Nm23-H1 Metastasis Suppressor Phosphorylation of Kinase Suppressor of Ras via a Histidine Protein Kinase Pathway*

Melanie T. Hartsough‡, Deborah K. Morrison§, Massimiliano Salerno, Diane Palmieri, Taoufik Ouatas, Michael Mair, Jilla Patrick, and Patricia S. Steeg†

From the Women’s Cancers Section, Laboratory of Pathology, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 20892 and the §ABL-Basic Research Program, NCI-Frederick, Frederick, Maryland 21702

Received for publication, April 1, 2002, and in revised form, June 24, 2002
Published, JBC Papers in Press, June 24, 2002, DOI 10.1074/jbc.M203115200

The metastasis-suppressive activity of Nm23-H1 was previously correlated with its in vitro histidine protein kinase activity, but physiological substrates have not been identified. We hypothesized that proteins that interact with histidine kinases throughout evolution may represent partners for Nm23-H1 and focused on the interaction of Arabidopsis “two-component” histidine kinase ERS with CTR1. A mammalian homolog of CTR1 was previously reported to be c-Raf; we now report that CTR1 also exhibits homology to the kinase suppressor of Ras (KSR), a scaffold protein for the mitogen-activated protein kinase (MAPK) cascade. Nm23-H1 co-immunoprecipitated KSR from lysates of transiently transfected 293T cells and at endogenous protein expression levels in MDAMB-435 breast carcinoma cells. Autophosphorylated recombinant Nm23-H1 phosphorylated KSR in vitro. Phosphoamino acid analysis identified serine as the major target, and two peaks of Nm23-H1 phosphorylation were identified upon high performance liquid chromatography analysis of KSR tryptic peptides. Using site-directed mutagenesis, we found that Nm23-H1 phosphorylated KSR serine 392, a 14-3-3-binding site, as well as serine 434 when serine 392 was mutated. Phosphorylated MAPK but not total MAPK levels were reduced in an nm23-H1 transfectant of MDA-MB-435 cells. The data identify a complex in vitro histidine-to-serine protein kinase pathway, which may contribute to signal transduction and metastasis.

Metastasis suppressor genes are credentialed by their ability to suppress metastatic potential in vivo upon injection of a transfected tumor cell line, without a concomitant reduction in primary tumor size (reviewed in Ref. 1). The nm23 gene family was described by its reduced expression in highly metastatic murine melanoma cell lines, as compared with related, tumorigenic but less metastatic cell lines (2) and consists of eight family members (reviewed in Ref. 3). In a recent review, 18 of 24 studies found a significant relationship between decreased Nm23 expression in primary human breast carcinomas and an aspect of aggressive clinical course (patient survival, lymph node metastasis, etc.), although Nm23 expression does not represent an independent prognostic or predictive factor (4). Ten transfection experiments have shown that nm23-transfected, metastatically competent cell lines are 40–98% less metastatic in vivo than control transfectants (5–14). For Nm23-H1, the most studied member, overexpression in human MDA-MB-435 breast carcinoma cells reduced colonization in soft agar, both unstimulated and transforming growth factor-β-stimulated (6), and invasion/motility to a variety of chemottractants (15–17). Nm23-H1 breast carcinoma transfectants exhibited morphological (ascus formation) and biosynthetic aspects of differentiation in three-dimensional culture (18), and this finding is supported by similar studies in neural cells (19–25).

Despite extensive work, the biochemical mechanism of action whereby Nm23-H1 suppresses the metastatic potential of cancer cells is unknown, and debate exists over several of its reported biochemical activities. Nm23 proteins form a histidine-phosphorylated intermediate, which is involved in its nucleoside diphosphate kinase (NDP kinase) (26), geranyl and farnesyl pyrophosphate kinase (27), histidine protein kinase (28–31), and possibly serine protein kinase activities (32, 33). An autophosphorylation on serine residues was also reported (34–37). Nm23-H2 has been reported to be a transcription factor and to cleave DNA (38–40); Nm23-H1 was recently reported to exert similar activity (41). Nm23-H1 proteins have been reported to bind to many proteins, including small and heterotrimeric G-proteins (42–45), their exchange factors (46), ROR/RZR receptors (47), centrosomes (48), glyceraldehyde-3-phosphate dehydrogenase (49, 50), cytoskeletal proteins (51–57), muscarinic receptors (58), heat shock proteins (59, 60), Prune (61), phytocromes (62, 63), phosphatases (64), menin (65), and Epstein-Barr virus proteins (66).

One method to link Nm23 biochemical and biological function is site-directed mutagenesis. We reported that alterations in Nm23 sequence affected one aspect of metastasis, suppression of in vitro motility. Mutation of Nm23-H1 proline 96 (P96S), involved in Drosophila development (67), or serine 120 (S120G), a site of mutation in human neuroblastomas (68), impaired its motility suppressive capacity to multiple chemottractants (15). Biochemical analysis of the recombinant proteins indicated that P96S resulted in a histidine protein kinase-deficient mutant in assays using sucinic thio kinase, Nm23-H2, and aldolase; the S120G mutant resulted in reduced Nm23-H1 histidine autophosphorylation and aldolase phosphorylation (28, 29, 69). These data prompted the hypothesis that a histidine protein kinase activity may contribute to the motility-suppressive effect of Nm23-H1. The Nm23-H1 P96S mutant also exhibited loss of function in the non-GTP regeneration interaction with the muscarinic receptor G-protein (58). Cho et al. (70) reported that the Nm23-H1 P96S and S120G
mutations exhibited transactivational activity comparable with that of wild type protein, separating this function and motility suppression. Other biochemical functions have not been reported in this model system to date.

We searched for proteins that interacted with histidine protein kinases, which might represent candidate signaling pathways for the Nm23-H1-suppressive capacity. Histidine protein kinases are prominent members of the “two-component” or histidyl-aspartyl pathways of prokaryotes and lower eukaryotes. In the simplest scenario, an external signal interacts with a receptor, which leads to a histidine autophosphorylation of a sensor kinase, the phosphate of which is passed to an aspartate of a response regulator protein, resulting in signal propagation (reviewed in Refs. 71–73). When cloned into an *Escherichia coli* “two-component system,” Nm23 functioned as a histidine sensor kinase (31). We conducted a search in *situ* for proteins interacting with “two-component” histidine sensor kinases and having mammalian homologs. None were found among bacterial response regulator proteins. In *Arabidopsis*, the ethylene receptor controls differentiation through a complex “two-component” pathway (74) (reviewed in Refs. 75–77). Whereas many of the *Arabidopsis* “two-component” proteins were hybrids, a single protein containing both the sensor histidine protein kinase and aspartate regulator domains, the *Arabidopsis* ERS histidine sensor kinase was found by two-hybrid analysis to interact with the N-terminal portion of CTR1 (78). CTR1 was not reported to be a typical aspartate-phosphorylated response protein, but led to a diminution of the mitogen-activated protein kinase (MAPK) pathway by an unknown mechanism and thus represented a novel suppressive pathway extending from the two-component system. Whereas it was originally reported to be homologous to mammalian Raf family members (78, 79), we report herein that CTR1 also shares significant homology to the kinase suppressor of Ras (KSR) (80), a putative scaffold protein for the MAPK pathway (reviewed in Ref. 81). In this paper, we present evidence for an interaction of Nm23-H1 and KSR in mammalian cells as well as evidence that Nm23-H1 can phosphorylate KSR in a complex manner in *vitro* via a histidine-dependent pathway. Our data provide evidence for a new type of signaling pathway in mammalian cells.

### EXPERIMENTAL PROCEDURES

**Reagents and Cell Lines**

FLAG-tagged Raf-1, N-terminal Raf-1, C-terminal Raf-1, and Pyo-tagged murine KSR, N-terminal KSR (amino acids 1–539), C-terminal KSR (amino acids 542–873), KSR-AA and KSR-AAA cDNA constructs, anti-Py, and rabbit anti-KSR were all previously described (82, 83). Anti-FLAG antibody was purchased from Sigma; anti-Erk1/2 MAPK and pErk1/2 MAPK was purchased from Transduction Laboratories. Nm23 antibodies (antibody 301 and anti-peptide 11) were described previously (84, 85). Human MDA-MB-435 breast carcinoma transfecants were cultured on Matrigel as described (86). 

**Recombinant Nm23-H1 Proteins**

Human wild type, P96S, S120G, and H118F Nm23 DNA (69) were excised from a pET3c vector (69) with *Nde* I restriction sites and transformed into E. coli strain BL21 (DE3). Recombinant protein was prepared as described (69), with the exception that the pellets were lysed in Bug Buster™ Protein Extraction Reagent (Novagen) and purified by nickel chromatography, as described by the manufacturer (Novagen).

**Cell Transfection**

293T cells were plated at a density of $6 \times 10^{5}$ cells/60 mm plate and transfected with $3 \mu$g of cDNA plasmids, utilizing the Fugene method, as per the manufacturer’s instructions (Roche Molecular Biochemicals). Forty-eight h post-transfection, cells were analyzed. Alternatively, 293T cells were transfected using the Effectene transfection reagent (Qiagen), using 3 $\mu$g of DNA by the manufacturer’s protocol. The cell culture medium was changed 24 h post-transfection, and cells were harvested 48 h post-transfection. MDA-MB-435 human breast carcinoma transfecants were previously described (6, 15) and were maintained in Dulbecco’s modified Eagle medium at 37°C, 5% CO2. MDA-MB-435 cells were also transfected using Effectene as described above.

**Lysates**

Cells were washed twice in 1× phosphate-buffered saline and lysed in Nonidet P-40 (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% glycerol, 1% Nonidet P-40, 2 mM EDTA, aprotinin (0.15 units/ml), 1 mM phenylmethylsulfonyl fluoride, 20 $\mu$M leupeptin, 5 mM sodium vanadate) or in radioimmunoprecipitation buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, aprotinin (0.15 units/ml), 1 mM phenylmethylsulfonyl fluoride, 20 $\mu$M leupeptin, 5 mM sodium vanadate). Particulate lysates were removed from the lysate by centrifugation at 14,000 × g at 4°C for 10 min. Cleared lysates were frozen at −80°C until use.

**Immunoprecipitation and Western Analysis**

Lysates were normalized to equivalent total protein (BCA; Pierce) prior to immunoprecipitation. Immunoprecipitation assays were performed by incubating cell lysates with either anti-Py, anti-Nm23 (antibody 301), rabbit anti-KSR, or anti-FLAG and either Protein A/G-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or Protein G-agarose (Roche Molecular Biochemicals) for 2 h at 4°C. The immunoprecipitated was washed four times in lysis buffer prior to separation by SDS-PAGE and transfer to nitrocellulose membrane. The membrane was then immunoblotted with anti-Py, rabbit anti-KSR, anti-Nm23 (anti-peptide 11) or anti-FLAG, followed by horseradish peroxidase-conjugated secondary antibody. Proteins were visualized by ECL (Amersham Biosciences).

**In Vitro Protein Kinase Assays**

**32P Labeling of Nm23 Proteins—Five $\mu$g of recombinant Nm23 proteins (wild type, P96S, S120G, H118F) were incubated in TMD buffer (20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol with 330 $\mu$Ci of [γ-32P]ATP (final concentration 0.33 $\mu$Ci/mL), P96S (S22) were previously described (69). Anti-FRAP antibody was purchased from Sigma; anti-Erk1/2 MAPK and pErk1/2 MAPK was purchased from Transduction Laboratories.

**Site-directed Mutation of KSR**

Site-directed mutagenesis of Pyo-tagged murine KSR constructs was performed using the QuikChange III kit (Stratagene) using the manufacturer’s directions. The following oligonucleotides were used to generate serine to alanine mutants: serine 297 to alanine (KSR297A), 5'-TCA CAC GGA GCA AGG CCC AGC AAG AGC-3'; serine 392

---

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; pMAPK, phosphorylated mitogen-activated protein kinase; KSR, kinase suppressor of Ras; HPLC, high performance liquid chromatography; rNm23-H1, recombinant Nm23-H1.
Phosphorylation of KSR by Nm23-H1

Phosphoamino Acid Analysis

Phosphoamino acid analysis was performed as previously described (82, 87). N-terminal KSR was phosphorylated with Nm23-H1 as described above. The phosphorylated N-terminal KSR was electrophoresed on a 8% SDS-PAGE, transferred to polyvinylidene difluoride, and excised from the membrane. Partial peptidic hydrolysis was achieved by heating in 6N HCl at 100 °C for 80 min. Hydrolysates were evaporated to dryness and then reconstituted in 12 ml of electrophoresis buffer that also contained unlabeled phosphoamino acid standards. Samples were spotted on a DEAE-cellulose-coated TLC plate. Constitutive phosphoamino acids were separated and eluted by reversed-phase high performance liquid chromatography (HPLC). HPLC fractions containing peaks of radioactivity were subjected to semiautomated Edman degradation in a spinning cup sequenator.

Phosphopeptide Analysis

Phosphopeptide analysis was conducted as previously described (82). Phosphorylated N-terminal KSR was subjected to SDS-PAGE, transferred to a nitrocellulose filter, and visualized by autoradiography. A membrane piece containing the phosphoprotein was excised and transferred to a nitrocellulose filter, and visualized by autoradiography. A similar experiment using full-length, N-terminal, or C-terminal KSR vectors.

RESULTS

Arabidopsis CTR1 Shares Homology with Raf and KSR—The Arabidopsis CTR1 protein sequence was used as bait to search the expressed sequence tag data base for homologs that could represent binding partners of histidine protein kinases. Initially, a Drosophila expressed sequence tag encoding KSR exhibited 43% identity and 50% homology to a 121-amino acid region of CTR1 (amino acids 543–724). KSR contains five domains, CA1 through CA5 (80). In agreement with Raf/CTR1 data (79), homology between KSR and CTR1 is principally found in the KSR “kinase” CA5 domain in the C terminal and extends upstream to include the serine/threonine-rich CA4 domain located in the N-terminal portion of the protein. Using Pep Tool version 1.0 analysis, the Arabidopsis CTR1 protein exhibited 25.1 and 25.9% identity to the kinase domains of mammalian KSR and c-Raf-1, respectively. The KSR serine/threonine-rich CA4 region exhibited 25% identity to CTR1.

Phosphopeptide analysis was conducted as previously described (82, 87). N-terminal KSR was phosphorylated with Nm23-H1 as described above. The phosphorylated N-terminal KSR was electrophoresed on a 8% SDS-PAGE, transferred to polyvinylidene difluoride, and excised from the membrane. Partial peptidic hydrolysis was achieved by heating in 6N HCl at 100 °C for 80 min. Hydrolysates were evaporated to dryness and then reconstituted in 12 ml of electrophoresis buffer that also contained unlabeled phosphoamino acid standards. Samples were spotted on a DEAE-cellulose-coated TLC plate. Constituent phosphoamino acids were separated and eluted by reversed-phase high performance liquid chromatography (HPLC). HPLC fractions containing peaks of radioactivity were subjected to semiautomated Edman degradation in a spinning cup sequenator.

Homology Searches

The initial homology between KSR and CTR1 was identified using expressed sequence tag encoding KSR Drosophila (X03484), and murine KSR (accession number NPO38599). We hypothesized that mammalian homologs of CTR1 could represent binding partners of histidine protein kinases. Initially, a Drosophila expressed sequence tag encoding KSR exhibited 43% identity and 50% homology to a 121-amino acid region of CTR1 (amino acids 543–724). KSR contains five domains, CA1 through CA5 (80). In agreement with Raf/CTR1 data (79), homology between KSR and CTR1 is principally found in the KSR “kinase” CA5 domain in the C terminal and extends upstream to include the serine/threonine-rich CA4 domain located in the N-terminal portion of the protein. Using Pep Tool version 1.0 analysis, the Arabidopsis CTR1 protein exhibited 25.1 and 25.9% identity to the kinase domains of mammalian KSR and c-Raf-1, respectively. The KSR serine/threonine-rich CA4 region exhibited 25% identity to CTR1. By comparison, the Raf-1 CR2 domain, which is homologous to KSR CA4, exhibited a 22% identity to the CTR1 sequence. These comparisons are similar to those previously reported between Raf-1 and CTR1 using other analysis programs (79).

We hypothesized that mammalian homologs of CTR1 could interact with Nm23-H1 but were unable to detect either co-immunoprecipitation of Nm23-H1 with Raf-1 (data not shown) or Nm23-H1 phosphorylation of Raf-1 (this paper). Similar questions were therefore asked for Nm23-H1 and KSR.

Co-immunoprecipitation of Nm23-H1 and KSR—In the experiment shown in Fig. 1A, 293T cells were transiently transfected with a murine Pyo-tagged KSR construct, the cells were

FIG. 1. Co-immunoprecipitation of Nm23 and KSR. A, 293T cells were transiently transfected with Pyo-tagged murine KSR, and lysates were immunoprecipitated with anti-Pyo, anti-Nm23-H1 (monoclonal antibody 301), or a control IgG. The immunoprecipitates were electrophoresed and transferred to a filter, and the filter was split and developed as a Western blot using anti-rabbit KSR or anti-Nm23-H1 (peptide 11). B, similar experiment using full-length, N-terminal, or C-terminal KSR vectors. C, control (C-100) or nm23-H1 transfectants (H1–177, wild type Nm23-H1; S-22, Nm23-H1-P96S) of human MDA-MB-435 breast carcinoma cells, previously reported (83, 221), were utilized. Lysates were immunoprecipitated with anti-Nm23-H1, electrophoresed, and developed as two split Western blots as described above. Data are representative of at least three experiments conducted.
lysed in an Nonidet P-40 containing buffer, and aliquots were immunoprecipitated with either a control IgG, anti-Pyo, or anti-Nm23-H1. Immunoprecipitates were electrophoresed and transferred to a membrane; the membrane was split and hybridized to anti-Nm23-H1 and anti-KSR, which was detected by chemiluminescence. Immunoprecipitation of Nm23-H1 co-immunoprecipitated KSR. In a similar experiment, Nm23-H1 was immunoprecipitated from lysates of 293T cells transiently transfected with full-length, N-terminal, or C-terminal KSR, and co-immunoprecipitation of KSR was detected (Fig. 1B). Thus, the interaction between Nm23-H1 and KSR is controlled by multiple regions of the KSR protein. The anti-Pyo immunoprecipitation in Fig. 1, A and B, indicated that only a small fraction of total KSR co-immunoprecipitated with Nm23-H1, which may explain in part the failure to detect Nm23-H1 in anti-KSR-immunoprecipitated lanes. In experiments not shown, detection of KSR in anti-Nm23-H1 immunoprecipitates was obtained when cells were lysed in either Nonidet P-40 or radioimmune precipitation buffers.

Lysates of human MDA-MB-435 breast carcinoma cells, previously transfected with a control vector (C-100) or constitutively overexpressing wild type Nm23-H1 (H1–177) (6) or kinase-deficient P96S-mutated Nm23-H1 (P96S) (15), were immunoprecipitated with anti-Nm23-H1 and subjected to Western blot (Fig. 1C). Co-immunoprecipitation of Nm23-H1 and KSR was observed in all cell lines. The data indicate an interaction of Nm23-H1 and KSR under physiological conditions, since the endogenous levels of expression of both the KSR and Nm23-H1 were unaltered in the C-100 cells. KSR is a low abundance cytoplasmic protein in human breast carcinoma cells and cannot be detected by Western blot of total cell lysates (data not shown). Thus, the amount of KSR detected, although low, is significant.

Phosphorylation of KSR by Nm23-H1 in Vitro—Given the interaction between Nm23-H1 and KSR as well as the correlation of a histidine kinase pathway with Nm23-H1’s metastasis-suppressive function, we asked whether autophosphorylated Nm23-H1 could phosphorylate KSR in vitro. Since KSR associates with several kinases, which could phosphorylate it in the presence of contaminating [γ-32P]ATP, and Nm23-H1 as a NDP kinase can generate ATP in the presence of ADP and NTPs, we utilized conditions in which an equivalent amount of free [γ-32P]ATP did not phosphorylate KSR in vitro. Briefly, rNm23-H1 was autophosphorylated; for all experiments, the autophosphorylated rNm23-H1 was purified by column chromatography, and the presence of contaminating [γ-32P]ATP was undetectable on 30-min autoradiographs of thin layer chromatography using 2 μl of purified protein as described (69). Autophosphorylated rNm23-H1 was incubated with Pyo-tagged murine KSR, N-terminal KSR, or C-terminal KSR proteins that were immunoprecipitated from transiently transfected 293T cells. Kinase reactions were electrophoresed, transferred to a filter, autoradiographed, and subsequently developed as a Western blot with rabbit anti-KSR (Fig. 2A). Full-length and N-terminal KSR were phosphorylated by Nm23-H1. The C-terminal portion of KSR was not phosphorylated by Nm23-H1, despite protein expression and immunoprecipitation (lower panel), providing a control for specificity. A high molecular weight form of autophosphorylated Nm23-H1, visible below the 44-kDa marker on the autoradiograph (upper panel), was verified as Nm23-H1 by mass spectrometry sequencing (data not shown) and indicated comparable loading of autophosphorylated protein (TLC).

Additional controls for this kinase reaction are shown in Fig. 2B, using N-terminal KSR. In this experiment, immunoprecipitated KSR from transiently transfected 293T cells was incubated with either autophosphorylated wild type rNm23-H1, the Nm23-H1 histidine-phosphorylated intermediate mutant H118F (69), or a flow-through fraction from the PD10 column (ATP). In the last sample, the same input amount of [γ-32P]ATP used in the Nm23-H1 autophosphorylation was incubated in buffer, but without added Nm23-H1. The reaction was applied to a PD10 column, and fractions were collected identically to other samples, as a control for the effects of contaminating [γ-32P]ATP. N-terminal KSR was phosphorylated by Nm23-H1 only under the specific conditions of transfection with Pyo-tagged N-terminal KSR, immunoprecipitation with anti-Pyo, and incubation with wild type autophosphorylated Nm23-H1 (upper panel). The Western blot demonstrates the presence of immunoprecipitated N-terminal KSR in the appropriate lanes devoid of phosphorylation (lower panel). Similar results were obtained when full-length KSR was used, and denatured Nm23-H1 was without kinase activity (data not shown). Immunoprecipitation of KSR from lysates containing either radioimmune precipitation buffer- or Nonidet P-40-based buffers gave comparable results in kinase assays (data not shown). Also, Nm23-H1 phosphorylated KSR when immunoprecipitated from 293T cells using a rat anti-KSR antibody, indicating that the results described above were not an artifact of the use of anti-Pyo (data not shown).

In the experiment shown in Fig. 3, aliquots of immunoprecipitated KSR from transiently transfected 293T cells were incubated with equivalent cpm of [γ-32P]ATP-autophosphorylated rNm23-H1, either wild type, P96S, or S120G (69); for the H118F mutant an equivalent amount of protein was used. The kinase reactions were electrophoresed and autoradiographed. Wild type Nm23-H1 phosphorylated KSR, and H118F was without kinase activity, providing a confirmation of the histidine dependence of this pathway. The P96S Nm23-H1 protein was deficient in KSR kinase activity; this protein was kinase-deficient in other histidine kinase reactions (28, 69), and transfection experiments showed impaired motility-suppressive activity (15). The presence of a strong autophosphorylated Nm23-H1 P96S band in this lane suggests that this is a kinase-deficient reaction as opposed to artifactual underloading of Nm23 protein. The S120G Nm23-H1 protein, which also showed impaired motility-suppressive activity (15), phosphorylated KSR at levels similar to wild type. The biochemical defect in this protein was attributed to the initial autophosphorylation part of the histidine kinase pathway (69) and was probably overcome by the addition of similar cpm of each Nm23-H1 protein. The NDP kinase activities of the wild type, P96S, and S120G rNm23-H1 proteins was determined as described (15) and were comparable (data not shown), separating Nm23-H1 NDP kinase activity and KSR phosphorylation. Thus, the Nm23-H1 P96S mutant exhibited binding to KSR comparable with that of wild type protein but was kinase-deficient.

Based on the homology of both KSR and Raf to CTR1, we asked whether Nm23-H1 also phosphorylated Raf-1 in vitro. 293T cells were transiently transfected with constructs for Pyo-tagged full-length, N-terminal, or C-terminal KSR or FLAG-tagged full-length, N-terminal, or C-terminal Raf-1. Lysates were immunoprecipitated with either anti-Pyo or anti-FLAG and processed for Nm23-H1 kinase assays and Western blots as previously described, with the exception that the Western blot was reprobed sequentially to anti-Pyo or -FLAG (Fig. 4). Nm23-H1 phosphorylation of full-length and N-terminal KSR occurred as previously shown. Phosphorylation of N-terminal KSR was stronger than that of full-length KSR, but the former contained more immunoprecipitated protein. Phosphorylation of c-Raf was essentially negative (Fig. 4, upper panel) despite vigorous protein expression (lower right panel). Thus,
the in vitro histidine kinase activity of Nm23-H1 for KSR did not extend to Raf in vitro. The phosphorylation of KSR, but not the closely related c-Raf, by Nm23-H1 indicates specificity in the kinase activity of Nm23-H1 and provides further evidence that it is not simply nonspecifically providing nucleotides.

**Fig. 2. Nm23-H1 phosphorylates KSR in vitro.** A, 293T cells were transiently transfected with Pyo-tagged full-length, N-terminal, or C-terminal KSR, and lysates were immunoprecipitated with anti-Pyo. The immunoprecipitants were incubated with purified $^{32}$P-autophosphorylated rNm23-H1, and the products were electrophoresed, transferred to a blot, autoradiographed (upper panel), and developed as a Western blot with anti-rabbit KSR (lower panel). The arrowheads indicate the positions of KSR proteins. B, similar experiment, in which 293T cells were transfected with a control vector or Pyo-tagged N-terminal KSR, immunoprecipitated with anti-Pyo or a control IgG, and incubated with autophosphorylated rNm23-H1, rNm23-H1-H118F, or ATP (a reaction in which the same amount of input [γ-$^{32}$P]ATP was processed identically to that used to autophosphorylate Nm23-H1, except in the absence of added protein). The arrowhead indicates the position of N-terminal KSR. Data are representative of at least four experiments conducted.
Phosphorylation on threonines 260, 274, and serine 443, which are MAPK phosphorylation sites. Volle et al. (88), using similar methods, identified the 14-3-3 sites and the MAPK sites at serines 260 and 274 as well as minor sites of phosphorylation on serines 190, 429, 434, and 516 and threonines 256 and 411.

Fig. 3. Nm23-H1-P96S is deficient in KSR phosphorylation. 293T cells were transiently transfected with Pyo-tagged murine KSR, and lysates were immunoprecipitated with anti-Pyo. Immunoprecipitants were incubated with \( \gamma^{32-P} \)ATP-autophosphorylated rNm23-H1 proteins (15, 69), the reactions were electrophoresed, and the gel was autoradiographed. The positions of KSR and Nm23-H1 are noted, and the amount of autophosphorylated Nm23-H1 indicated equal loading. Data are representative of seven experiments conducted.

Fig. 4. Nm23-H1 does not phosphorylate Raf-1. 293T cells were transiently transfected with Pyo-tagged murine KSR (full-length, N-terminal, and C-terminal) or FLAG-tagged murine Raf-1 (full-length, N-terminal, and C-terminal), and the lysates were immunoprecipitated with anti-tag antibodies. The immunoprecipitants were incubated with \( \gamma^{32-P} \)ATP-autophosphorylated rNm23-H1, and the kinase reactions and Western blots were processed as described in the legend to Fig. 2, with the exception that the Western blot was split and hybridized to anti-tag antibodies as shown. Phosphorylated KSRs are noted by the arrowheads. Data are representative of two experiments conducted.

Lysates from 293T cells transiently transfected with Pyo-tagged N-terminal KSR were immunoprecipitated with anti-Pyo and phosphorylated \textit{in vitro} by autophosphorylated rNm23-H1. The kinase reaction was electrophoresed and transferred to a membrane, and KSR was excised. Phosphoamino acid analysis was performed, an autoradiograph of which is shown on Fig. 5A. The overwhelming majority of
Nm23-H1-phosphorylated KSR was phosphoserine, with trace amounts of phosphothreonine.

Peptide mapping of Nm23-H1 phosphorylated N-terminal KSR is shown in Fig. 5B. Immunoprecipitated N-terminal KSR from 293T cells was incubated with autophosphorylated Nm23-H1; KSR was excised from a gel, digested with trypsin, and subjected to reverse phase HPLC analysis as previously reported (82). The radioactive profile of the HPLC was compared with another sample processed side-by-side, in which immunoprecipitated N-terminal KSR was incubated with [γ-32P]-ATP, permitting associated kinases to phosphorylate KSR (Fig. 5C). This arm of the experiment controlled for potential nonspecific contribution of ATP by the rNm23-H1 and enabled the distinction of Nm23-H1 phosphorylation pattern from that of other physiologic binding partners. Two peaks of radioactivity were identified in the KSR sample phosphorylated by Nm23-H1 (fraction 44) was mutated to an alanine. 293T cells were transiently transfected with Pyo-tagged murine KSR. Lysates were immunoprecipitated with anti-Pyo, phosphorylated by [γ-32P]-autophosphorylated recombinant purified Nm23-H1, electrophoresed, and transferred to a filter; the filter was autoradiographed as a kinase assay and processed as a Western blot using anti-rabbit KSR as described in the legend to Fig. 2. B, similar experiments were performed with S392A, identified in Fig. 5B. Data are representative of three experiments conducted.

Site-directed Mutagenesis of Potential KSR Phosphorylation Sites—Site-directed mutagenesis of KSR was used to confirm and extend these findings. The experiments shown in Fig. 6A test the effect of mutation of each of 14 serines in the amino acids 419–467 region of the KSR CA4 domain, identified as phosphorylated in fraction 44. Immunoprecipitates of transiently transfected Pyo-tagged KSR were incubated with autophosphorylated rNm23-H1, and the reaction products were electrophoresed, transferred to a filter, and autoradiographed; subsequently, the filters were developed as a Western blot to confirm KSR expression. The vector alone versus wild type KSR control is shown in the first panel. None of the individual site-directed mutants consistently reduced the kinase activity of Nm23-H1. We conducted a similar round of experiments with a KSR-AAA-mutated construct (82), which eliminated three MAPK phosphorylation sites (Ser443, Thr260, and Thr274), and did not observe reduced phosphorylation (data not shown).

Using a similar strategy, mutation of KSR Ser392 to alanine was tested for Nm23-H1 kinase activity (Fig. 6B). Ser392 was the site of a Nm23-H1-induced phosphorylation in fraction 23 and represents one of two 14-3-3 binding sites. In this case, mutation of Ser392 to alanine consistently reduced Nm23-H1 phosphorylation, confirming it as a site of Nm23-H1 kinase activity.

Interaction of the KSR 14-3-3 Binding Sites and Serine 434—Given the demonstration of KSR Ser392 as a site of Nm23-H1 phosphorylation activity but the lack of a clear identification of a Nm23-H1 phosphorylation site predicted in the CA4 domain (fraction 44; Fig. 5B), we investigated the hypothesis that interactions between these sites existed. The KSR-AA construct (82), in which both 14-3-3 binding sites were mutated to alanines, was utilized. In the experiment shown in Fig. 7, 293T cells were transiently transfected with Pyo-tagged murine KSR, a KSR-AA mutant, and KSR-AA containing additional...
mutations in the CA4 domain, KSR-AA-S429A and KSR-AA-S434A. The additional mutations were chosen on the basis of previous reports indicating them as sites of in vivo KSR phosphorylation. Two technical approaches were used to observe KSR phosphorylation levels below that of KSR-AA. In order to maximize the sensitivity of the kinase assay, half of the immunoprecipitated KSR proteins were incubated with autophosphorylated rNm23-H1 and electrophoresed, and the gel was directly autoradiographed (upper panel); the other half was processed as a Western blot for total protein (lower panel) (Fig. 7A). In the experiment shown in Fig. 7B, the kinase reaction was transferred to a blot and then autoradiographed (upper panel), after which the filter was developed as a Western blot (lower panel). Lanes marked with asterisks (B) included the immunoprecipitated KSR proteins noted to the left plus an equivalent amount of $\gamma^{-32}$P]ATP as was used for the Nm23-H1 autophosphorylation, without Nm23-H1. Data are representative of three experiments conducted.

Phosphorylation of KSR by Nm23-H1

Two technical approaches were used to observe KSR phosphorylation levels below that of KSR-AA. In order to maximize the sensitivity of the kinase assay, half of the immunoprecipitated KSR proteins were incubated with autophosphorylated rNm23-H1 and electrophoresed, and the gel was directly autoradiographed (upper panel); the other half was processed as a Western blot for total protein (lower panel) (Fig. 7A). In the experiment shown in Fig. 7B, the kinase reaction was transferred to a blot and then autoradiographed (upper panel), after which the filter was developed as a Western blot (lower panel). In both cases, phosphorylation of KSR-AA by Nm23-H1 was quantitatively reduced as compared with wild type protein, confirming the S392A data from Fig. 6B and extending it to conditions eliminating binding of 14-3-3 at both sites. Mutation of serine 434, in addition to the AA mutation, was accompanied by a further reduction in Nm23-H1 phosphorylation as compared with KSR-AA (Fig. 7C). KSR-AA-S429A data provided a control, since this represented a site of in vivo phosphorylation that was not reduced as compared with KSR-AA. The data suggest that Nm23-H1 phosphorylation of KSR serine 434 may occur on a subpopulation of KSR, determined by the binding or phosphorylation status of 14-3-3 sites.

MAPK Levels in nm23-H1 Transfectants

Since CTR1 functioned to reduce MAPK signaling in Arabidopsis cells, we asked whether control and nm23-H1 transfectants of the human
MDA-MB-435 breast carcinoma cell line exhibited varying Erk1/2 MAPK activities. In the experiment shown in Fig. 8A, the C-100 control transfectant and H1–177 nm23-H1 transfectant cell lines were cultured on tissue culture plastic or Matrigel. Inclusion of the latter culture condition was prompted by the observation that most biological assays of Nm23-H1 function have not used tissue culture plastic, such as motility, soft agar colonization, and differentiation (6, 16, 18). Western blots of lysates from these cultures indicated that the nm23-H1 transfectant exhibited basal phospho-MAPK (pMAPK) levels below that of the control transfectant under either culture condition. Total MAPK levels were comparable. The graph in Fig. 8B presents a comparison of three MDA-MB-435 transfectants grown on tissue culture plastic: the vector-transfected C-100, the nm23-H1 transfectant H1–177, and the nm23-H1 P96S mutant cell line S22. The ratio of pMAPK/total MAPK in cell lysates is shown, determined by densitometry of Western blots. The nm23-H1 transfectant H1–177 exhibited ~2-fold less pMAPK/MAPK, confirming data from Fig. 8A. The P96S Nm23-H1 mutant transfectant, which is kinase-deficient for KSR, exhibited relatively high pMAPK/MAPK levels, suggesting that Nm23-H1 overexpression diminishes MAPK activity by a mechanism requiring its protein kinase activity.

**DISCUSSION**

We present evidence herein for the in vivo association of Nm23-H1, a metastasis suppressor for breast and other cancers, with KSR. The association of KSR and Nm23-H1 was observed in two different cell lines: 293T cells in which one Nm23 was transfectantly and MDA-MB-435 human breast carcinoma cells at endogenous protein expression levels. Auto-phosphorylated rNm23-H1 phosphorylated transiently transfected, immunoprecipitated KSR in vitro on serine 392 and serine 434 in combination with a mutated serine 392. The Nm23-H1 P96S mutant, which failed to suppress breast carcinoma motility in vitro (15), was deficient in KSR phosphorylation. If confirmed and extended by additional in vitro and in vivo studies, the data suggest a new kinase pathway that may regulate signal transduction and cancer metastasis.

KSR was identified in *Drosophila melanogaster* and *Caenorhabditis elegans* systems, in which inactivating mutations suppressed the phenotypic effects of activated Ras (80, 89, 90). These genetic studies placed KSR either upstream of or parallel to Raf in signal transduction; biochemical and molecular studies are still investigating its precise role(s). KSR and Raf display sequence similarities in domain content. Whereas both proteins exhibit C-terminal “kinase” domains, the biological contribution of each has been debated (80, 91–93). KSR differs from Raf in lacking a Ras-binding domain. KSR has been investigated in mammalian model systems primarily in growth factor or ceramide/tumor necrosis factor-α signaling systems in proliferation or differentiation, which tie to the MAPK pathway (82, 93–99). Co-immunoprecipitation studies have shown that KSR can bind Raf-1, MEK1/2, Erk1/2, 14-3-3, Hsp90, rsy (30, 69), and the gamma subunit of heterotrimeric G proteins; binding of some proteins is modulated by serine/threonine phosphorylation (82, 91, 99–103). These and other studies have led to the hypothesis that KSR serves as a scaffolding protein for the MAPK pathway (reviewed in Ref. 81). Scaffolds are thought to contribute to the specificity and stabilization of a pathway as well as enhancing the rate of phosphate transfer (reviewed in Refs. 104 and 105).

Our data indicate that Nm23-H1 phosphorylated KSR in vitro in a complex manner, including the utilization of a Nm23-H1 phosphohistidine intermediate. Nm23 proteins were previously reported to function as histidine protein kinases. In these experiments, Nm23 proteins phosphorylated the histidines of succinic thiokinase, ATP citrate lyase, and other Nm23 proteins (30, 69) and the aspartate of aldolase (28, 29). The latter protein was of particular interest, since it did not auto-phosphorylate on aspartate. In *vivo*, Nm23 was cloned into a bacterial “two-component” system and functioned as a histidine protein kinase (31). This activity has been questioned, since the NDP kinase activity of Nm23 permits ATP generation in the presence of contaminating ADP and NTPs, and the distinction of transphosphorylation from generation of ATP/autophosphorylation is difficult (106). Our data concerning KSR are compelling in several respects. 1) The removal of \( ^{32}P \)ATP from kinase reactions was accomplished via column chromatography and was undetectable in long exposures of thin layer chromatographs. In multiple experiments, the initial input amount of \( ^{32}P \)ATP was also used in the kinase assays in the absence of Nm23 protein, to control for trace amounts of ATP that may have escaped column purification or to provide an estimate of the effect of ATP potentially generated by trace amounts of NDPs from the cell lysates. In all cases, these reactions failed to phosphorylate KSR. 2) The HPLC pattern of the KSR tryptic digests phosphorylated by Nm23-H1 protein versus that phosphorylated by copious amounts of \( ^{32}P \)ATP was distinct, particularly in the enrichment of fraction 44 (serine 434) in the former. These data indicate a specificity in the pattern of Nm23-H1 phosphorylation of KSR inconsistent with the simple provision of nucleotides. 3) Similarly, the phosphorylation of KSR, but not the closely related c-Raf protein, by Nm23-H1 indicated a similar level of specificity. Other concerns in evaluating the potential significance of a histidine protein kinase activity of Nm23 concern its x-ray crystallography structure, which demonstrated a phosphohistidine-containing active site pocket of sufficient size to accommodate a NDP but which appeared too small to accommodate a protein substrate (reviewed in Ref. 107). Two observations may be germane to this question. 1) Studies in *Drosophila*, where the awd null germ line was replaced with various nm23 constructs, indicated that only a minor percentage (~4%) of Nm23 protein (measured by NDP kinase activity) was sufficient to restore normal development and differentiation (108). These data predict that the biological effects of Nm23 may be mediated by a small percentage of the total available protein, possibly with an altered conformation. 2) The first indication of the existence of another Nm23 protein conformations was reported in denaturation-renaturation studies, in which the S120G mutant of Nm23 formed a “molten globule” (109). A recent review suggested that wild type Nm23 proteins may assume this structure (107). A unique complex of Nm23 with glyceraldehyde-3-phosphate dehydrogenase was also reported (49) and stands as evidence of another alternative conformation. Further investigation should ascertain the Nm23 structure in solution, investigate the kinetics of its kinase activity using purified proteins, and demonstrate such a kinase activity in vivo. We have been unable to produce recombinant KSR for such studies (data not shown) to date. Precedent exists for a histidine-to-serine phosphotransfer pathway. Nm23 autophosphorylation on serine may lie down-stream of histidine (34–37). Other proteins that exhibit similar activity include pyruvate dehydrogenase kinase. The Arabidopsis form of this histidine kinase phosphorylated serines on mitochondrial pyruvate dehydrogenase (110).

Our in vitro data suggest that KSR is phosphorylated by Nm23-H1 in a complex manner. We demonstrate that serine 392 mutation, either alone or in combination with serine 297 (the other 14-3-3 binding site), reduced Nm23-H1 phosphorylation. Whereas phosphopeptide mapping identified fraction 44, site-directed mutagenesis of the 14 serines in this region failed to consistently reduce Nm23-H1 phosphorylation, as compared with wild type KSR. The concomitant phosphorylation of KSR...
Phosphorylation of KSR by Nm23-H1

REFERENCES


2 M. Salerno and P. S. Steeg, unpublished results.
Phosphorylation of KSR by Nm23-H1
Nm23-H1 Metastasis Suppressor Phosphorylation of Kinase Suppressor of Ras via a Histidine Protein Kinase Pathway

Melanie T. Hartsough, Deborah K. Morrison, Massimiliano Salerno, Diane Palmieri, Taoufik Ouatas, Michael Mair, Jilma Patrick and Patricia S. Steeg


doi: 10.1074/jbc.M203115200 originally published online June 24, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203115200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 116 references, 50 of which can be accessed free at http://www.jbc.org/content/277/35/32389.full.html#ref-list-1