Expression of Kinase Suppressor of Ras in the Normal Adult and Embryonic Mouse

Susan M. Giblett, David J. Lloyd, Yvonne Light, Richard Marais, and Catrin A. Pritchard

Department of Biochemistry, University of Leicester, Leicester LE1 7RH [S. M. G., D. J. L., C. A. P.], and Institute of Cancer Research, London SW3 6JB [V. L., R. M.], United Kingdom

Abstract
Recent studies indicate that kinase suppressor of Ras (KSR) is a scaffold protein for the Ras/Raf/MEK/ERK signaling cascade in mammals. To help determine the in vivo function of KSR, we have examined the tissue-specific distribution of this protein in the embryonic and adult mouse using a rat monoclonal antibody raised against the mouse protein. Western blot analysis indicates that the protein is expressed at highest levels in the adult brain. It is also expressed at low levels in bladder, ovary, testis, and lung, but the protein is not detectable in any other adult tissue. However, reverse transcription-PCR analysis shows that Ksr transcripts are detected in all adult tissues except the liver. A variant containing a differentially spliced exon in the CA4 domain is observed in brain, cerebellum, ovary, and intestine. The protein is also expressed throughout the E6.5 embryo and at high levels in the neuroepithelium of the E10.5 embryo. At this embryonic stage, expression is also detected at lower levels in the limb and tail buds as well as in the myocardium.

Introduction
Ras proteins mediate extracellular signals to control cell growth and development through the highly conserved Ras/Raf/MEK/ERK pathway (1). Activated Ras alleles are among the most common oncogenes mutated in human tumors, particularly tumors of the colon and lung (2). Therefore, there has been a great deal of interest in identifying and characterizing agents that suppress Ras activity. Recent genetic studies in Drosophila melanogaster and Caenorhabditis elegans identified a novel regulator of oncogenic Ras that encodes a protein with a putative kinase domain (3–5). Loss-of-function mutations in this gene suppress the effects of oncogenic Ras proteins; this protein is therefore referred to as KSR. KSR homologues have been described in humans and mice and represent a novel family of proteins (5). In structure, KSR is more closely related to the Raf kinase than to any other known kinase, although it lacks the Ras binding domain (5) and does not bind Ras (6). It contains five conserved domains (CA1–5): CA1–3 comprise the NH2-terminal regulatory region, CA4 is a region rich in serine/threonine residues that is a target for regulatory phosphorylation and a docking site for MAPK (7), and CA5 contains the putative kinase domain.

Recent evidence suggests that KSR in mammals may be involved in the regulation of the Ras/Raf/MEK/ERK pathway through acting as a scaffold for several components of the pathway (8) and that this function may be independent of any KSR kinase activity (9). KSR can interact with several components of this cascade including Raf-1 (10), MEK1/2 (6, 12), and ERK1/2 (12) as well as other scaffold proteins known to bind to these signaling proteins including 14-3-3 (13), heat shock protein 90, and p50cdc37 (9). Consistent with it being a scaffold protein, its effect on the MAPK signaling cascade can vary, depending on its expression level, because at low expression levels, it appears to be a positive regulator of the ERK cascade, whereas at high expression levels it appears to inhibit this cascade (14). KSR is phosphorylated on multiple sites, and this may be involved in regulating the subcellular distribution of KSR, which may have significant effects on ERK signaling (15). The recent generation of mice with a knockout mutation of KSR support a role for this protein in the regulation of ERK activation (16). Despite there being a reduced level of ERK activation in Ksr−/− MEFs and T lymphocytes, the phenotype of Ksr−/− mice was relatively normal, although tumor development was suppressed.

Because the expression level of KSR appears to be a critical determinant of whether it activates or represses the MAPK signaling pathway, it is important to explore the physiological expression levels of KSR in the normal adult and embryonic mouse. Our studies show that, whereas Ksr mRNA is ubiquitously expressed, expression of the KSR protein is highly tissue restricted. Using Western blot analysis, the protein is only detected in the cerebral cortex, cerebellum, ovary, testis, lung, and bladder. In support of previous data (17), a differentially spliced exon is present in brain, cerebellum, and ovary, which gives rise to the addition of 14 amino acids to the CA4 domain of KSR. The KSR protein is expressed in the embryonic and extraembryonic cells of the E6.5 embryo and at highest levels in the embryonic brain of the E10.5 embryo. Of three cell lines tested, the KSR protein is only present in ES cells but not MEFs or PC-12 cells.
Results

Generation of KSR Monoclonal Antibody and Western Blot Analysis of KSR Expression in Adult Mouse Tissues and Cell Lines. A rat monoclonal antibody was raised against the full-length human KSR protein fused to GST by standard procedures. To investigate the specificity of this antibody, COS cells were transfected with a vector expressing myc-Ksr or with the vector control, and protein lysates were prepared and analyzed with the KSR antibody. The antibody detects a single protein species of M, 108,000 only in the cells transfected with the myc-Ksr vector. B, analysis of adult mouse tissues. Western blots were prepared using 20 μg of tissue lysate and analyzed with the anti-KSR rat monoclonal antibody (top panel). To control for protein loading, the Western blots were analyzed with an anti-vinculin mouse monoclonal antibody (bottom panel). Only the faster migrating isoform (B-KSR1) is detected in cerebral cortex and cerebellum, and only the slower migrating isoform (KSR1) is detected in bladder and lung. The ovary expresses both isoforms. C, analysis of three commonly used cell lines. Western blots were prepared using 20 μg of cell lysate and analyzed with the anti-KSR rat monoclonal antibody (top panel) and an anti-vinculin mouse monoclonal antibody (bottom panel). Expression of KSR1 is only detected in ES cells.

RT-PCR Analysis of Ksr Expression in Adult Mouse Tissues. It has been reported previously that the variant observed in brain arises as a result of two differential splicing events, one within the CA4 domain resulting in the addition of 14 amino acid residues and another at the COOH terminus resulting in the removal of 24 residues. As a result, the brain isoform (B-KSR1) is 10 amino acids smaller than the larger isoform (KSR1; Ref. 17). We designed a RT-PCR strategy to amplify transcripts corresponding to the Ksr gene such that the first differential splicing event could be detected (Fig. 2A). RNA was isolated from a variety of adult mouse tissues and cell lines, reverse transcribed using random primers, and then amplified with primers A and B. To confirm that equal amounts of RNA template were included in the RT-PCR reaction, the samples were amplified with primers specific for the ubiquitously expressed Raf-1 gene (18).

PCR products of 838 and 796 bp were observed, and sequence analysis with primers C and D confirmed that the larger product corresponds to B-KSR1 and arises as a result of the differential splicing event in the CA4 domain that was previously reported (Fig. 2A; Ref. 17). A great deal of variability in the level of amplification between different tissues was observed, and in general, the level of amplification correlated with the level of expression of KSR protein observed in each tissue (Fig. 2B). No RT-PCR products were detected in liver, consistent with the observation that no protein is expressed in this tissue. However, for heart, spleen, kidney, and the intestinal tract, low levels of RT-PCR products were detected, whereas these samples did not contain detectable levels of KSR protein (Fig. 1). Consistent with the Western data, PCR products corresponding to the B-KSR1 isoform were detected in brain, cerebellum, and ovary, and a small
The cell-specific expression of B-KSR1 and KSR1 in tissues. A, diagrammatic illustration of the differential splicing event resulting in the inclusion of exon 9a. The additional 42 nucleotides and 14 amino acids included as a result of this event are indicated in uppercase and bold, respectively. The location of primers used for PCR (A and B) and for sequencing (C and D) are indicated by arrows. B, RT-PCR analysis of adult mouse tissue RNAs, E10.5 embryo RNA, and cell line RNA. RNA was reverse transcribed with random primers and then PCR amplified with primers A and B (top panel). Amplification of the 796-bp fragment is indicative of the KSR1 isoform, whereas amplification of the 838-bp fragment is indicative of the B-KSR1 isoform containing exon 9a. As a control, the reverse-transcribed cDNAs were also amplified with primers for the raf-1 gene that generate a 357-bp product in all samples (bottom panel).

amount was detected in the bladder and the large intestine. PCR products corresponding to the KSR1 isoform were detected in E10.5 embryo, cerebellum, heart, spleen, thymus, lung, kidney, testis, uterus, ovary, bladder, and the intestinal tract. Surprisingly, RT-PCR products corresponding to KSR1 were predominantly detected in the cerebellum, whereas by Western analysis this tissue expressed predominantly the B-KSR1 isoform (Fig. 1B).

Of the cell lines tested, ES cells demonstrated the largest degree of amplification of the KSR1 isoform, consistent with the Western data. Although neither MEFs nor PC-12 cells expressed detectable levels of KSR protein, Ksr transcripts were detectable at low levels in both cell lines. MEFs predominantly expressed both isoforms, although KSR1 was predominant, whereas PC-12 cells only expressed the B-KSR1 isoform (Fig. 1B).

To determine the origins of these additional exon sequences, we analyzed the structure of the mouse Ksr gene. The sequence of the mouse Ksr gene was obtained by performing a BLAST search with the mouse cDNA sequence against the unfinished high-throughput genomic sequence database in GenBank. Clone RP23-240G13, derived from mouse chromosome 11, was found to contain the Ksr gene. Table 1 provides a summary of the organization of the mouse Ksr gene based on the analysis of the sequence of this clone. The size of each exon, the amino acid residues they encode, and the CA domain within which each exon is located are presented. We found that the additional exon sequences arise by differential splicing of a new exon, named exon 9a, rather than by the addition of sequences to exons 9 or 10 by the use of cryptic splice acceptor/donor sites. Intron sequences flanking exon 9a are in good agreement with the consensus for donor and acceptor splicing sites.

Immunohistochemical Staining of KSR in the Adult Tissues. The cell-specific expression of B-KSR1 and KSR1 in cerebral cortex, cerebellum, ovary, lung, and testis was analyzed by immunohistochemical techniques using the KSR antibody. Intense B-KSR1 expression was detected in the cell bodies of the pyramidal neurons of the cerebral cortex (Fig. 3A) and in the Purkinje cells of the cerebellum (Fig. 3B). In the ovary, staining of KSR proteins (either B-KSR1 or KSR1) was detected in the granulosa cells in the maturing follicles (Fig. 3C). Staining was also observed in the ciliated columnar epithelia of the infundibulum (data not shown). Staining of KSR1 was detected throughout the lung in the squamous epithelial cells of the alveoli (Fig. 3D). In the testis, expression of KSR1 was highest in the primary and secondary spermatocytes of the seminiferous tubules (Fig. 3E). Interestingly, the KSR immunostaining in tissues expressing predominantly the B-KSR1 isoform (brain and cerebellum) was found to be entirely cytoplasmic (Fig. 3, A and B). By contrast for all tissues expressing the KSR1 isoform (testis, lung, and ovary), as determined by Western and RT-PCR analysis, staining of the protein appeared to be primarily localized to the plasma membrane region, as exemplified by KSR immunostaining of lung and testis at high magnification (Fig. 3, D and E, right panels).

Analysis of KSR Expression in the Developing Embryo. Embryos at E6.5 in the decidua were embedded, sectioned, and stained with the KSR antibody. Expression was detected throughout the embryo in all three germ layers as well as in the cell layers of the amnion and the extraembryonic membranes (Fig. 4A). Embryos at E10.5 were either stained whole with the antibody (Fig. 4B) or embedded, sectioned, and then stained with the antibody (Fig. 4, C and D). Consistent with the adult staining pattern, a high level of KSR staining was also observed in the neuroepithelium of the developing brain. High levels of staining were also observed in the embryonic limbs and tail buds as well as in the developing heart. To control for staining specificity, the KSR immunogen (full-length mouse KSR protein fused to GST) was used as a blocking peptide by inclusion in the primary antibody incu-
The location of exons was determined by analysis of the mouse Ksr gene sequence obtained from the unfinished high-throughput genomic sequence database in GenBank. Clone RP23-240G13, derived from mouse chromosome 11, was found to contain the Ksr gene. The Ksr gene contains 19 exons spanning 4094 nucleotides and coding for 873 amino acids. The exons are differentially spliced so that the KSR1 isoform contains exon 17 but not exon 9a, and the B-KSR1 isoform contains exon 9a but not exon 17.

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*CA, conserved area; NC, not conserved.

Discussion
Studies of the physiological expression pattern of KSR in the mammalian organism are important because this protein has been identified as a suppressor of oncogenic Ras (3–5). There is now good evidence to suggest that mammalian KSR provides this function by acting as a scaffold for components of the Raf/MEK/ERK signaling cascade, promoting the assembly of a multiprotein signaling complex for these proteins in a Ras-dependent manner (8, 16). In recently generated Ksr−/− mice, ERK activation is reduced in MEFs and T lymphocytes, confirming that KSR regulates ERK activation in vivo (16). As with all scaffold proteins, fulfillment of this role is critically dependent on expression level. Therefore, we examined the physiological expression pattern of the KSR protein in the mouse by performing Western and immunohistochemical analysis with a newly created antibody for KSR as well as by RT-PCR. Our Western data are consistent with that reported previously that showed highest levels of expression of KSR in neuronal tissue (17). Similarly, we detected lower levels of KSR expression in ovary, testis, lung, and bladder but unlike the previous report, we did not detect expression of KSR in spleen. Throughout this work, we found the KSR protein extremely difficult to detect, even in neuronal tissue. Similarly, Muller et al. (17) required the combined use of immunoprecipitation and Western analysis with two different antibodies to detect the KSR protein. Although this may reflect on the poor strength of the antibody-antigen interactions, a low level of KSR protein expression is consistent with the fact that Ksr mRNA is also expressed at low levels in the brain as determined by Northern analysis (data not shown).

Of three commonly used cell lines, we only detected KSR1 protein in ES cells and not PC-12 cells or MEFs. However, RT-PCR analysis shows the presence of Ksr transcripts corresponding to KSR1 in MEFs and low levels of B-KSR1 in MEFs and PC-12 cells (Fig. 2B), suggesting that if KSR protein is expressed, then it is beyond the level of detection used here. By using immunoprecipitation combined with Western analysis, Muller et al. (17) were able to detect low levels of KSR1 in immortalized fibroblasts and low levels of B-KSR1 in PC-12 cells. Given the higher level of expression of KSR1 in ES cells, our results suggest that these cells are the most appropriate cell type in which to study the physiological functions of KSR1, rather than fibroblasts that have been more commonly used to date.

In contrast to the Western data, transcripts for Ksr are detected in many adult mouse tissues (Fig. 2B). This suggests that either the protein is expressed at extremely low levels in these tissues or that the transcripts are not translated in the tissues that do not express detectable levels of the protein. In this respect, the expression pattern of KSR bears many similarities to that of B-Raf. Similar to KSR, the B-Raf protein is expressed at highest levels in neuronal tissue, at lower levels in testis and spleen, and is not detectable in many other tissues and cell lines (18, 19). These overlapping expression patterns may be significant because recent studies have shown that B-Raf is a more important MEK activator than Raf-1, even in tissues and cell lines that do not contain detectable levels of the B-Raf protein but contain high levels of the Raf-1 protein (20, 21). In Drosophila melanogaster, the D-raf homologue is more closely related to
mammalian B-raf than Raf-1 (22), and an association between D-KSR and D-Raf has been detected in D. melanogaster embryos (10). Despite this, mammalian KSR does not appear to interact with mammalian B-Raf in the yeast two-hybrid interaction assay (6), in PC-12 cells, or in brain tissue (16, 17). Although a number of studies have suggested that KSR may interact with Raf-1 (10, 11), a recent report has shown that multiprotein signaling complexes containing MEK and ERK are disrupted in Ksr+/− tissues, but that complexes containing B-Raf and Raf-1 are not (16). Therefore, the role of KSR in promoting the assembly of an active signaling complex involving B-Raf and/or Raf-1 is not clear and needs to be addressed in light of recent data.

The RT-PCR and Western analysis support the observation that there is a differentially spliced isoform expressed in brain that contains an additional 14 amino acids in the CA4 domain in neuronal tissue (17). We have determined that this B-KSR1 isoform arises partly by the inclusion of an additional exon, named exon 9a, between exons 9 and 10, and that it is also expressed in ovary and the large intestine. In all other tissues, ES cells and embryos the KSR1 isoform is expressed. The presence of this additional exon results in the addition of a binding site for MAPK. This is likely to lead to an increased affinity of B-KSR1 for ERK, which may have important consequences for assembly of the Raf/MEK/ERK signaling complex, possibly through regulation of its subcellular localization (15).

Immunohistochemical staining of adult mouse tissues shows expression of the B-KSR1 isoform in the pyramidal cells of the neurones and the Purkinje cells of the cerebellum (Fig. 3, A and B). KSR immunostaining was also observed in the epithelial cells of the lung and ovary as well as in the developing spermatocytes of the testis. In the E6.5 embryo, KSR was detected in embryonic and extraembryonic tissues. However, by E10.5, a more restricted pattern of expression of the protein was observed with it being detected primarily in the neuroepithelium of the developing brain as well as in the limb and tail buds. Interestingly, immunostaining of the

Fig. 3. KSR immunohistochemical staining of adult mouse tissues. A, B-KSR1 immunostaining in the cerebral cortex showing expression in pyramidal neurones. PN, pyramidal neurones. Bar, 100 μm. B, B-KSR1 and KSR1 immunostaining in the cerebellum showing expression in Purkinje cells. PC, Purkinje cells. Bar, 100 μm. C, Immunostaining of KSR1 and B-KSR1 in ovary. F, ovarian follicle. Bar, 200 μm. D, KSR1 immunostaining in lung at lower magnification (left panel; bar, 50 μm) and higher magnification (right panel; bar, 25 μm). A, alveolus; AS, alveolar sac; AC, alveolar cell. E, KSR1 immunostaining in testis at lower magnification (left panel; bar, 50 μm) and higher magnification (right panel; bar, 25 μm). St, seminiferous tubule; IC, interstitial cells; Sp, developing spermatocytes. Individual arrows, cells in testis and lung expressing KSR in the periplasma membrane region.
testis and lung clearly showed predominant KSR1 staining in the plasma membrane region, whereas the B-KSR1 staining in the cerebral cortex and cerebellum appeared to be cytoplasmic (Fig. 3). The significance of this finding is not clear. Previous investigations have indicated that KSR1 is located in the cytoplasm and translocates to the membrane in response to Ras activation, where it forms part of a multiprotein signaling complex with other components of the ERK signaling cascade (9, 13, 23). KSR has also been detected in the nucleus, where it could interact with MEK and ERK, and it has been suggested that the main function of KSR is to regulate MEK localization (15). Given the important consequences this would have for ERK signaling, it is important to further address the differential localization of the two KSR isoforms and MEK/ERK in these different tissues.

**Materials and Methods**

**Generation of Antibody and Western Blot Analysis.** Full-length mouse KSR-GST fusion protein was injected into rats, and monoclonal antibodies were prepared by standard procedures. Triton X-100 soluble lysates and Western blots were prepared by the methods described previously (24). The blots were incubated with a 1:200 dilution of anti-KSR rat monoclonal antiserum or with a 1:1000 dilution of the F9 vinculin monoclonal antibody (a gift from Dr. V. Koteliansky, Centre National de la Recherche Scientifique, Paris, France) overnight at 4°C and then washed in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween 20. For KSR, the filters were incubated with a 1:10,000 dilution of either a rabbit antirat antibody (Sigma), filters were washed with TBS-Tween and then incubated with a 1:10,000 dilution of an antirabbit antibody coupled to horseradish peroxidase for 1 h at room temperature. For vinculin, filters were incubated with a 1:10,000 dilution of an antimouse (vinculin) secondary antibody coupled to horseradish peroxidase (Sigma) for 1 h at room temperature. After washing with TBS/Tween, the enhanced chemiluminescence detection system (Pierce) was used to visualize antigen-antibody complexes.

**RT-PCR.** RNA was prepared from homogenized mouse tissues and cells using the Qiagen RNeasy mini kit according to the manufacturers’ instructions. cDNA was synthesized from 2.5 μg of RNA in a 25-μl reaction containing 0.5 μg of random primers, 0.5 mM deoxynucleotide triphosphates, 40 units of RNasin, and 200 units of mouse Moloney leukemia virus (Life Technologies, Inc.) in the appropriate buffer for 2 h at 42°C. The samples were heated to 95°C for 5 min and then subjected to PCR. PCR reactions were performed with 1 μl of cDNA in 10 μl of Reddy-MIX PCR mix (Advanced Biotech-
nologies) containing primers A (5′-CTCCACACCT-CATCGGCGG-3′) and B (5′-GCCGAAAGGTTGATGACCC-3′) for the murine Ksr gene. For theraf-1 PCR, primers corresponding to the 3′ untranslated region were used: 5′-AACCCTGACCTGACCTGTC-3′ and5′-GTGCAAGCTTGTACCTC-3′. All PCR reactions were performed at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles. The PCR reactions were analyzed by gel electrophoresis on a 1% (w/v) agarose gel. PCR reactions were sequenced using primers C (5′-GCACCTTCTCGACCTGATC-3′) or D (5′-CTCTGTCCTGGAACACCT-3′) using an automated sequencer.

Immunohistochemical Staining. MF-1 embryos were collected from pregnant females at 10.5 or 6.5 days postcoitum, and tissues were collected from 6-week-old MF-1 mice. For sectioning, embryos and tissues were either embedded in Tissue-Tek OCT compound (Sakura) in cryogenic molds and solidified on a dry ice-hexane bath or fixed in Methacarn (10% (v/v) glacial acetic acid, 30% (v/v) chloroform, 60% (v/v) methanol), and paraffin embedded. Cryostat sections at 10-μm thickness or paraffin sections at 5 μm were mounted onto microscope slides pretreated with silane. Sections were either stained with H&E or processed for KSR immunostaining. After blocking in 30% (v/v) hydrogen peroxide in methanol for 30 min, sections were incubated with a 1:10 dilution of the KSR rat monoclonal antibody at room temperature overnight. Antigen-antibody complexes were visualized with biotinylated secondary antibody and with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). For whole mount staining, E10.5 embryos were harvested and fixed in 4% paraformaldehyde overnight at 4°C. Embryos were processed and stained as described previously using a 1:10 dilution of the KSR antibody (25).

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References