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

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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 e-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-immuno-precipitated KSR from lysates of transiently transfected 293T cells and at endogenous protein expression levels in MDA-MB-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 KSR 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 members, 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 chemotactants (15–17). Nm23-H1 breast carcinoma transfectants exhibited morphological (ascinus 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), phytochromes (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 chemotactants (15). Biochemical analysis of the recombinant proteins indicated that P96S resulted in a histidine protein kinase-deficient mutant in assays using aucistic 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 transactivation 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 silico 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)3 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-Pyo, and rabbit anti-KSR were all previously described (82, 83). Anti-FLAG antibody was purchased from Sigma; anti-Erk1/2 MAPK and anti-Pyo, and rabbit anti-KSR were all previously described (82, 83). Human MDA-MB-435 breast carcinoma transfec-
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 blocked with 1.5% PVP-40 for 1 h at 37°C. The membrane piece containing the phosphoprotein was excised and separated and eluted by reversed-phase high performance liquid chromatography (HPLC). HPLC fractions containing peaks of radioactivity were subjected to semi-automated 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 phosphopeptide was excised and blocked with 1.5% PVP-40 for 1 h at 37°C. KSR protein was subjected to enzymatic digestion with trypsin. The resulting tryptic peptides 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 the Blast program. To further investigate homology between protein domains, the Pep Tool version 1.0 program was used, with Arabidopsis CTR1 (accession number NP195993), human c-Raf-1 (accession number X03484), and murine KSR (accession number NP038599).

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 extend 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% identity to KSR by

A 293-T cells

| Immunoprecipitation | Western Blot | α-KSR | α-Nm23 |
|---------------------|-------------|-------|--------|
| KSR                 | α-KSR      | α-Nm23|
| KSR N-term          | α-KSR      | α-Nm23|
| KSR C-term          | α-KSR      | α-Nm23|
| MDA-MB-435 cells    |             |       |        |
|                     | Western Blot | α-KSR | α-Nm23 |

B 293-T cells

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 MDA-MB-435; S-22, MDA-MB-435-P96S) of human MDA-MB-435 breast carcinoma cells, previously reported (3, 22), 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.

comparison, the Raf-1 CR2 domain, which is homologous to KSR CA4, exhibited a 22% identity to the CRA1 sequence. These comparisons are similar to those previously reported between Raf-1 and CRA1 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...
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 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 Nm23 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.
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.

Lysates from 293T cells transiently transfected with Pyo-tagged N-terminal KSR were immunoprecipitated with anti-Pyo. Immunoprecipitants were incubated with \(^{32}P\)-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. 5.** Phosphoamino acid and phosphopeptide analysis of phosphorylated KSR. A. lysates from 293T cells 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 \(^{32}P\)-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.
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 auto-phosphorylated 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 (Fig. 5B): fraction 23, which was previously demonstrated to correspond to serine 392, a 14-3-3 binding site (82), and fraction 44, corresponding to amino acids 419–466 of the serine-rich CA4 domain of KSR. By comparison with the profile of [γ-32P]-ATP-incubated KSR, where all associated kinases can participate (Fig. 5C), several conclusions are apparent. First, both Nm23-H1 and all associated kinases phosphorylated the 14-3-3 binding site at serine 392, consistent with the recent identification of C-TAK1 as a kinase for this site (87). Second, the fraction 44 peak was enriched in the Nm23-H1 kinase reaction, indicating specificity in Nm23-H1 kinase activity and indicating that Nm23-H1 was not simply supplying ATP. Fraction 44 corresponded to a shoulder of a more slowly migrating peak in fraction 43 on Fig. 5C. Two-dimensional gel electrophoresis was performed on fraction 44 from the Nm23-H1 reaction and was found to be distinct from that produced by the associated kinases (data not shown), providing further support for the presence of an enriched Nm23-H1-mediated phosphorylation site.

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 auto-phosphorylated 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 of Fig. 5B 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 Ser434 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 ala-nines, 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 [γ-32P]ATP as was used for the Nm23-H1 autophosphorylation, without Nm23-H1. Data are representative of three experiments conducted.

FIG. 7. Synergistic effects of mutations at KSR 14-3-3 binding sites and Serine 434. A, 293T cells were transiently transfected with vector or Pyo-tagged murine KSR constructs: wild type; KSR-AA, containing S392A and S297A (35); KSR-AA also containing S429A; and KSR-AA also containing 434A. Lysates were immunoprecipitated with anti-Pyo; the immunoprecipitants were incubated with 32P-autophosphorylated recombinant, purified Nm23-H1; and the products were electrophoresed. The gel was directly autoradiographed (upper panel), and the positions of KSR and Nm23-H1 are noted by the arrowheads. Autophosphorylated Nm23-H1 indicates equal loading. A second aliquot of the immunoprecipitation was electrophoresed and developed as a Western blot using anti-rat KSR (lower panel). B and C, two experiments encompassing the mutants from Fig. 7A, except that the kinase reactions were transferred to a blot after electrophoresis, autoradiographed, and then developed as a Western blot. Lanes marked with asterisks (B) included the immunoprecipitated KSR proteins noted to the left plus an equivalent amount of [γ-32P]ATP as was used for the Nm23-H1 autophosphorylation, without Nm23-H1. Data are representative of three experiments conducted.

FIG. 8. MAPK levels of control and nm23-H1 transfectants. A, control (C-100) and nm23-H1-transfected (H1–177) lines of the MDA-MB-435 human breast carcinoma cell line were cultured for 3 days on either tissue culture plastic or matrigel. Cells were harvested and lysed, and equivalent protein concentrations were electrophoresed. Western blots were processed using antibodies to pMAPK (pErk1/2) (upper panel) or total MAPK (Erk1/2) (lower panel). B, densitometric analysis of pMAPK/total MAPK from Western blots of lysates of C-100, H1–177, and S-22 (Nm23-H1 P96S mutant) transfectants. Data from replicate experiments were normalized to the C-100 control transfectant as 0.5 pMAPK/MAPK, and the mean and S.E. of three replicate experiments is shown.
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 KSR was transiently transfected 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 Drosofila 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). Raf and KSR 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, p50/cdc37, and the γ 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 [γ-32P]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 [γ-32P]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 [γ-32P]ATP was distinct, particularly in the enrichment of fraction 44 (serine 434) in the former. These data indicate a specificity in the pattern of phosphorylation 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 oud 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 other 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 downstream 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
serine 392 by Nm23-H1 in phosphopeptide mapping studies suggested that this site may influence phosphorylation at other sites. Indeed, elimination of the 14-3-3 sites in the KSR-AA construct reduced Nm23-H1 phosphorylation, and mutation of serine 343 further reduced phosphorylation in this context. Mutation of serine 429 in the KSR AA construct failed to diminish phosphorylation levels as compared with the AA construct, an important control. Both Ser392 and Ser434 have been identified as sites of KSR phosphorylation in vivo (82, 88). S392 is not conserved in Drosophila KSR, whereas Ser434 is conserved in Drosophila and murine KSR (80). The data suggest that Nm23-H1 phosphorylates a proportion of total KSR in concert with an alteration at its serine 392 14-3-3 binding site. In an analogous system, the absence of 14-3-3 binding permitted Raf-1 to be activated but in a different, Ras-independent manner (83). These data suggest the hypothesis that KSR, with altered phosphorylation or binding patterns at its 14-3-3 site, and phosphorylated at Ser434, may exert a distinct function. These data also suggest that more than one kinase is active at KSR Ser392: c-TAK (87) and Nm23-H1. Similar results were observed for the 14-3-3 binding site of Cdc25C, in that c-TAK and the Chk1 kinase phosphorylated this site (87, 111, 112). These data do not eliminate several additional possibilities, including 1) other KSR phosphorylation sites in the 419–465 CA4 region, below the limits of detection on our gels; 2) other sites of KSR phosphorylation in conjunction with the 14-3-3 sites; and 3) combinatorial effects of amino acids in the CA4 domain. We have been unable for technical reasons to confirm Nm23-H1 phosphorylation of recombinant KSR, since production of recombinant KSR also precludes conclusions as to whether the kinase experiments using immunoprecipitated KSR Ser 392: c-TAK (87) and Nm23-H1. Similar results were identified as sites of KSR phosphorylation and metastasis-suppressive activity. The unique interaction may exist kinase activity of Nm23-H1 suggests the hypothesis that this signaling pathways may also exist.

In vitro stream of the multiple receptors involved. The identification of Ish signal transduction, for instance by reducing motility to a known to bind and be modulated by many other proteins. The lack of recombinant KSR also precludes conclusions as to whether the co-immunoprecipitation and kinase interactions are direct or whether other proteins are also involved.

We have long hypothesized that Nm23-H1 served to diminish mass signal transduction, for instance by reducing motility to a variety of chemotacticants (serum, platelet-derived growth factor, autotaxin, fibroblast growth factor) at a point downstream of the multiple receptors involved. The identification of a MAPK pathway protein as an in vitro substrate of a histidine kinase activity of Nm23-H1 suggests the hypothesis that this interaction may exist in vivo and be functionally involved in its signaling and metastasis-suppressive activity. The unique phosphorylation pattern of KSR by Nm23-H1 will be the subject of further investigation to determine its effects on KSR protein binding, subcellular localization, response to various signals, etc. Our data indicate that nm23-H1-transfected H1–177 cells exhibit less pMAPK (pErk1/2) than control transfectants under basal (Fig. 8) and stimulated conditions.2 Furthermore, the kinase-deficient F96S Nm23-H1 transfectant exhibited relatively high pMAPK levels, suggesting that the diminution of MAPK activity by elevated Nm23-H1 expression requires its protein kinase activity. If confirmed and extended, these observations suggest that similar non-“two-component” histidine kinase pathways have survived through evolution and contribute to signal transduction pathways in mammalian cells. Histidine phosphorylation events are difficult to detect. Other mammalian signaling proteins have been reported to exhibit histidine phosphorylation, including the β subunit of heterotrimeric G proteins (113–115), annexin I (116), and the 20 S proteasome (117), and suggest that more involved signaling pathways may also exist.

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2 M. Salerno and P. S. Steeg, unpublished results.
Phosphorylation of KSR by Nm23-H1

54. Engel, M., Seifert, M., Theisinger, B., Seyfert, U., and Welter, C. (1998) J. Biol. Chem. 273, 20658–20665
55. Wep, L., Liderau, R., Leone, A., Liscia, D., Cappa, A., Campbell, G., Barker, E., Doussal, V., Steeg, P., and Callahan, R. (1994) J. Natl. Cancer Inst. 86, 1167–1169
56. Barcellino-Hoff, M. H., Aggerler, J., Ram, T. G., and Bissell, M. J. (1989) Dev. Biol. 133, 55–67
57. Muller, J., Ory, S., Copeland, T., Piwaca-Worms, H., and Morrison, D. (2001) Mol. Cell 8, 983–993
58. Otero, A., Fulton, J., Chaika, O., Mc Dermott, K., Huang, H., Steinke, L., and Lewis, R. (1998) Biochemistry 37, 5130–5137
59. Kornfeld, K., Hum, D., and Horvitz, H. (1995) Cell 83, 903–913
60. Sundaram, M., and Han, M. (1995) Cell 82, 889–901
61. Stewart, S., Sundaram, M., Zhang, Y., Lee, J., Han, M., and Guan, K. (1999) Mol. Cell. Biol. 19, 5523–5534
62. Huser, M., Luccket, J., Chiboech, M., Ziver, I., Wohbi, M., Gisette, S., Sun, X., Brown, M., Rivas, R., and Pritchard, C. (2001) EMBO J. 20, 1840–1851
63. Michaud, N., Therrien, M., Caacace, A., Edsall, S., Speigel, S., Rubin, G., and Morrison, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12792–12796
64. Yan, F., and Polk, B. (2001) Cancer Res. 61, 963–989
65. Muller, J., Caacace, A., Lyons, W., McGill, C., and Morrison, D. (2000) Mol. Cell. Biol. 20, 5529–5534
66. Sugimoto, T., Stewart, S., Han, M., and Guan, K.-L. (1998) EMBO J. 17, 1717–1727
67. Joneson, T., Fulton, J., Velle, D., Chaika, O., Barb-Sagi, D., and Lewis, R. (1998) J. Biol. Chem. 273, 7743–7748
68. King, H., Lezann, J., and Kolesnicky, R. (2000) J. Biol. Chem. 275, 17276–17280
69. Denouel-Galy, A., Dovville, E., Warne, P., Papin, C., Laugier, D., Calotby, G., Downward, J., and Eychene, A. (1997) Curr. Biol. 8, 46–55
70. Yu, W., Fanil, W., Harrowe, G., and Williams, L. (1997) Curr. Biol. 8, 56–64
71. Jacobs, D., Glossip, D., Xing, H., Muslin, A., and Kornfeld, K. (1999) Genes Dev. 13, 163–175
72. Bell, R., Xing, H., Yan, K., Gatum, N., and Muslin, A. (1999) J. Biol. Chem. 274, 7792–7796
73. Xing, H., Kornfeld, K., and Muslin, A. (1997) Curr. Biol. 7, 294–300
74. Babcock, L., and Shapley, A. (2000) Curr. Opin. Cell Biol. 12, 211–216
75. Pawson, T., and Scott, J. (1997) Science 278, 2075–2080
76. Levit, M., Abramczky, B., Stock, J., and Postel, E. (2002) J. Biol. Chem. 277, 5163–5168
77. Lascul, I., Giartosio, A., Ransac, S., and Erent, M. (2000) J. Bioenerg. Biopharmac. 32, 227–236
78. Chi, J., Liu, D., Deng, F., Timmons, L., Hersperger, E., Steeg, P., Veren, M., and Shearn, A. (1996) Dev. Biol. 177, 544–557
79. Lascul, I., Scharer, S., Wang, C., Sager, C., Giartosio, A., Briand, G., Lacombe, M.-L., and Kornfeld, N. (1999) J. Biol. Chem. 272, 15599–15602
80. Theisen, J., Miernyk, J., and Randall, D. (2000) Biochem. J. 349, 195–201
81. Sanchez, Y., Wong, C., Thoma, R., Richman, R., Wu, Z., Piwaca-Worms, H., and Elledge, S. (1997) Science 277, 1497–1501
82. Peng, C., Graves, P., Ogg, S., Thoma, R., III, W., Wu, Z., Stephenson, M., and Piwaca-Worms, H. (1998) Cell Growth Differ. 9, 197–208
83. Nederkoorn, P., Timmerman, H., Timms, D., Wilkinson, A., Kelly, D., Broadley, K., and Davies, R. (1998) J. Mol. Struct. 452, 25–47
84. Hahneuemger, M., Mitterauer, T., Voss, C., Frensimm, M. (1996) Mol. Pharmacol. 49, 73–80
85. Kowlux, A., Seayev, C., Rhodes, C., and Metz, S. (1996) Biochem. J. 313, 97–107
86. Muto, A., hurnickova, Z., Riement, C., Gerke, L., Matthews, H., and Mehta, A. (2000) J. Biol. Chem. 275, 36632–36636
87. Yano, M., Mori, S., and Kudo, H. (1999) J. Biol. Chem. 274, 34375–34382