Egr-1, known to be up-regulated 2 h after PH and still induced at 12 h. Egr-1 is a known transcriptional target of SRF, whereas c-myc is not. c-myc expression was not differently induced 90 min and 12 h after PH in mutant and control animals (Fig. 5A). In contrast, Egr-1 was only induced in control animals and not in mutant ones (Fig. 5B).

We looked at other IEGs that are known targets of SRF and are normally induced at early points after PH. Mutant mice showed a differential IEG activation 2 h after PH when compared with wildtype littersmates, as evidenced by the reduced levels of JunB, c-fos, and pip92 (Fig. 5C). In contrast, Cyr61 expression was not affected. Impairment in the activation of these IEGs is consistent with the induction of SRF gene expression shortly after liver resection. Together, these observations suggest that the early induction of SRF after PH is required for the normal onset of liver regeneration.

DISCUSSION

SRF is a transcription factor with a dual specificity (28): on the one hand, it plays a major role in muscle and heart developmental programs (5, 17, 22, 23); on the other, it is essential for the induction of various IEGs (25). During liver regeneration, IEGs have been shown to direct growth factor-dependent hepatocellular reentry and progression through the cell cycle. We studied the expression of SRF during liver regeneration and showed that it is induced in the early steps of this process. We then performed a targeted invalidation of SRF in the liver to determine whether its increased expression was detrimental for regeneration. Mutant mice, in which disruption of the SRF gene occurred during embryogenesis before E12.5, harbor a normal liver architecture, indicating that SRF hepatocyte expression is dispensable for liver ontogenesis. We then performed PH as a model of liver regeneration. Mutant animals showed an impaired regeneration after PH, in correlation with a delayed induction of cyclins A and E. We found a blunted induction of various transcriptional IEGs that are normally upregulated in the first steps of liver regeneration, and that could have contributed to the delayed progression through hepatocyte cell cycle observed in mutant mice after surgery. Among them, Egr-1, JunB, pip92, and c-fos, which are known targets of SRF, were not induced in SRF mutant regenerating livers. It would be interesting to compare SRF+/+Cre−/− mutants with mice lacking c-fos to establish whether the delayed regeneration is related to the decrease in c-fos. However, only 40% of c-fos null mice survive, showing osteopetrosis, growth retardation, and abnormal hematopoiesis (13, 29), and no liver-specific c-fos invalidation has yet been pub-
Fig. 3. Kinetics study of liver regeneration. A: proportion of bromodeoxyuridine (BrdUrd)-positive hepatocytes at different times after PH (n = 4 animals/group). All data are means ± SE. *P < 0.05. B: BrdUrd immunostaining of representative liver sections at different times after surgery in SRFf/fCre− and Cre+ mice, counterstained with hematoxylin (original magnification, ×400). C: hematoxylin-eosin staining of representative liver sections 48 h after PH in Cre− and Cre+ animals (original magnification, ×400). Arrowheads indicate figures of mitosis. D: proportion of hepatocytes undergoing mitosis at different times after PH (n = 4/group). All data are means ± SE. *P < 0.05.
lished. In the same line, the specific roles of JunB, a component of the activator protein-1 factor known to play a role in liver regeneration (2, 8), and pip92, involved in the initial mechanism for cellular adaptation to stress (6), during liver regeneration have never been studied. Blunted Egr-1 induction could also have contributed to the observed phenotype of SRFf/fCre mice. Egr-1 is a zinc finger transcription factor induced in various models of cellular proliferation during the transition from G0 to G1. Egr-1-deficient mice showed impaired liver regeneration with lower hepatocellular BrdUrd labeling and reduced hepatocellular mitotic body frequency 48 h after PH (18). The first wave of DNA synthesis occurs normally in Egr1−/− livers, but progression through the cell cycle was impaired thereafter. The livers of SRFf/fCre+ mice also showed a delayed regeneration with a reduced peak of mitosis 48 h after PH. However, the kinetics of hepatocellular proliferation was slightly different between SRFf/fCre+ and Egr1−/− livers, SRF mutant livers showing an earlier delay in regeneration with impaired induction of cyclins involved in the progression through the G1/S transition phase. This difference can be explained by the incomplete inactivation of Egr-1 in the liver of SRF mutant mice and/or by the participation of other SRF target genes. A combined effect of reduced IEG expression, such as c-fos, Egr-1, pip92, and JunB, may have contributed to the reduced regenerative response in SRF−/− livers. Further analysis of gene expression, combining, for example, microarrays with chromatin immunoprecipitation, will help unveil other SRF target genes involved in liver regeneration. In contrast, Cyr61, a gene critical to the formation of new blood vessels (15, 16, 20), seemed not to be affected by SRF liver deletion. However, this is not surprising, since this gene is mostly expressed in the vasculature and endothelial cells, where SRF has not been deleted. Multiple pathways contribute to the completion of the regeneration process, explaining why the loss of an individual gene rarely leads to complete inhibition of liver regeneration (3, 10, 18, 19, 21).

In conclusion, our data indicate that SRF is a new player in this complex network that is liver regeneration, triggering the early phases of this process through the activation of various IEGs.
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