Deficiency of Kinase Suppressor of Ras1 Prevents Oncogenic Ras Signaling in Mice

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ABSTRACT

In Drosophila and Caenorhabditis elegans, kinase suppressor of ras (KSR) positively modulates Ras/Raf/mitogen-activated protein kinase (MAPK) signaling. The precise signaling mechanism of mammalian KSR1 and its role in Ras-mediated transformation, however, remain uncertain. To gain insight into KSR1 function in vivo, we generated mice homozygous null for KSR1. ksr1−/− mice are viable and without major developmental defects. However, an unusual disorganized hair follicle phenotype manifests in epidermal growth factor receptor knockout mice is recapitulated in ksr1−/− mice, providing genetic support for the notion that epidermal growth factor receptor, Ras, and KSR1 are on the same signaling pathway in mammals. Furthermore, ksr1−/− mice allow for the definition of KSR1-dependent and -independent mechanisms of c-Raf-1 activation. In embryonic fibroblasts, epidermal growth factor and 12-O-tetradecanoylphorbol-13-acetate activated the MAPK cascade to a similar extent, yet only c-Raf-1 activation by epidermal growth factor depended on KSR1. Moreover, whereas the genesis of polymavirus middle T antigen (MT)-driven mammary cancer appears independent of KSR1, KSR1 is obligate for v-Ha-ras-mediated skin tumor formation. The growth of MT-driven mammary tumor was moderately slowed in ksr1−/− mice, however, consistent with a decreased rate of proliferation of ksr1−/− cells (T cells and embryonic fibroblasts). Nonetheless, all ksr1−/− animals succumbed to mammary cancer. In contrast, papilloma formation in Tg.AC mice, resulting from skin-specific v-Ha-ras expression, was completely abrogated in the ksr1−/− background. Hence, MT-driven mammary tumor genesis, which is signaled through src and phosphatidylinositol 3-kinase, appears KSR1 independent, whereas v-Ha-ras-mediated skin cancer, signaled through the Raf-1/MAPK cascade to a similar extent in MEFs, yet only c-Raf-1 activation by mitogenic doses of EGF depended on KSR1. The KSR1 knockout mouse thus allows the delineation of KSR1-dependent and -independent mechanisms of c-Raf-1 activation. Furthermore, tumor formation in Tg.AC mice resulting from skin-specific v-Ha-ras expression, which utilizes MAPK signaling for transformation (18), was abrogated in the ksr1−/− background. These defects in proliferation, transformation, and tumor formation suggest that KSR1 transduces some forms of Ras-mediated neoplasia.

INTRODUCTION

KSR4 was identified in Drosophila melanogaster and Caenorhabditis elegans as a positive modulator of Ras/MAPK signaling either upstream of or parallel to Raf (1–3). Although an intensive effort has been directed at elucidating the biochemical properties of mammalian KSR1, its precise signaling mechanism remains uncertain. In particular, its role in Ras-mediated transformation has not been addressed convincingly. Some groups have reported that KSR1 inhibits MAPK activation and Ras-induced transformation (4–6), whereas others have observed enhancing effects (7–9). These experiments used cell systems overexpressing recombinant KSR1 to levels far beyond endogenous KSR1, and evidence suggests these discrepancies might reflect gene dosage (10). Whereas we and others argue the necessity of both the kinase and scaffolding functions of KSR1 for its optimal activation of the Raf-MAPK cascade (11–15), others believe that KSR1 signals solely via its scaffolding function (8, 16, 17).

To gain insight into the in vivo function of KSR1, we generated a mouse homozygous null for KSR1. ksr1−/− mice are viable and without major developmental defects. Newborn mice, however, display a unique hair follicle phenotype previously observed in EGFR-deficient mice. MEFs from ksr1−/− animals displayed diminished proliferative potential and impaired oncogenic v-Ha-Ras-dependent transformation. Moreover, EGF and TPA activated the MAPK cascade to a similar extent in MEFS, yet only c-Raf-1 activation by mitogenic doses of EGF depended on KSR1. The KSR1 knockout mouse thus allows the delineation of KSR1-dependent and -independent mechanisms of c-Raf-1 activation. Furthermore, tumor formation in Tg.AC mice resulting from skin-specific v-Ha-ras expression, which utilizes MAPK signaling for transformation (18), was abrogated in the ksr1−/− background. These defects in proliferation, transformation, and tumor formation suggest that KSR1 transduces some forms of Ras-mediated neoplasia.
Northern and Western Blot Analysis of KSR1 Gene Expression. Poly(A)^+ RNA was prepared from adult mouse tissues using the Oligotex kit from Qiagen Inc. Blots were hybridized with a specific ^32P-labeled probe corresponding to the CA2-CA4 domains of murine ksr1 cDNA (1.47 kb). For embryonic tissues, we used a Mouse Embryo MTN Blot (BD Biosciences). Protein homogenates were prepared from ksr1^+/− and ksr1^−/− tissues or MEFs in radiolabelling assay buffer and fractionated by SDS-PAGE (100 µg protein/lane). KSR1 expression was detected by Western blot with a mouse monoclonal anti-KSR1 antibody (BD Biosciences) or a goat polyclonal anti-KSR1 antibody generated to amino acids 855–871 of KSR1 (c-19; Santa Cruz Biotechnology). Total amount of MEK, MAPK, and activated MEK and MAPK in MEFs was detected by Western blot with the following antibodies from Cell Signaling: polyclonal anti-MEK; polyclonal anti-p44/42 MAPK; polyclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204); and polyclonal anti-phospho-MEK1/2 (Ser217/Ser223).

Histology. Skin tissues were collected from 10-day old ksr1^+/+ and ksr1^−/− mice (kindly provided by Dr. Laura Hansen) and fixed for 15–18 h in 10% neutral buffered formalin, washed for 2 h in 70% ethanol, and embedded in paraffin blocks. The blocks were sectioned 4–6 µm thick, placed on glass slides, and stained with H&E.

MEF Studies. MEFs, derived from ksr1^+/+ and ksr1^−/− day 12–13 embryos, were prepared as described previously (19). Early passage MEFs (population doublings < 6; 0.6 x 10^6) were seeded in 6-well plates and grown in DMEM supplemented with 10% fetal bovine serum for 24 h at 37°C. After 48 h in serum-free medium, cells were stimulated with 0.01–100 ng/ml EGF for 3 min or with 10 nM to 1 µM TPA for 10 min, washed with PBS, and lysed in 0.2 ml of NP40 lysis buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 1% NP40 plus protease and phosphatase inhibitors]. RAF-1 activity assay was performed as described previously (12). Briefly, 30 µg of total lysate was immunoprecipitated with a polyclonal anti-Raf-1 antibody (Upstate Biotechnology), washed with NP40 buffer containing 0.5 M NaCl, and incubated with the kinase-dead GST-MEK-1 (K97M). Activated MEK-1 was visualized by Western blot with a polyclonal anti-phospho-MEK-1 antibody (Cell Signaling). To analyze cell proliferation, 0.15 x 10^6 ksr1^+/+ or ksr1^−/− low-passage MEFs were seeded on 60-mm plates and counted at the indicated times by hemocytometer. Data (mean ± SD) are compiled from three independent experiments. To assess transformation capacity, MEFs from ksr1^+/+ and ksr1^−/− mice were infected sequentially with retroviral plasmids pWZL-Hygro-c-myc and pBabe-Puro-H-RasV12 (kindly provided by Scott Lowe; Cold Spring Harbor Laboratories), resuspended in 0.3% noble agar, and seeded in 60-mm plates as described previously (19, 20). Colonies consisting of at least 50 cells were counted after 3 weeks.

Generation of ksr1^+/−/+/+ and ksr1^−/−/− Mice. Mice with homozygous male and female Tg.AC transgenic mice (21) were obtained at 3–4 weeks of age from Charles River Laboratories Inc. To produce the target population, ksr1^+/− mice were first bred to hemizygous Tg.AC mice containing the v-Ha-ras transgene. The resulting F1 females and males, heterozygous for ksr1 and hemizygous for the Tg.AC transgene, were then bred to obtain offspring in the ksr1 background. Nonresponder Tg.AC mice (22) were excluded from the study group. Presence of the Tg.AC transgene was determined by PCR amplification as follows: initial denaturation of 1 min and 10 s at 94°C, followed by 30 cycles with annealing at 55°C for 1 min, extension at 72°C for 3 min, and denaturation at 94°C for 1 min. The sequence of the forward primer was 5'-GGAGAAGTCTACCTCTGTTGGTTCTGAGC-3', and the sequence of the reverse primer was 5'-ATCGAACGCTTGTACGTCGTTGC-3'. PCR results were confirmed by Southern blot analysis as described previously (22).

Skin Tumor Experiments. Mice were treated twice weekly with 5 µg of TPA (Sigma Chemical Co., St. Louis, MO) for 15 weeks and observed for papilloma development as described previously (21). Offspring from the original Tg.AC mice in the FVB/N background from Charles River Laboratory were used as controls. Papillomas were counted weekly for 20 weeks. v-Ha-ras transgene expression in skin after TPA treatment was assessed by nested PCR as described previously (23).

RESULTS AND DISCUSSION

Generation of ksr1^−/− Mice. To investigate the in vivo function of mammalian KSR1, we targeted the mouse ksr1 locus to obtain mice deficient in KSR1 expression. ksr1^−/− mice were generated by homologous recombination in ES cells using the pPF targeting vector shown in Fig. 1A. The targeted region included the starting methionine (ATG codon at nucleotide 83 in ksr1 cDNA) and the following 74 amino acids encompassing 85% of the KSR1 unique CA1 domain. Two targeted ES clones (Fig. 1B) were microinjected into C57BL/6 blastocysts, and both resulted in chimeric mice that transmitted the mutated ksr1 allele through to the germ line. Crosses of the ksr1^−/− mice generated progeny with genotypes of the expected Mendelian frequencies. A PCR-based screening strategy was developed to detect both the wt and targeted alleles from mouse genomic DNA (Fig. 1C).

As reported previously (24), Northern blot analysis revealed wt KSR1 transcripts of 6.4 and 7.4 kb. The smaller transcript was detected by embryonic day 7, whereas the larger transcript was observed from day 11 on (Fig. 1D). In the adult, numerous tissues expressed ksr1 transcripts including the heart, spleen, lung, thymus, and brain (data not shown). Kidney displayed little if any ksr1 mRNA, whereas the larger transcript was restricted to brain. The existence of this larger mRNA was recently reported by Muller et al. (24) to represent a splice variant of murine KSR1 named B-KSR1. Importantly, ksr1^+/− mice did not express detectable levels of either ksr1 mRNA in any tissue tested (data not shown).

In a recent report (25), using a rat monoclonal antibody raised against full-length GST-KSR1, KSR1 protein was detected mainly in embryos and at a low level in a limited number of adult tissues (brain, bladder, ovary, testis, and lung), despite appreciable levels of ksr1 mRNA in most adult tissues. Here, a mouse monoclonal antibody raised against amino acids 90–203 of mouse KSR1 (BD Biosciences) was used. KSR1 was readily detected in adult mouse brain, spleen, lung, thymus, and testis (Fig. 1E; data not shown) and in the tissues of the gastrointestinal tract (stomach, duodenum, small intestine, and colon). Our results are in agreement with those of Nguyen et al. (26), who also showed that various adult tissues express KSR1 protein. These findings were confirmed using another anti-KSR1 polyclonal antibody raised against the COOH terminus of mouse KSR1 (amino acids 855–871; c-19; Santa Cruz Biotechnology). The discrepancy between our results and those of Giblett et al. (25) likely reflects the different amount of lysates used for the Western analysis [100 µg/ml in our study versus 20 µg/ml in that of Giblett et al. (25)] or differential sensitivity of the antibodies, with the mouse monoclonal antibody used in our studies being more sensitive. Consistent with the lack of detectable ksr1 transcript in the liver according to Giblett et al. (25), we did not detect KSR1 protein in the wt adult mouse liver. Furthermore, neither full-length nor truncated forms of KSR1 and B-KSR1 proteins were detectable in any adult tissue or in the MEFs from ksr1^−/− mice (Fig. 1E). This lack of KSR1 expression was also confirmed by reverse transcription-PCR analysis with primers specific for the 3'-untranslated region of ksr1 cDNA (data not shown). Our data thus suggest that replacement of the 5' region of ksr1 including the start coding site and most of the CA1 domain successfully abolished expression of both forms of murine KSR1.

ksr1^−/− Mice Manifest a Disorganized Hair Follicle Phenotype Similar to egfr^−/− Mice. KSR1 knockout mice were viable and fertile, with no major developmental defects. No gross histological abnormalities of the major organs were apparent in young mice or in adults up to 1 year of age. Animal weight, behavior, and brood size were also unaffected in the KSR1 knockout. These findings were consistent with a recent report of the generation of another mouse null for KSR1 (26). The lack of developmental defects in KSR1-deficient C. elegans and mice (1, 2, 26) could be due to compensation by a recently discovered second KSR allele, KSR-2 (17, 27), because

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B. Polk, unpublished observations.
deletion of both ksr1 genes is necessary for embryonic lethality in C. elegans. Alternatively, epigenetic factors may be determinant in the penetrance of the cell growth-related phenotype (see below).

Histological examination of the skin of our 10-day-old ksr1−/− mice revealed noticeably fewer hair follicles, which were disorganized in dermal location (depth) and orientation (direction) and manifested asynchronous growth (Fig. 2, A versus B and C). Furthermore, a significant proportion displayed a serpentine morphology (Fig. 2B, black arrows). In other follicles, the inner root sheath separated from the hair shaft, resulting in formation of blisters or cysts (Fig. 2C, black arrow). Strikingly, this phenotype closely resembles that found in the skin of EGFR-deficient mice (Ref. 28; Fig. 2D). Grossly, egf−/− mice display short, wavy pelage hair and curly whiskers during the first weeks of age, with pelage and vibrissa hairs becoming progressively sparser and atrophic over time, eventually leading to alopecia (28). Although these gross phenotypes were not seen in ksr1−/− mice, increased alopecia and sparse hair growth were observed after treatment with the phorbol ester TPA compared with similarly treated ksr1+/− controls (data not shown). The manifestation of this unique hair follicle phenotype by both the EGFR and KSR1 knockouts supports the contention that EGFR and KSR1 might be on the same signaling pathway in mice.

MAPK Activation in Response to Mitogenic Doses of EGF Requires KSR1 Activation of c-Raf-1.

Whereas in COS-7 cells overexpressing FLAG-KSR1, Raf-1 activation by mitogenic doses of EGF (10 ng/ml EGF) appears to require KSR1, Raf-1 activation by pharmacological EGF concentrations may bypass KSR1 by using a tyrosine kinase-dependent pathway (13). Wennstrom and Downward (29) have similarly demonstrated distinct mechanisms for Raf-1/MAPK activation by mitogenic and pharmacological doses of EGF. To further elucidate the effect of KSR1 disruption on activation of the EGFR/Ras-Raf-MAPK pathway, MEFs were generated from ksr1−/− mice.
To examine Raf-1 activation under conditions of MAPK inhibition, endogenous Raf-1 was evenly immunoprecipitated from all MEF lysates (data not shown), and activity was assayed using kinase-inactive MEK (K97M) as a substrate. Whereas Raf-1 activity was greatly inhibited (>90%) in ksr1−/− MEFs in response to mitogenic doses of EGF (Fig. 3B, top panel, Lanes 4 and 6), no inhibition was observed when stimulated with 100 ng/ml EGF (Fig. 3B, top panel, Lane 8). Thus, the partial inhibition of MAPK activation in response to 100 ng/ml EGF in ksr1−/− MEFs is independent of Raf-1 activation, likely resulting from the known MEK scaffolding function of KSR1. These results indicate that EGF-stimulated Raf-1 activation in MEFs is dose dependent and may occur via KSR1-dependent and -independent mechanisms, consistent with our previous findings (13).

The requirement for KSR1 for TPA-induced c-Raf-1 activation differed from that of mitogenic doses of EGF. In contrast to complete inhibition of c-Raf-1 activation after stimulation with mitogenic doses of EGF upon deletion of ksr1, TPA-induced Raf-1 activation was not altered in ksr1−/− MEFs (Fig. 3B, bottom panel). A similar finding for TPA was reported recently by Nguyen et al. (26). Thus, the use of the KSR1 knockout MEFs allows for the definition of two mechanisms for c-Raf-1 activation, a KSR1-dependent mechanism necessary for mitogenic EGF stimulation, and a KSR1-independent mechanism used by TPA, and perhaps pharmacological doses of EGF. Loss of KSR1 thus can impact MAPK activation by two mechanisms, via loss of c-Raf-1 activation as well as the MEK scaffolding function of KSR1.

To examine the biological consequence of MAPK inhibition on cell proliferation in vivo, a proliferation assay was performed using MEFS in the exponential phase of cell growth. Consistent with reduction in signaling through the MAPK mitogenic pathway, which provides proliferative signals, a 50% reduction in growth rate in ksr1−/− MEFs was observed (Fig. 3C). Similarly, Nguyen et al. (26) showed that T cells proliferate slower in the absence of KSR1.

**KSR1 Is Required for v-Ha-ras-mediated Skin Tumorigenesis.** To determine the potential impact of KSR1 inactivation in Ras-mediated transformation, c-Myc and Ha-rasV12 constructs were transduced into ksr1+/+ and ksr1−/− early-passage MEFS using high-titer retroviruses, and the ability to grow as colonies in soft agar was assessed as described previously (19, 20). Whereas ksr1+/+ MEFS did not form colonies in soft agar, they did so in the presence of Myc and Ras oncogenes (data not shown). In contrast, ksr1−/− MEFS could not be transformed by Ha-rasV12, even though they were immortalized by c-Myc. Taken together, all these results show that inactivation of KSR1 by genetic deletion attenuates signaling through the EGFR/Ras/MAPK pathway.

The participation of oncogenic ras in human cancers is estimated to be 30% (30), and approximately 25% of skin lesions in humans involve mutations of Ha-Ras (25% for squamous cell carcinoma and 28% for melanomas (31, 32)]. Because ksr1−/− mice exhibited a defect in normal development of the hair follicle, presumably via impairment of EGFR signaling, we examined the role of KSR1 in gain-of-function Ras signaling in the skin. Studies in the mouse two-stage tumorigenesis model identified mutations in codons 12, 13, or 61 of c-Ha-ras (90%) as the initial and predominant oncogenic event (33, 34). In our studies, we used Tg.AC mice, which harbor oncogenic v-Ha-ras (containing a codon 12 mutation) fused to the ç-globin promoter (21, 22, 35), a standardized model for the study of two-stage skin carcinogenesis. The v-Ha-ras transgene of Tg.AC mice is transcriptionally silent until induced in latent neoplastic cells (putative stem cells) closely associated with the outer root sheath cells of the hair follicle (36), a site consistent with our localization of KSR1 in mouse skin (data not shown). Tg.AC mice (FVB/N strain background) were crossed with ksr1−/− mice (in a mixed C57BL/6:129sv

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**Fig. 2.** Skin phenotype in newborn ksr1−/− mice. Full-thickness skin cuts of 10-day-old ksr1+/+, ksr1−/− and egfr−/− mice were sectioned 4–6-µm thick, placed on glass slides, and stained with H&E, x, serpentine; bl, blister; do, disoriented.

and ksr1−/− littermates and evaluated for responses to EGF and TPA, two growth stimuli known to activate the MAPK cascade. Disruption of KSR1 gene expression had no apparent effect on Ras activation (Ref. 26; data not shown), indicating that signaling events upstream of Ras activation were intact. After 48 h of serum starvation, MAPK activation in response to various doses of EGF (0.01–100 ng/ml) or TPA (10 nm to 1 µM) was determined by Western blot analysis using the monoclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody. ksr1−/− MEFS displayed a significant reduction in EGF- and TPA-induced MAPK (extracellular signal-regulated kinase 1/2) activation at all doses examined, whereas total MAPK content remained largely unchanged (Fig. 3A). For EGF stimulation, inhibition of MAPK activation was manifest at doses as low as 0.01 ng/ml (data not shown), whereas at 100 ng/ml EGF, MAPK activation was partially restored (Fig. 3A, top panel, Lane 8).
background). F1 offspring heterozygous for the ksr1 gene were then interbred to obtain F2 offspring carrying the v-Ha-ras transgene in the ksr1+/+ and ksr1−/− background. To determine whether disruption of ksr1 might influence tumorigenesis in this model, we topically treated the dorsum of F2 mice twice weekly for 15 weeks with vehicle (acetone) or with 5ng of TPA. Animals were monitored for development of skin malignancies for 20 weeks.

Initial control studies using reverse transcription-PCR to detect the v-Ha-ras transgene mRNA showed that loss of KSR1 function in ksr1−/−/− mice had no impact on TPA-induced expression of the oncogenic v-Ha-ras transgene in the skin (data not shown). This is consistent with the lack of an identifiable AP1 transcriptional regulatory site, which is classic for MAPK, within the 900-bp β-globin promoter of the Tg.AC transgene.6 However, 70% of Tg.AC transgenic mice in a ksr1+/+ background developed papillomas, whereas only 10% in a ksr1−/− background displayed papillomas (Fig. 4). The average number of papillomas in our study was 2–4 papillomas/mouse in each group. These studies with Tg.AC mice demonstrate that KSR1 is necessary for oncogenic v-Ha-Ras-mediated skin tumorigenesis.

This result contrasts with the recent report by Nguyen et al. (26) demonstrating that mouse mammary tumor virus MT-dependent mammary tumorigenesis, which is driven by the polyomavirus MT, did not require KSR1. In that study, which was performed in a different KSR1 null mouse strain, all of the mice developed tumors and succumbed to mammary cancer, although the tumors grew at a somewhat slower rate (onset of tumor formation at 65 days versus 35 days). The moderately slowed growth rate of this mammary tumor when developing in a ksr1−/− background is consistent with the decreased rate of proliferation of ksr1−/−/− low-passage MEFs (0.15 × 10^5) were seeded on 60-mm plates and grown as described in “Materials and Methods.” Cells were trypsinized every other day and counted by hemocytometer. Data (mean ± SD) are compiled from three independent experiments.

6 R. Cannon, unpublished observations.
KSR1 DEFICIENCY PREVENTS ONCOGENIC RAS SIGNALING

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REFERENCES


Fig. 4. Disruption of ksr gene abrogated oncogenic Ras-mediated tumorigenesis in ksr+/− mice. Mice, grouped according to genotype (10 mice/group), were treated with 5 μg of TPA twice a week for 15 weeks. Papillomas were counted weekly for 20 weeks.

7 R. Xing and R. Kolesnick, unpublished observations.
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