Caspase-dependent Cleavage Disrupts the ERK Cascade Scaffolding Function of KSR1*\textsuperscript{**}\textsuperscript{†}

Received for publication, March 29, 2007, and in revised form, June 20, 2007 Published, JBC Papers in Press, July 5, 2007, DOI 10.1074/jbc.M702692200

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Kinase suppressor of Ras 1 (KSR1) is a protein scaffold that facilitates ERK cascade activation at the plasma membrane, a critical step in the signal transduction process that allows cells to respond to survival, proliferative, and differentiative cues. Here, we report that KSR1 undergoes caspase-dependent cleavage in apoptotic cells and that cleavage destroys the scaffolding function of the full-length KSR1 protein and generates a stable C-terminal fragment that can inhibit ERK activation. KSR1 is cleaved in response to multiple apoptotic stimuli and occurs in vitro during the involution of mouse mammary tissues, a morphogenic process requiring cellular apoptosis. In addition, we find that in comparison with KSR1\textsuperscript{−/−} mouse embryonic fibroblasts expressing wild type KSR1 (WT-KSR1), cells expressing a cleavage-resistant KSR1 protein (DEVA-KSR1) exhibit reduced apoptotic signaling in response to tumor necrosis factor-α/cycloheximide treatment. The effect of DEVA-KSR1 expression was found to correlate with increased levels of active phosphoERK and could be significantly reversed by treating cells with the MEK inhibitor U0126. In contrast, reduced phosphoERK levels and enhanced apoptotic signaling were observed in cells constitutively expressing the C-terminal KSR1 fragment (CTF-KSR1). Moreover, we find that cleavage of WT-KSR1 correlates with a dramatic reduction in active phosphoERK levels. These findings identify KSR1 as a caspase target and suggest that cleavage of the KSR1 scaffold represents another mechanism whereby caspases down-regulate ERK survival signaling to promote cellular apoptosis.

Death by apoptosis is an evolutionarily conserved mechanism whereby diseased, damaged, or unwanted cells are eliminated from an organism. A hallmark feature of apoptosis is the activation of aspartate-directed, cysteineyl proteases known collectively as caspases (1). These enzymes can be broadly divided into two groups: the initiator caspases (caspase-2, -8, -9, -10), which mediate the activation of downstream caspases, and the effector caspases (caspase-3, -6, -7), which cleave cellular proteins to induce biological and morphological changes that ultimately result in cell death (2). In mammals, caspase activation and apoptosis can be triggered either through the engagement of cell surface death receptors (known as the extrinsic death receptor pathway) or by the release of apoptogenic factors from mitochondria (known as the intrinsic mitochondrial pathway) (3, 4).

Apoptosis plays an important role in essential biological processes such as embryogenesis, tissue remodeling, and the immune response, and its deregulation has been linked with a variety of human pathologies including cancer, myocardial ischemia, and neurodegenerative diseases (5–7). In addition, many cancer chemotherapies activate the apoptotic machinery to prevent tumor growth, whereas inhibition of the apoptotic response contributes to the emergence of chemoresistance (8, 9). Thus, the regulation of apoptosis is critical for ensuring the homeostasis of an organism. As such, the cell has derived various mechanisms to precisely control the balance between survival and apoptotic signaling. For example, caspases not only cleave and activate substrates that contribute to the apoptotic process (e.g. caspases themselves, Bid, MEKK1), they can also cleave certain signaling proteins (e.g. Raf, lamin, focal adhesion kinase) and inactivate their function in cell survival (2, 10). In addition, phosphorylation of specific targets (e.g. Rsk, Elk-1) by prosurvival kinases can promote cell survival, whereas the phosphorylation of other targets (e.g. BAD and caspase-9) can inhibit apoptosis (11, 12).

The ERK/MAPK\textsuperscript{2} cascade is a well documented mediator of survival signaling (12). This three-tiered kinase cascade functions downstream of the RasGTPase and is composed of the Raf, MEK, and ERK kinases (13, 14). Although signal transmission between these kinases is mediated via direct phosphorylation, recent studies have demonstrated the importance of scaffolding proteins in regulating the timing, location, strength, and duration of ERK cascade signaling (15, 16). One such scaffold, kinase suppressor of Ras 1 (KSR1), associates with all the core kinase components of the ERK cascade and is a conserved positive regulator of Ras/ERK signaling (17–21). KSR1 interacts

* This work was supported by a grant from the NCI, National Institutes of Health/Department of Health and Human Services. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{**} This article was selected as a Paper of the Week.

\textsuperscript{†} The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

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\textsuperscript{2} The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; KSR, kinase suppressor of Ras; CTF, C-terminal fragment; CHX, cycloheximide; MEF, mouse embryonic fibroblast; WT, wild type; TNFα, tumor necrosis factor-α; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Ac, acetyl; pNA, p-nitroanilide; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone.
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constitutively with MEK and translocates from the cytosol to the plasma membrane upon Ras activation (22–25). At the membrane, KSR1 coordinates the assembly of a multiprotein complex that colocalizes MEK with its upstream activator Raf and downstream substrate ERK, thus facilitating the phosphorylation reactions required for ERK activation (23, 24, 26, 27).

Here, we report that KSR1 undergoes caspase-dependent cleavage in apoptotic cells and that cleavage destroys the ability of KSR1 to promote ERK survival signaling. KSR1 is cleaved in response to multiple apoptotic stimuli, and cleavage of endogenous KSR1 is observed in an in vivo apoptotic model system, involving mouse mammary tissue. Cleavage of KSR1 destroys its ERK scaffolding potential, results in the generation of a stable C-terminal fragment that can inhibit ERK signaling, and correlates with a pronounced reduction in active phosphoERK levels. In addition, we find that in comparison with KSR1+/− MEFs expressing WT-KSR1, cells expressing a cleavage-resistant KSR1 protein (DEVA-KSR1) have elevated phosphoERK levels and reduced apoptotic signaling following TNFα/CHX treatment, whereas cells constitutively expressing the C-terminal KSR1 fragment exhibit increased apoptotic signaling and reduced basal phosphoERK levels. Moreover, the effects of DEVA-KSR1 expression can be significantly reversed by treatment of cells with the MEK inhibitor U0126. From these data, we conclude that alterations in the scaffolding activity of KSR1 can negatively impact ERK survival signaling to facilitate the apoptotic process.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies recognizing caspase-3 (clone 46), Bid (clone 40), MEK1 (clone 25), and cytochrome c (clone 6H2.B4) were obtained from BD Biosciences, antibodies to caspase-9 (clone C9), caspase-8 (mouse-specific), and phosphoERK were purchased from Cell Signaling Technology (Beverly, MA), and antibodies to actin, ERK1/2 (C-14), and KSR1 (C-19 and M-18) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Pyo epitope tag antibody has been previously described (28), and antibodies recognizing either the N-terminal or the C-terminal domains of KSR1 were generated against amino acids 118–248 or 847–861 of murine KSR1, respectively.

KSR1 Constructs and Generation of Stable Cell Lines—Pyo-tagged WT-KSR1 has been previously described (29), DEVA (D529A)-KSR1 was generated by site-directed mutagenesis, and CTF-KSR1 (encoding amino acids 530–873) was generated by PCR amplification. Sequences encoding the KSR1 proteins were subcloned into the pBabe-puro retroviral vector, and stable cell lines were generated by puromycin selection (2.5 μg/ml) of KSR1+/− MEFs (a kind gift from Dr. Robert Lewis, Eppley Cancer Center, Omaha, NE) (18) infected with recombinant retroviruses.

Induction of Apoptosis—Apoptosis was induced by serum starvation or treatment with 1 μM staurosporine (EMD Biosciences, La Jolla, CA) or 10 ng/ml murine TNFα (Invitrogen) and 1 μg/ml CHX (Sigma-Aldrich) for the indicated times. In some experiments, cells were treated with 100 μM z-VAD-fmk (Bachem, Torrance, CA) or 10 μM U0126 (Cell Signaling Technology) for 30–60 min prior to and then during stimulation with TNFα/CHX. Cells were then harvested (including those in the medium), washed with PBS, and either examined directly by FACS analysis or lysed in Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.15 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 5 mM sodium vanadate) for immunoblot analysis or lysed in CHAPS buffer (0.1% CHAPS, 0.2 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 0.15 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin in PBS, pH 7.4) for caspase activity assays.

Caspase Activity Assays and in Vitro Caspase Cleavage Assays—For in vivo caspase activity assays, crude lysate (~100 μg of protein) was incubated with Ac-DEVDP-NH2 (200 μM final concentration, BIOMOL, Plymouth Meeting, PA), and the rate of pNA release was determined by plotting the change in absorbance at 405 nm over time. For in vitro caspase-cleavage assays, Pyo-tagged KSR1 proteins that had been affinity-purified from 293T cells as described previously (25) were incubated with 200 units of recombinant caspase-3 or caspase-8 (BIOMOL) in caspase buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol) for 1 h at 30 °C, following which the samples were resolved by SDS-PAGE and analyzed by immunoblotting. The activities of recombinant caspase-3 and caspase-8 were confirmed by measuring the rate of pNA release from Ac-DEVDP-pNA or Ac-IETD-pNA substrates (200 μM final concentration), respectively.

FACS Analysis—Cellular levels of active caspase-3 or H2A.X phosphorylation were determined by flow cytometry using the fluorescein isothiocyanate-conjugated monoclonal active caspase-3 apoptosis kit I (BD Biosciences) and the H2A.X phosphorylation assay kit (Millipore, Billerica, MA), respectively. Flow cytometry was also used to examine cells for annexin V staining using the Vybrant apoptosis assay kit 11 (Invitrogen) and for TUNEL staining using the Apo-direct kit (BD Biosciences). Data were collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using Cell Quest Software.

Cytochrome c Release—MEFs were treated with TNFα/CHX, harvested, washed with PBS, and permeabilized for 5 min in PBS containing 100 μg/ml digitonin and 100 mM KCl (Note: control cells were incubated in PBS alone). All cells were then fixed in 4% paraformaldehyde for 20 min at room temperature and washed twice with PBS. Cells were incubated in blocking buffer (3% bovine serum albumin + 0.05% saponin in PBS) for 1 h with rocking followed by incubation overnight at 4 °C with anti-cytochrome c antibodies at 1:200 with shaking. Cells were washed once with blocking buffer and incubated with Alexa Fluor 488 goat anti-mouse secondary antibodies (Invitrogen) at 1:200 for 1 h. Cells were washed once with blocking buffer and PBS, following which they were subjected to FACS analysis. For immunofluorescence, cells were grown on glass coverslips, fixed with 4% paraformaldehyde for 20 min at room temperature, and stained for cytochrome c as described for FACS analysis except that washes were increased to three times per step. Cells were also stained with 4′,6-diamidino-2-phenylindole (300 nM in PBS for 5 min) prior to mounting in Prolong Gold antifade solution (Invitrogen).
Analysis of Endogenous KSR1 Cleavage in Involuting Mouse Mammary Tissue and KSR1+/+ MEFs—For mammary tissue isolation, pups were removed from female C57BL/6 mice on day 7 of lactation, and the mammary glands of the females were then harvested on day 0 and day 3 following removal of pups. Tissue lysates were prepared as described previously (30).

KSR1+/+ MEFs were lysed in Nonidet P-40 buffer following treatment with TNFα/CHX for 6 h. Endogenous KSR1 proteins were immunoprecipitated using antibodies recognizing either the KSR1 N terminus or the KSR1 C terminus and examined by immunoblot analysis.

RESULTS

KSR1 Is Cleaved in Apoptotic Cells—The caspase-dependent cleavage of anti-apoptotic/pro-survival signaling molecules is an important mechanism for facilitating the onset of apoptosis. Within their substrates, caspases recognize a core tetrapeptide motif (P4P3P2P1) that contains an essential aspartic acid residue required for the cleavage reaction at position P1 (31). One well defined substrate recognition motif for effector caspases is the sequence DEXD (32), and analysis of the KSR1 protein sequence indicates that KSR1 contains a putative caspase cleavage site (DEVD) at amino acid residues 526–529 (Fig. 1A). Significantly, cleavage at the DEVD site would be expected to destroy the ERK scaffolding activity of KSR1 in that cleavage would separate the C-terminal region, which mediates MEK binding, from the N-terminal region, which contains the ERK binding site and the C1 domain required for plasma membrane localization. Therefore, to investigate whether KSR1 is a caspase target, we examined the electrophoretic mobility of KSR1 in cells undergoing apoptosis. For this study, KSR1−/− MEFs stably expressing Pyo-tagged WT-KSR1 (PyoWT-KSR1 MEFs) were subjected to various conditions known to induce apoptosis, including staurosporine treatment, serum starvation, and stimulation with TNFα/CHX. Cell lysates were then prepared and examined by immunoblot analysis using various KSR1 antibodies and an antibody recognizing the pro- and cleaved forms of

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![Diagram showing KSR1 cleavage](image)

**Figure 1.** KSR1 is cleaved in apoptotic cells. A, shown are schematic representations of full-length (FL) KSR1 and the CTF released by cleavage at the DEVD motif. U represents a domain unique to KSR proteins, whereas P, C1, and S/T indicate domains rich in proline, cysteine, and serine/threonine residues, respectively. The ERK and MEK binding sites are indicated. B, KSR1−/− MEFs stably expressing Pyo-tagged WT-KSR1 (PyoWT-KSR1 MEFs) were serum-starved or were treated with Me2SO (DMSO), TNFα/CHX, or staurosporine for 6 h prior to lysis. Cleavage of KSR1 and caspase-3 (Casp-3) was determined by immunoblot analysis using the designated antibodies. The o indicates a nonspecific protein band detected by the KSR1 C-terminal antibody. Equal protein loading was confirmed by visualization of actin levels. C, WT-KSR1 MEFs were treated with CHX, TNFα, or TNFα/CHX for 6 h and analyzed as described in B. D, KSR1−/− MEFs were treated with TNFα/CHX for 6 h prior to lysis. Endogenous KSR1 proteins were immunoprecipitated and examined by immunoblot analysis as indicated. Caspase-3 cleavage was monitored by immunoblot analysis.
FIGURE 2. Analysis of apoptotic signaling in WT-KSR1 MEFs. A, in the extrinsic death receptor pathway, ligand binding to a cell surface death receptor triggers activation of initiator caspase-8, which activates effector caspase-3, resulting in the cleavage of proteins required for apoptosis. The extrinsic death receptor pathway can also engage the intrinsic mitochondrial pathway through the caspase-8-mediated cleavage of Bid, resulting in the release of cytochrome c and activation of initiator caspase-9, which can also activate effector caspase-3. B, WT-KSR1 MEFs were treated with TNFα/CHX for the indicated times, and the cleavage of KSR1, caspase-8, caspase-9, caspase-3, and Bid was assessed by immunoblot analysis. Actin levels confirmed equal protein loading. FL-KSR1, full-length KSR1. • indicates a nonspecific protein band detected by the KSR1 C-terminal antibody. C, WT-KSR1 MEFs were treated as indicated, following which they were examined for cytochrome c release by indirect immunofluorescence and FACS analysis. Cytochrome c staining is shown in green; nuclei are visualized by 4',6-diamidino-2-phenylindole staining in blue. Shown is a representative FACS experiment, indicating the mean and range of duplicate samples. In the absence of digitonin treatment, no change in cytochrome c release was observed between untreated and TNFα/CHX-treated cells (data not shown). D, WT-KSR1 MEFs were treated as indicated and analyzed for annexin V staining. Shown is a representative experiment, indicating the mean and range of duplicate samples. E, WT-KSR1 MEFs were treated as indicated and analyzed for TUNEL positive cells by indirect immunofluorescence and FACS analysis. TUNEL staining is shown in green; nuclei are visualized by propidium iodide staining in red. Shown is a representative FACS experiment, indicating the mean and range of triplicate samples.
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As shown in Fig. 1B, the appearance of the cleaved/activated form of caspase-3 confirmed that serum starvation or treatment with either staurosporine or TNFα/CHX for 6 h were all capable of inducing apoptotic signaling, although the extent of caspase-3 cleavage did vary. Under these apoptotic conditions, a reduction in the level of full-length KSR1 was observed, as was the appearance of a 42-kDa protein recognized by an antibody directed against the C terminus of KSR1 (Fig. 1B). In addition, only treatment with TNFα in combination with CHX was able to induce apoptotic signaling and the appearance of the 42-kDa protein recognized by the KSR1 C-terminal antibody (Fig. 1C). The molecular weight of this protein is consistent with the predicted size of the C-terminal fragment that would be generated if KSR1 were cleaved at the DEVD site. The 42-kDa protein was not observed in lysates of non-apoptotic cells (untreated or Me2SO-treated, Fig. 1B; CHX alone-treated or TNFα alone-treated, Fig. 1C), and its appearance correlated with the reduction in full-length KSR1, as well as the degree of apoptosis observed in cells (Fig. 1, B and C, and data not shown). Interestingly, using antibodies recognizing various N-terminal regions of KSR1, no stable accumulation of an N-terminal fragment was detected (Fig. 1B and data not shown). Finally, to confirm that cleavage of KSR1 was bio-logically relevant and not a unique property of the Pyo-tagged WT-KSR1 protein, the cleavage of endogenous KSR1 was examined in KSR1+/+ MEFs. As shown in Fig. 1D, a reduction in the level of full-length endogenous KSR1 was observed in cells treated with TNFα/CHX, as was the appearance of the 42-kDa C-terminal fragment (CTF).

Given that TNFα/CHX treatment efficiently induced apoptosis and KSR1 cleavage in the WT-KSR1 MEFS, these conditions were used as the apoptotic inducer in subsequent experiments. TNFα is known to activate the extrinsic death receptor pathway by binding to the TNF1 receptor, thus inducing the cleavage and activation of caspase-8, which in turn cleaves and activates the effector caspase-3 (33). In many cell types, however, the ability of the extrinsic death receptor pathway to activate caspase-3 and induce apoptosis is amplified by engaging the intrinsic mitochondrial pathway (34, 35). Under these circumstances, caspase-8 mediated cleavage of Bid, a Bcl2 family member, promotes cytochrome c release from mitochondria, thereby trig-gering the cleavage and activation of caspase-9, an initiator caspase that can also cleave and activate caspase-3 (Fig. 2A). To further char-acterize the apoptotic pathways induced by TNFα/CHX in the WT-KSR1 MEFS, cells treated with TNFα/CHX for a time course of 0–6 h were examined by immuno-blot analysis for the cleavage/activa-tion of various apoptosis effectors. As indicated in Fig. 2B, either by the loss of the full-length protein or by the appearance of a cleaved fragment, TNFα/CHX treatment induced the cleavage of caspase-8, Bid, caspase-9, and caspase-3, demonstrating that both the extrinsic and the intrinsic apoptotic pathways were activated. Cytochrome c release was also observed in TNFα/CHX cells, and its release could be blocked by the general caspase inhibitor z-VAD-fmk, consistent with the model that cytochrome c release is induced by the caspase-8-mediated cleavage of Bid (Fig. 2C).

Moreover, the WT-KSR1 MEFS exhibited multiple markers of cellular apoptosis following TNFα/CHX treatment, including exposure of phosphatidylserine on the cell surface (as determined by annexin V staining, Fig. 2D), double-strand DNA breaks (as determined by TUNEL staining, Fig. 2E and Ser-139 phosphorylation of histone H2A.X (36) (see Fig. 5C)), and the presence of active caspase-3 (Figs. 3 and 5A).

Over the time course of TNFα/CHX treatment, loss of the full-length KSR1 protein and appearance of the 42-kDa CTF correlated with the kinetics of caspase activation (Fig. 2B), sug-gesting that KSR1 cleavage is caspase-dependent. However, to confirm that KSR1 cleavage does require caspase activity, cells were treated with the general caspase inhibitor z-VAD-fmk prior to and during TNFα/CHX stimulation. As shown in Fig. 3, z-VAD-fmk inhibited the TNFα/CHX-induced cleavage of caspase-3 (panel A), as well as the induction of DEVD-directed caspase activity (panel B) and the detection of cells containing active caspase-3 (panel C). Moreover, z-VAD-fmk completely blocked the cleavage of KSR1 induced by TNFα/CHX treatment (Fig. 3A).

Caspase-dependent Cleavage of KSR1 Occurs at the DEVD Motif—The above findings implicate KSR1 as a caspase target. To determine whether KSR1 cleavage is indeed caspase-mediated and to confirm that cleavage occurs at the DEVD motif, we first generated a KSR1 mutant in which the aspartic acid residue (Asp-529) predicted to be required for caspase cleavage was mutated to alanine (DEVA-KSR1). This construct was then sta-
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**A**

WT KSR1  
DEVA KSR1  

|   | 0h | 3h | 5h |
|---|----|----|----|
| TNFα/CHX |   |    |    |
| FL-KSR1 |   |    |    |
| CTF |   |    |    |
| Actin |   |    |    |

**B**

WT KSR1  
DEVA KSR1  

|   | Control | Casp-3 | Casp-8 | Control | Casp-3 | Casp-8 |
|---|---------|--------|--------|---------|--------|--------|
| Input FL-KSR1 |   |        |       |   |        |       |
| CTF |   |        |   |   |        |       |

**Figure 4. Caspase-dependent cleavage of KSR1 occurs at the DEVD site.**

- **A:** KSR1−/− MEFs stably expressing either Pyo-tagged WT-KSR1 or Pyo-tagged DEVA-KSR1 were treated with TNFα/CHX as indicated prior to lysis. KSR1 cleavage was assessed by immunoblot analysis, and actin was used as a protein loading control. FL-KSR1, full-length KSR1, indicates a nonspecific protein band detected by the KSR1 C-terminal antibody. B, affinity-purified Pyo-tagged WT- and DEVA-KSR1 proteins were incubated in vitro with purified, recombinant caspase-3 (Casp-3) or caspase-8 (Casp-8), and the cleavage of KSR1 was assessed by immunoblot analysis. Activity of the recombinant caspases was verified using the colorimetric substrates Ac-DEVD-pNA (caspase-3) and Ac-IETD-pNA (caspase-8). Shown is a representative experiment, indicating the mean and range of triplicate samples. Abs, absorbance.

- **B:** Casp-3 and caspase-8 were confirmed using colorimetric peptide substrates (32). Although the activities of both caspase-3 and caspase-8 were confirmed using colorimetric peptide substrates (32), only caspase-3 was able to cleave WT-KSR1 and generate the 42-kDa CTF (Fig. 4B, upper panel). No cleavage or CTF generation was observed when DEVA-KSR1 was incubated with caspase-3 (Fig. 4B). These results confirm that KSR1 is a target of the effector caspase-3 and that cleavage occurs at the DEVD site.

**Effects of KSR1 Cleavage on Apoptotic Signaling—** As the above results indicate, caspase-dependent cleavage of KSR1 destroys the full-length scaffold, which interacts with all the ERK cascade components to facilitate ERK activation and results in the stable accumulation of a 42-kDa C-terminal fragment, which has the potential to function in a dominant inhibitory manner given that it contains the MEK binding region but not the domains required for ERK binding or membrane localization. Therefore, we initiated experiments to determine whether KSR1 cleavage might alter ERK survival signaling and thus impact the apoptotic process. For these studies, KSR1−/− MEFs stably expressing either WT-KSR1 or DEVA-KSR1 or constitutively expressing the 42-kDa CTF (CTF-KSR1) were treated with TNFα/CHX and then examined for markers of apoptotic signaling (caspase-3 activation and double-strand DNA breaks). (Note that the KSR1 proteins were stably expressed in KSR1−/− MEFs such that the effect of these proteins could be evaluated without interference from endogenous KSR1.) By FACS analysis, no significant difference in active caspase-3 levels was observed in untreated cells; however, the percentage of cells exhibiting caspase-3 activation at 4 and 6 h of TNFα/CHX treatment was significantly lower in the DEVA-KSR1 line than was detected in the WT- and CTF-KSR1 lines (Fig. 5A and supplemental Fig. 1). The reduction in caspase-3 activation could also be observed in the DEVA-KSR1 MEFs if cleavage of pro-caspase-3 was monitored by immunoblot analysis (Fig. 5B). In contrast, when WT- and CTF-KSR1 MEFs were compared, the percentage of cells showing caspase-3 activation was elevated for the CTF-KSR1 line (Fig. 5A). Similar results were also observed when the cell lines were examined for double-strand DNA breaks following TNFα/CHX treatment. Specifically, when compared with WT-KSR1-expressing cells, Ser-139 H2A.X phosphorylation and TUNEL staining were reduced in DEVA-KSR1 MEFs (Fig. 5C and data not shown), whereas Ser-139 H2A.X phosphorylation and TUNEL staining were elevated in CTF-KSR1 MEFs (Fig. 5C and data not shown).

Next, to investigate whether the effects of the KSR1 proteins might be related to their ERK scaffolding function, we examined the level of active phosphoERK present in the WT-,
DEVA-, and CTF-KSR1 MEFs (Fig. 6A). In untreated cycling cells, significant levels of phosphoERK were detected in both the WT-KSR1 and the DEVA-KSR1 lines, whereas phosphoERK levels were dramatically reduced in cells expressing CTF-KSR1. Strikingly, following TNFα/CHX treatment, a significant reduction in phosphoERK levels was observed in WT-KSR1 MEFs that was not observed in DEVA-KSR1-expressing cells, suggesting that cleavage of WT-KSR1 disrupts its scaffolding properties and thus its ability to promote ERK activation. In addition, consistent with the model that ERK survival signaling antagonizes the apoptotic process, using the MEK inhibitor U0126 to block ERK activation resulted in a significant increase in the percentage of both WT-KSR1 and DEVA-KSR1 MEFs exhibiting caspase-3 activation following TNFα/CHX treatment (Fig. 6B). Of note, however, the level of caspase-3 activation in U0126-treated DEVA-KSR1 MEFs was still lower than that observed in similarly treated WT-KSR1-expressing MEFs.

One well defined target of ERK signaling that could directly impact caspase-3 activation and apoptotic signaling is caspase-9 (12). In previous studies, caspase-9 has been identified as an in vivo ERK substrate, and the phosphorylation of pro-caspase-9 by ERK has been shown to block its processing and activation (37). Therefore, because TNFα/CHX-treatment was found to engage the intrinsic mitochondrial pathway and activate caspase-9 in the WT-KSR1 MEFs and given that caspase-9 contributes to the cleavage and activation of caspase-3, we next examined the effects of WT-, DEVA-, and CTF-KSR1 expression on the processing of pro-caspase-9 (Fig. 6A). By immunoblot analysis, cleavage of pro-caspase-9 was observed in TNFα/CHX-treated cells expressing WT- or CTF-KSR1. However, in DEVA-KSR1 MEFs, which exhibit high levels of active phosphoERK, the cleavage of caspase-9 was significantly reduced (Fig. 6A), consistent with the model that ERK survival signaling can inhibit apoptotic processes such as the cleavage and activation of caspase-9. In further support of this model, treating DEVA-KSR1 MEFs with U0126 to block ERK activation and reduce their levels of active phosphoERK resulted in increased caspase-9 cleavage following TNFα/CHX treatment (Fig. 6C).

**KSR1 Cleavage Occurs in Involuting Mouse Mammary Tissue**—If caspase-dependent cleavage of KSR1 does contribute to the inhibition of survival signaling in apoptotic cells, we would predict that KSR1 cleavage might be observed in an in vivo apoptotic system. An extensively characterized model for studying apoptosis is that of involuting mouse mammary tissue (38). After pregnancy and upon weaning, the mammary glands of mice undergo a complex tissue remodeling process, called involution, which restores the tissue to its pre-pregnancy state (39). Extensive apoptosis occurs in the early stages of this process (day 1–3 post-weaning) such that the milk-producing epithelial cells are removed. As shown in Fig. 7, KSR1 is present as a full-length protein in mammary tissue from lactating mice (day 0) but is cleaved following the induction of apoptosis (post-weaning day 3). Interestingly, cleavage of KSR1 appears complete as no full-length protein remains on post-weaning day 3, and only the 42-kDa CTF is observed. In addition, consistent with the C-terminal domain of KSR1 mediating constitutive MEK binding, the 42-kDa CTF observed at day 3 was found to be associated with MEK (Fig. 7).

**DISCUSSION**

The onset of apoptosis requires not only the up-regulation of pro-death pathways but also the corresponding down-regulation of proliferative/survival pathways. As such, the caspase-dependent cleavage of pro-survival/anti-apoptotic signaling pro-
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In this study, we find that KSR1 is cleaved in response to multiple apoptotic stimuli and that KSR1 cleavage occurs in an in vivo apoptotic model system, involving involuting mouse mammary tissue. For many caspase targets, cleavage results in the separation of functional domains (2), and KSR1 is no exception. Cleavage occurs at a consensus DEVD motif and destroys the ERK scaffolding function of KSR1.

Interestingly, although the KSR1 N-terminal domain appears to undergo further degradation following cleavage at the DEVD motif, stable accumulation of the C-terminal domain, which contains the ERK binding site as well as the C1 domain required for localizing KSR1 to the plasma membrane, where it interacts with the Raf family kinases. As a result of cleavage, the ability of KSR1 to associate with all members of the ERK cascade and thereby facilitate ERK activation is lost. Interestingly, although the KSR1 N-terminal domain appears to undergo further degradation following cleavage at the DEVD motif, stable accumulation of the C-terminal domain, which contains the ERK binding site as well as the C1 domain required for localizing KSR1 to the plasma membrane, where it interacts with the Raf family kinases. As a result of cleavage, the ability of KSR1 to associate with all members of the ERK cascade and thereby facilitate ERK activation is lost.

FIGURE 6. Analysis of the ERK scaffolding activities of WT-, DEVA-, and CTF-KSR1 in TNFα/CHX-treated cells. A, WT-, DEVA-, or CTF-KSR1 MEFs were treated with TNFα/CHX for 4 h prior to lysis. Levels of KSR1, activated phosphoERK, total ERK2, and caspase-9 (Casp 9) were determined by immunoblot analysis. FL KSR1, full-length KSR1. * indicates a nonspecific protein band detected by the KSR1 C-terminal antibody. B, WT- and DEVA-KSR1 MEFs were treated with TNFα/CHX in the presence or absence of the MEK inhibitor U0126 as indicated. Levels of activated phosphoERK, total ERK2, and caspase-9 were determined by immunoblot analysis.

FIGURE 7. Cleavage of KSR1 in involuting mouse mammary tissue. Protein extracts were prepared from mouse mammary glands harvested at 0 and 3 days post-weaning. Endogenous KSR1 proteins were immunoprecipitated and examined by immunoblot analysis as indicated. Mammary tissue extracts from KSR1−/− mice were used as a negative control. Caspase-3 (Casp-3) cleavage was monitored by immunoblot analysis. IP, immunoprecipitation; FL, full-length.

proteins contributes to the shift from cell survival to cell death. KSR1 is a conserved component of the Ras pathway that functions as a molecular scaffold to facilitate ERK cascade signaling. Here, we report that KSR1 is a target of caspase-cleavage in apoptotic cells and that cleavage destroys the ERK scaffolding function of KSR1.

Because these C-terminal domain proteins constitutively interact with MEK but cannot bind ERK or translocate to the plasma membrane, they are thought to inhibit ERK activation by sequestering MEK in the cytosol, thus preventing MEK from interacting with Raf or ERK. From our studies, it appears that the 42-kDa CTF generated by caspase cleavage has the characteristic properties of other KSR1 C-terminal domain proteins. In particular, the 42-kDa CTF is competent to bind MEK, as indicated by the presence of MEK in CTF complexes isolated from involuting mammary tissues. Moreover, we found that KSR1−/− MEFs stably expressing CTF-KSR1 had dramatically reduced levels of active phosphoERK in comparison with cells expressing WT-KSR1, suggesting that the CTF can inhibit ERK activation. Therefore, the consequences of KSR1 caspase-dependent cleavage appear to play a more
critical role in disrupting ERK survival signaling is currently unclear and could potentially vary depending on the cell type as well as the endogenous expression level of KSR1 and MEK.

The ERK cascade plays an important role in transducing survival signals, and previous studies have shown that increased signaling through the Raf/MEK/ERK cascade can inhibit apoptosis in a manner that is dependent on ERK activity (41, 42). Consistent with the model that ERK survival signaling impedes the apoptotic process, we found that DEVA-KSR1-expressing cells, which had elevated levels of active phosphoERK in comparison with WT-KSR1 MEFs, exhibited reduced apoptotic signaling following TNFα/CHX treatment. Specifically, H2A.X phosphorylation was reduced, as was cleavage and activation of effector caspase-3. TNFα/CHX-induced processing of pro-caspase-9 was also reduced in DEVA-KSR1 MEFs and is of interest given that processing/activation of caspase-9 contributes to caspase-3 activation under these conditions and is an event inhibited by ERK phosphorylation. In contrast, cells constitutively expressing the 42-kDa CTF (CTF-KSR), which had much lower basal phosphoERK levels than WT-KSR1 MEFs, exhibited increased apoptotic signaling following TNFα/CHX treatment.

Providing further evidence that ERK survival signaling antagonizes the apoptotic process, we found that using the MEK inhibitor U0126 to block ERK activation significantly reversed the effects of DEVA-KSR1 expression in TNFα/CHX-treated cells (levels of caspase-3 activation were increased, as was the processing of pro-caspase-9). In addition, caspase-3 activation was further elevated in WT-KSR1 MEFs. Of note, however, U0126 treatment did not restore caspase-3 activation levels in DEVA-KSR1 MEFs to that of WT-KSR1-expressing cells, suggesting that KSR1 may contribute to cell survival in ways that are distinct from its ability to promote ERK activation. KSR1 proteins also interact with B-Raf and C-Raf (27), Raf kinase family members that initiate the phosphorylation cascade, resulting in ERK activation. Interestingly, C-Raf has been shown to possess anti-apoptotic activity that is independent of its ability to activate the ERK cascade (43–45). Thus, it is interesting to speculate that KSR1 might also contribute to survival signaling through a mechanism involving C-Raf.

Taken together, our results indicate that destruction of the ERK scaffolding ability of KSR1 antagonizes ERK survival signaling and thus impacts the apoptotic process. Significantly, a role for KSR1 in the regulation of apoptotic signaling has also been implicated in previous reports. More specifically, loss of KSR1 in mouse intestinal epithelial cells and inhibition of KSR1 function in young adult mouse colon cells has been found to cause increased TNFα-mediated apoptosis that is associated with a failure to activate cell survival pathways including the ERK cascade (46, 47). In the mouse epithelial cell system, the effect of KSR1 loss on both ERK signaling and apoptosis could be reversed by restoring KSR1 protein levels, supporting the model that the ability of KSR1 to modulate the ERK pathway can impact apoptotic signaling.

In conclusion, we find that the KSR1 scaffold is another in a growing list of pro-survival signaling molecules that are cleaved during the apoptotic process. Given the importance of KSR1 in Ras-mediated ERK cascade regulation, we propose that disrupting the scaffolding ability of KSR1 by caspase cleavage may serve as a mechanism to down-regulate ERK activity in cells undergoing apoptosis and thus facilitate the transition toward cell death.

Acknowledgments—We thank Cryil Berthet, Howard Fearnhead, Shikha Sharan, Esta Sterneck, and members of the Morrison laboratory for helpful advice and technical assistance.

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