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Genetics

SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis

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ABSTRACT **SEK1 (MKK4/JNKK) is a mitogen-activated protein kinase activator that has been shown to participate *in vitro* in two stress-activated cascades terminating with the SAPK and p38 kinases. To define the role of SEK1 *in vivo*, we studied stress-induced signaling in SEK1$^{-/-}$ embryonic stem and fibroblast cells and evaluated the phenotype of SEK1$^{-/-}$ mouse embryos during development. Studies of SEK1$^{-/-}$ embryonic stem cells demonstrated defects in stimulated SAPK phosphorylation but not in the phosphorylation of p38 kinase. In contrast, SEK1$^{-/-}$ fibroblasts exhibited defects in both SAPK and p38 phosphorylation, demonstrating that crosstalk exists between the stress-activated cascades. Tumor necrosis factor α and interleukin 1 stimulation of both stress-activated cascades are severely affected in the SEK1$^{-/-}$ fibroblast cells. SEK1 deficiency leads to embryonic lethality after embryonic day 12.5 and is associated with abnormal liver development. This phenotype is similar to c-jun null mouse embryos and suggests that SEK1 is required for phosphorylation and activation of c-jun during the organo-genesis of the liver.**

Three vertebrate mitogen-activated protein kinase (MAPK) cascades have been shown to regulate signal transduction necessary for the development and function of many tissues (1–3). The stress-activated kinase cascade involving MEKK, SEK1, and SAPK leads to the phosphorylation of c-jun and is activated by stress stimuli such as interleukin 1 (IL-1), tumor necrosis factor α (TNFα), heat shock, UV irradiation, and protein synthesis inhibition. The p38 MAPK cascade is activated by similar stimuli. The upstream kinases MKK3 and MKK6 function to activate p38(4–7). The MAPK SEK1 (also called MKK4 or JNKK) is capable of phosphorylating and activating SAPK *in vitro*. Furthermore, kinase inactive mutants of SEK1 specifically prevent SAPK activation by stress stimuli (4). SEK1 also has been shown to phosphorylate and activate p38 MAPK *in vitro* (4), suggesting that SEK1 may participate in the regulation of two independent MAPK cascades.

Two recent reports have evaluated the function of SEK1 in murine embryonic stem (ES) cells (8, 9). These targeted gene disruption experiments demonstrated a significant defect in SAPK activity in response to stress signals such as anisomycin and heat shock. The activation of SAPK in response to UV irradiation was affected less dramatically in these SEK1-deficient ES cells, suggesting the existence of another kinase that activates SAPK. A candidate could be the recently cloned SEK1 family member MKK7 (10), which has been shown to specifically activate SAPK but not p38. Although these reports show that the activation of SAPK is affected in SEK1$^{-/-}$ ES cells, there are no defects seen in p38 kinase activity, implying that SEK1 only regulates the SAPK cascade *in vivo* and that the activation of p38 by SEK1 is an *in vitro* phenomenon.

These reports do not address the role of SEK1 in TNFα and IL-1 signaling. ES cells do not express receptors for TNFα and IL-1. To evaluate this, we generated SEK1$^{-/-}$ embryonic fibroblast lines and studied the response of these cells after treatment with different stimuli. Surprisingly, a significant defect in IL-1 and TNFα stimulation of both SAPK and p38 phosphorylation was detected and demonstrated that crosstalk between the SAPK and p38 MAPK cascades can occur through SEK1. The difference between these results obtained in SEK1$^{-/-}$ fibroblasts with the studies of SEK1$^{-/-}$ ES cells suggests that the signal-dependent activation of the cascades is distinct in different cell types. As reported (8, 9), SEK1 deficiency leads to an embryonic lethality some time before embryonic day (E) 14.5, but no information exists as to the function of SEK1 in development before this time or to the cause of death. Our analysis of the SEK1$^{-/-}$ mouse embryos demonstrates abnormal liver formation and hemorrhage. The similarity of this phenotype with that of c-jun-deficient embryos suggests that the stress-activated MAPK cascades lead to c-jun activation, which may be involved in normal liver development *in vivo.*

MATERIALS AND METHODS

Targeted Disruption of the SEK1 Gene. W9.5 ES cells were used for homologous recombination as described (11, 12) by using pGKneo/TK for construction of the targeting vector. The targeting vector was produced by insertion of a 6-kb *Bgl*II fragment into the *Bam*HI site of pGKneo/TK followed by insertion of a 1-kb *Eco*RI fragment into the *Pme*I site of the resulting vector. Initial selection of targeted ES cells was carried out first in 175 mg/ml G418 and later in 2 mg/ml G418 to obtain SEK1$^{-/-}$ cells.

ES Cell Culture. The ES cell lines were adapted by passage on gelatin plates and cultured in DMEM (Gibco/BRL) containing 15% fetal calf serum (HyClone), 2 mM glutamine (Gibco/BRL), 1× nonessential amino acids (Gibco/BRL), 0.1 mM β-mercaptoethanol (Sigma), 100 units/ml penicillin–streptomycin (Gibco/BRL), and 1,000 units/ml lymphocyte inhibitory factor (Gibco/BRL) at 37°C and in 5% CO_2.

Mouse Embryonic Fibroblast (MEF) Cultures. E13.5 embryos were isolated from an outbred mouse strain, from wild-type129Sv mice, or from chimeric embryos created by

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Abbreviations: MAPK, mitogen-activated protein kinase; ES, embryonic stem; TNFα, tumor necrosis factor α; IL-1, interleukin 1; E, embryonic day; MEF, mouse embryonic fibroblast.

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injection of nontransgenic blastocysts with SEK1$^{-/-}$ ES cells. Wild-type and SEK1$^{-/-}$ embryonic fibroblasts were cultured in 10% fetal bovine serum (HyClone), 2 mM glutamine (Gibco/BRL), and 1 mM nonessential amino acids (Gibco/BRL). SEK1$^{-/-}$ embryonic fibroblasts were maintained in 400 μg/ml G418 and were passaged at least twice to eliminate wild-type fibroblasts.

Western Blot Analysis. Immunoblot analysis of cell lysates was performed by probing with polyclonal antibodies to SAPK/JNK, phosphorylated SAPK/JNK (Thr 183/Tyr 185) (New England Biolabs), p38 and phosphorylated p38 (New England Biolabs), MKK3 [Santa Cruz Biotechnology, MEK-3 (C-19)] and phosphorylated MKK3/MKK6 (Ser189/207) (New England Biolabs), and SEK1 (Santa Cruz Biotechnology). Immune complexes were detected by enhanced chemiluminescence (Amersham).

Immunohistochemistry and *in Situ* Hybridization. Specimens were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2) at 4°C overnight, were dehydrated through a graded alcohol series, and were embedded in paraffin. Sections of 4 μm were prepared and stained with Harris hematoxylin and eosin according to standard procedures.

In Situ hybridization of specimens were carried out as reported (13). Apoptosis assays were carried out by using an ApopTag kit (Oncor) as recommended by the manufacturer. For counting cells stained with the cytokeratin antibody or by ApopTag, three high power fields (×63) of two different individual animals were evaluated.

RESULTS AND DISCUSSION

To generate a targeted mutation of the SEK1 locus, we disrupted two exons encoding part of the catalytic domain (Fig. 1). The modification introduces a frame shift that causes a deletion mutation. This modified gene should produce only a truncated SEK1 protein that would not function. Several independent heterozygous (SEK1$^{+/-}$) ES lines were generated. Multiple distinct SEK1$^{-/-}$ ES cell lines also were isolated by culture in increased G418 concentration (14) (Fig. 1*B*) as well as by retargeting the second allele (data not shown). Western blot analysis demonstrated that SEK1 protein was not detected in these ES cell lines (Fig. 1*C*). Compared with wild-type ES cells, heterozygous lines expressed about one-half the amount of SEK1 protein.

To examine the effect of SEK1 gene disruption on the phosphorylation and activation of SAPK and p38 in ES cells, the following experiments were performed. Wild-type and SEK1$^{-/-}$ ES cells were treated with anisomycin, heat shock, and UV irradiation. SEK1$^{-/-}$ ES cells treated with anisomycin or heat shock exhibited no SAPK phosphorylation compared with wild-type cells, as shown by western blot analysis with a phospho-SAPK antibody (Fig. 2 *a* and *b*). No difference in p38 phosphorylation was seen in ES cells. Thus, SEK1 has a specific role in the SAPK signal transduction pathway in response to these stimuli whereas the p38 signaling cascade in ES cells is not dependent on SEK1 activity. In contrast, UV irradiation of the SEK1$^{-/-}$ ES cell lines led to activation of SAPK phosphorylation, but the activation of SAPK was lower than in wild-type ES cell lines (Fig. 2*b*), supporting a model where alternative pathways exist that lead to the phosphorylation of SAP kinases. Another MAPK activator, MKK7, has been described recently (10) and could suffice in this role. To demonstrate that c-jun activity is affected by the lack of SAPK phosphorylation in the SEK1$^{-/-}$ cells, we used a c-jun dependent reporter system (PathDetect, Stratagene). The PathDetect *in vivo* reporter system includes the fusion activator plasmids that consist of the DNA binding domain of the yeast GAL4 fusion activator and the activation domain of the c-jun transcription factor. Stimulation of cells with anisomycin or by MEKK transfection led to activation of the reporter construct in wild-type ES cells; in contrast, activity was stimulated poorly

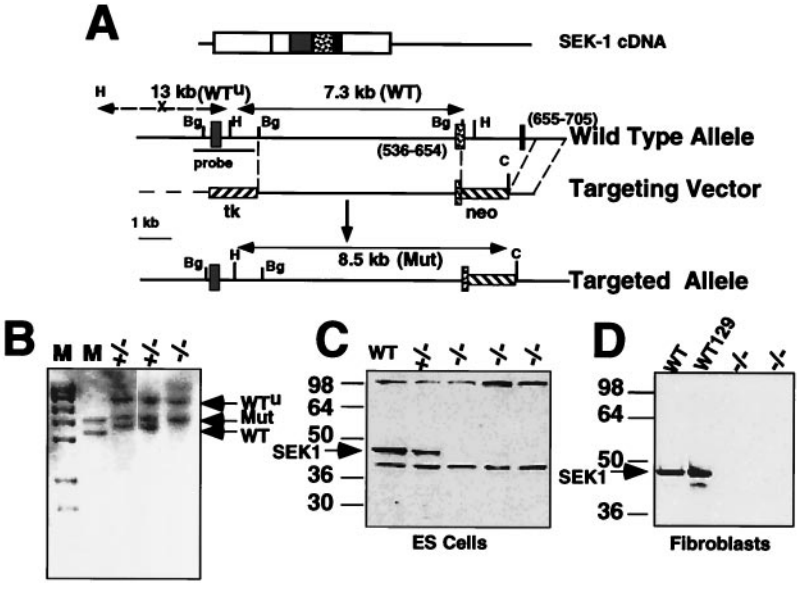

FIG. 1. Targeted disruption of the SEK1 gene. (*A*) Targeting Strategy. The SEK1 cDNA is shown at the top; the coding region is represented by boxes, and the internal catalytic domain is represented by separate filled boxes corresponding to specific exons also shown on the genomic map. The structure of the wild-type and targeted alleles are shown. The SEK1 cDNA nucleotides representing the mutated exons are shown in parenthesis. Genomic DNA was analyzed for targeting events by Southern blot analysis by using the indicated probe and *Hpa*I (H) and *Cla*I (C) digested DNA. The probe recognizes two wild-type fragments: a 13-kb 5′ (WT^{u}) fragment whose structure is not affected by the SEK1 mutation and a 3′ 7.3-kb fragment (WT). An 8.5-kb fragment appears after homologous recombination with the targeting vector. (*B*) Identification of SEK1$^{-/-}$ ES cells. A Southern blot is shown of the parental SEK1$^{+/-}$ ES cell lines and an example of one SEK1$^{-/-}$ line derived by increasing the dose of G418. M represents markers. (*C*) Western blot analysis of SEK1 expression in targeted ES cells. Shown are SEK1 levels present in either the parental W9.5 ES cell line (WT) or one of the heterozygous targeted lines (+/−) and three independent SEK1$^{-/-}$ ES cell lines. No SEK1 protein was detected in any of the double knockout cell lines. (*D*) Western blot analysis of SEK1 expression in primary fibroblast cultures. No SEK1 was detected in any of the SEK1$^{-/-}$ fibroblast cultures.

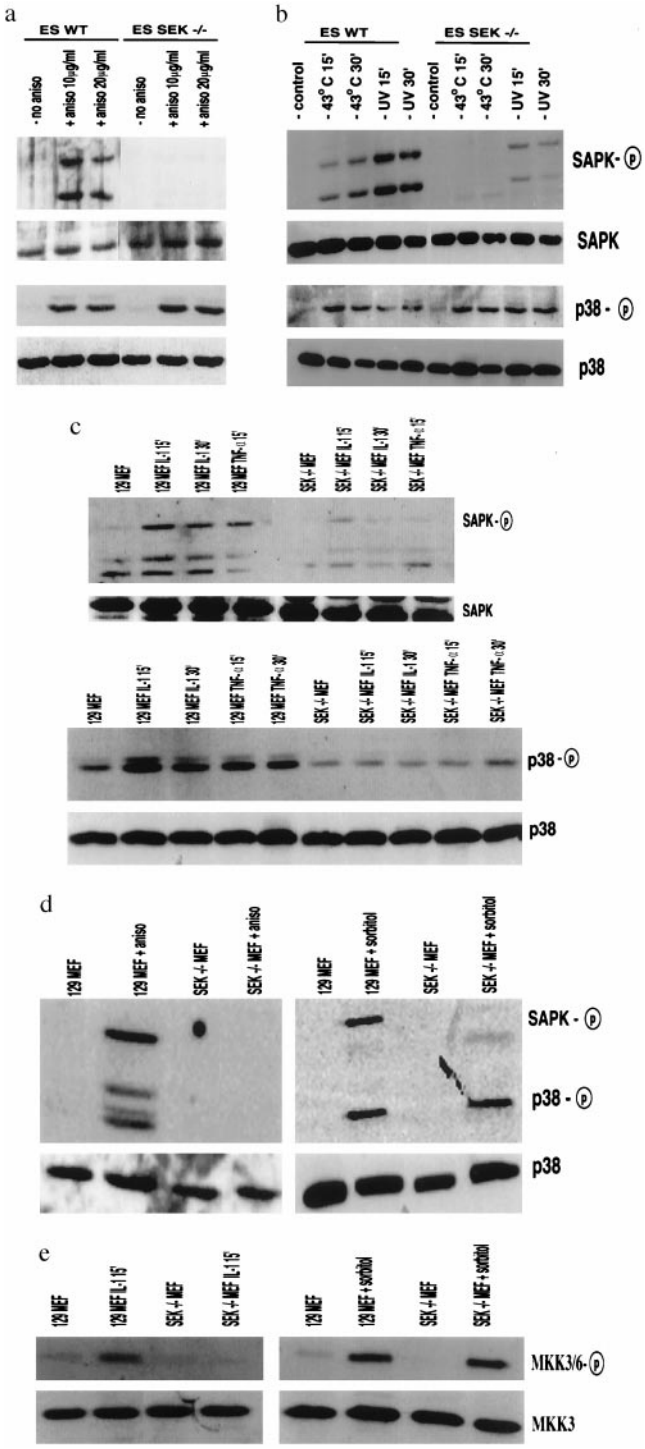

FIG. 2. Effect of SEK1 gene disruption on the phosphorylation and activation SAPK/JNK and p38 in ES cells and mouse embryonic fibroblasts. (*a*) Western blot analysis of wild type (ES WT) and SEK1$^{-/-}$ (ES SEK$-/-$) ES cells either untreated or activated with anisomycin (10 and 20 μg/ml, 30 min). Western blots were probed with specific antisera to phospho SAPK/JNK and p38. Control Western blots for levels of SAPK and p38 protein also are shown. (*b*) Wild-type (ES WT) and SEK1$^{-/-}$ (ES SEK$-/-$) ES cells were either untreated or UV treated (80 J/m^2 and then incubated for 15 min, 30 min) or heat shocked (43°C, 15 min, 30 min). Western blot analysis for phospho SAPK/JNK and phospho p38 is shown. Control Western blots for levels of SAPK and p38 protein also are shown. (*c*) 129 MEFs and SEK1$^{-/-}$ MEFs were stimulated with IL-1 (10 ng/ml, 15 min and 30 min) or TNFα (50 ng/ml, 15 min). Western blot analysis for phospho SAPK/JNK and phospho p38 is shown. Control western blots for levels of SAPK and p38 protein also are shown. (*d*) 129 MEFs and SEK1$^{-/-}$ MEFs stimulated with anisomycin (40 μg/ml, 30 min.) and sorbitol (2.5 mM, 15 min). Western blot analysis for phospho SAPK/JNK and phospho p38 is shown. Control Western blots for levels of SAPK and p38 protein also are shown. (*e*) 129 MEFs and SEK1$^{-/-}$ MEFs stimulated with IL-1 (10 ng/ml, 15 min) or sorbitol (2.5 mM, 15 min). Western blot analysis for phospho MKK3/6 is shown. Control Western blots for levels of MKK3 protein also are shown.

in the SEK1$^{-/-}$ cells (data not shown). These results are similar to those reported for SEK1$^{-/-}$ ES cells (8, 9).

Because ES cells do not express the receptors for IL-1 or TNFα, the role of cytokine signaling was investigated in SEK1-deficient MEFs. Western blot analysis confirmed that these lines were deficient in SEK1 protein (Fig. 1*D*). Wild-type fibroblast cells responded to IL-1 or TNFα by increased phosphorylation of SAPK (Fig. 2*c*). In contrast, SEK1$^{-/-}$ fibroblasts were low or undetectable in SAPK phosphorylation in response to IL-1 or TNFα . SAPK phosphorylation also was low or undetectable in response to anisomycin or sorbitol in these cells (Fig. 2*d*). Surprisingly, p38 phosphorylation also is reduced in response to IL-1, TNFα, and anisomycin (Fig. 2 *c* and *d*), whereas sorbitol-stimulation leads to an equivalent level of p38 phosphorylation in wild-type and SEK1$^{-/-}$ fibroblast cells. Because SEK1 has been shown to phosphorylate p38 *in vitro* (15), our data supports a model in which crosstalk exists between the two stress-activated cascades. As MKK3/6 have been shown to be the upstream activators of p38, we examined the effect of MKK3/6 activation by these cytokines in the SEK1 deficient embryonic fibroblasts. IL-1 does not stimulate MKK3/6 phosphorylation in the absence of SEK1 (Fig. 2*e*) whereas sorbitol leads to MKK3/6 phosphorylation. This finding, taken together with the lack of SAPK phosphorylation in IL-1 or sorbitol treatment of SEK1 deficient fibroblasts, demonstrates that SEK1 regulates MKK3/6 activation independent of SAPK activity. This could occur directly through a protein interaction between SEK1 and MKK3/6 or indirectly by SEK1 regulation of upstream activators of MKK3/6. Alternatively, it is possible that there is an indirect effect of targeting SEK1. For example, the fibroblasts that are SEK1 deficient grow more slowly compared with the wild-type cells, perhaps supporting prior evidence for effects of the stress-activated cascades on the cell cycle (16–18). These results demonstrate that SEK1 can affect p38 activity *in vivo* and that crosstalk between the SAPK and p38 cascades occurs. The differences in p38 phosphorylation in SEK1$^{-/-}$ ES and fibroblast cells suggests that the stimulation and crossregulation of these two stress-activated cascades is cell-type dependent.

To determine whether SEK1 is required for normal development, SEK1$^{+/-}$ ES cells were injected into host blastocysts, and chimeric mice were derived. Through matings, heterozygous mice were obtained, and these mice were bred to obtain SEK1 null animals. Deficiency of SEK1 is embryonically lethal. No live births were found in >150 offspring. Yang *et al.* (8) also noted an embryonic lethality with no SEK1$^{-/-}$ animals surviving beyond E14.5 (8). In our studies, timed matings of heterozygotes produced viable SEK1$^{-/-}$ embryos at E12.5. These embryos exhibited a severe phenotype that included hemorrhage in the region of the liver (Fig. 3*a*). Examination of SEK1$^{-/-}$ embryos from E11.5–12.5 demonstrated that the liver bud forms but is considerably reduced in size compared with wild-type embryos. Livers from SEK1$^{-/-}$ embryos exhibit abnormal histology, including enlarged sinuses (large arrows), and many embryonic livers have two small lobes (small arrows). To further examine the embryonic liver, tissue sections were stained with various antibodies such as cytokeratin and Factor VIII (Dako) to look at the hepatocytes and the liver architecture in general. The liver abnormalities appeared to be caused by a decrease in hepatocyte

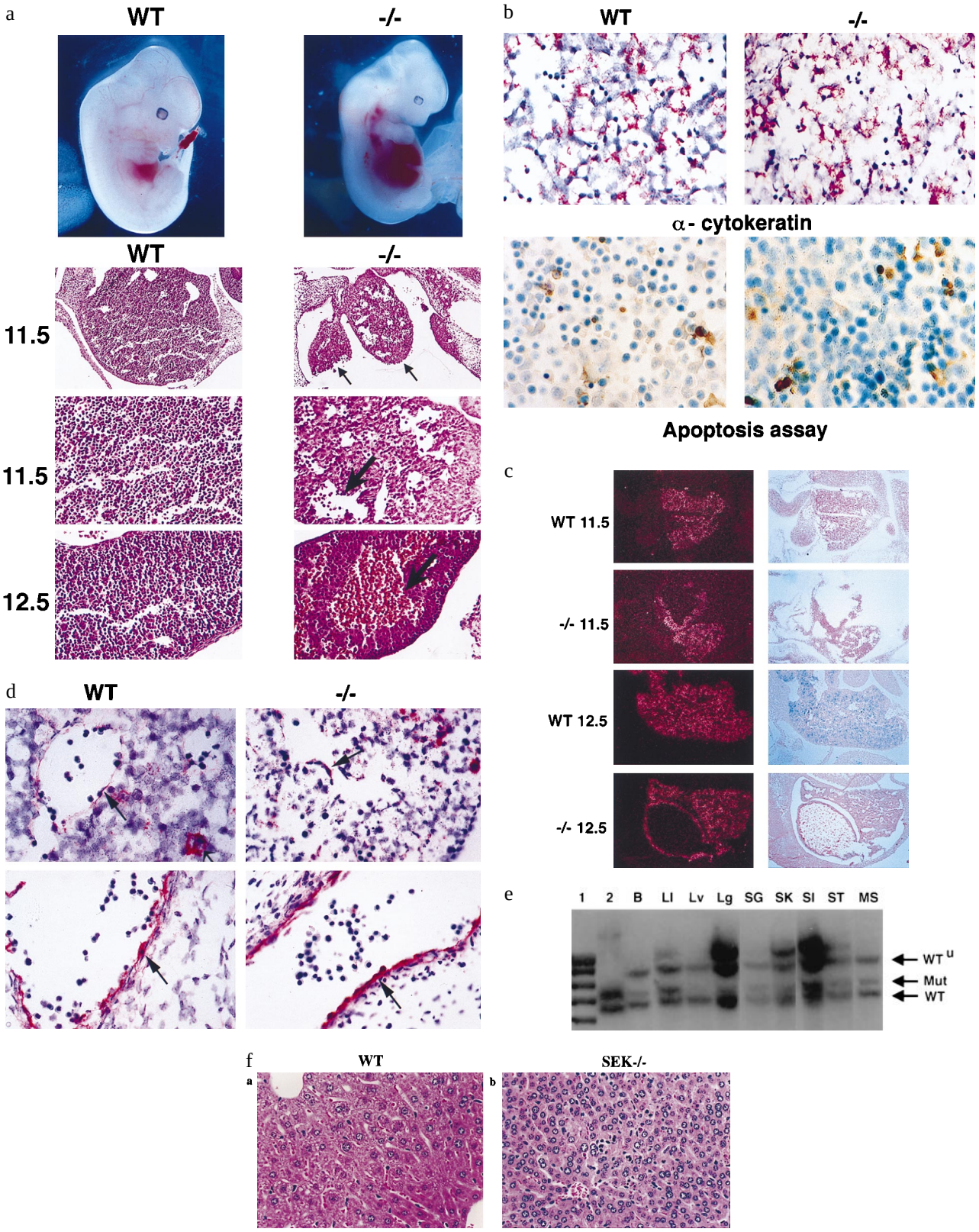

FIG. 3. Characterization of SEK1$^{-/-}$ embryos. (*a*) (*Top*) Wild-type (WT) and SEK1$^{-/-}$ (−/−) embryos at E12.5. (*Bottom*) Histological analysis with hematoxylin and eosin staining. Wild-type and SEK1$^{-/-}$ liver at E11.5 (×10 and ×25) and E12.5 (×25). (*b*) (*Top*) Immunohistochemical analysis of wild type (WT) and SEK1$^{-/-}$ (−/−) embryos at E11.5 with mAb to cytokeratin (Dako) (×63). The red color indicates specific staining. The numbers of cytokeratin-expressing cells per high power field are 476 ± 34, 318 ± 63, 350 ± 44, and 318 ± 17 for livers from wild-type E12.5, SEK1$^{-/-}$ E12.5, wild-type E11.5, and SEK1$^{-/-}$ E11.5 embryos, respectively. (*Bottom*) ApopTag staining of sections from wild-type (WT) and SEK1$^{-/-}$ (−/−) embryos at E12.5 (×63). The brown color indicates specific staining. The number of apoptotic cells per high power field was 34 ± 1 and 66 ± 13 for wild-type and SEK1$^{-/-}$ embryos, respectively. (*c*) *In situ* hybridization studies for transthyretin mRNA expression at day 11.5 and 12.5. (*Right*) Hematoxylin and eosin staining of corresponding sections. (*d*) Immunohistochemical analysis of wild-type (WT) and SEK1$^{-/-}$ (−/−) embryos at E11.5 with polyclonal antisera to Factor VIII (Dako) (×63). (*Top*) Liver. The small arrow delineates megakaryocytes. The large arrow shows the endothelial cells. (*Bottom*) Control section on other regions of the embryo. (*e*) Representative Southern blot analysis of tissues

number. Cytokeratin staining (19) at E11.5 demonstrated a reduced number of hepatocytes per embryo in SEK1$^{-/-}$ animals (Fig. 3*b Top*). The liver size increased between E11.5 to E12.5 in the SEK1$^{-/-}$ embryos, almost reaching an equivalent size as the wild-type liver. However, the number of hepatocytes per high power field in the liver of SEK1$^{-/-}$ embryos was lower than in the wild-type controls (see Fig. 3 legend). The sinuses of the liver continued to enlarge, and hemorrhage occurred into the cavity (Fig. 3*a*). To examine whether increased hepatocyte death was contributing to the liver degeneration, apoptosis assays were performed on the embryonic liver sections. There was almost a 2-fold increase in the number of apoptotic hepatocytes in the SEK1$^{-/-}$ livers (Fig. 3*b Bottom* and figure legend). Increased apoptosis could contribute to the liver abnormality; however, a 2-fold increase may not be sufficient to explain the cause of death. Although the exact cause of embryo death is unknown, the pale appearance and massive bleeding suggest that the embryos may die of anemia. Although stress-induced MAPK cascades are activated by hematopoietic cytokines (20, 21), yolk sac progenitor studies from E10.5 SEK1$^{-/-}$ embryos exhibit normal hematopoiesis (S.G. and L.I.Z., unpublished material). *In situ* hybridization studies demonstrated only slightly lower expression of liver-specific markers, transthyretin (Fig. 3*c*) (22), and α-fetoprotein (data not shown) (23) in the SEK1$^{-/-}$ liver bud, suggesting that there was no obvious problem with differentiation. Factor VIII staining of tissue sections demonstrated that SEK1$^{-/-}$ sinusoids frequently were lined with loose endothelial cells (Fig. 3*d Top*). Because endothelial cells in other tissues appeared grossly normal (Fig. 3*d Bottom*), it is likely that a primary defect in SEK1$^{-/-}$ livers is the hepatocyte number and architecture and not abnormalities in endothelial cells in general. To further define the defect in liver formation of SEK1$^{-/-}$ cells, we performed chimeric analysis. SEK1$^{-/-}$ ES cells were injected into wild-type blastocysts, and DNA prepared from isolated tissues was subjected to Southern blot analysis. The only tissue that consistently shows extremely low or no contribution from the SEK1$^{-/-}$ ES cells was the liver (Fig. 3*e*). Histological analysis of a liver from one high-level chimera demonstrated abnormal liver architecture, including hepatocyte hypoplasia and nuclear fragmentation of hepatocytes characteristic of apoptosis (Fig. 3*f*). These data suggest that SEK1 is cell autonomous for hepatocyte growth.

The phenotype of the SEK1-deficient mouse is strikingly similar to the c-jun-deficient mouse (24–26). It is possible that other genes important in liver development such as *relA* (27) and *Hlx* (28) may be targets of the SAPK or p38 cascades and thus may account for the defect in liver formation. As c-jun has been proposed to be a target of the SEK1/SAPK cascade, it is likely that the kinase cascade regulates c-jun phosphorylation during hepatogenesis. SAPK has been shown to be activated in liver extracts by metabolic stress (29), and SAPK signaling has been postulated to control fetal hepatocyte proliferation (30). Partial hepatectomy and subsequent liver regeneration also lead to SAPK activation and c-jun phosphorylation (31). p38 also has been shown to be activated in regenerating adult rat hepatocytes (32). In addition, p38 has been shown to activate transcription factors such as MEF2 (33), which may ultimately regulate tissue differentiation and proliferation. Stimulation of the *met* oncogene, which is the receptor for hepatocyte growth factor, or the *met*-related STK/RON receptor, which is the receptor for macrophage stimulating protein, recently has been shown to activate the SAPK pathway (34, 35). Furthermore, defects in mice deficient in hepatocyte growth factor or c-met (11, 12) are similar to defects in mice deficient in SEK1 or c-jun, perhaps indicating the existence of a ligand-stimulated cascade of events responsible for liver formation *in vivo*. Our studies of the SEK1$^{-/-}$ ES cells and embryos formally demonstrate that SEK1 is required for normal liver development *in vivo* and suggests a model in which the SEK1/SAPK kinase cascade is required for c-jun phosphorylation during embryogenesis.

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from a highly chimeric mouse. SEK1$^{-/-}$ cells fail to contribute to the liver. Five Chimeras were analyzed. Lanes: 1, marker; 2, phage DNA containing the SEK1 gene; B, bladder; LI, large intestine; Lv, liver; Lg, lung; SG, salivary gland; SK, skin; SI, small intestine; ST, stomach; MS, muscle. (*f*) Histopathology of liver derived from a highly chimeric adult mouse. Stained with hematoxylin and eosin. (a) Normal liver (×40). (b) SEK1$^{-/-}$ chimera (×40). Note the nuclear fragmentation and abnormal hepatocyte lobule structure.

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