Site-directed Mutagenesis of nm23-H1

MUTATION OF PROLINE 96 OR SERINE 120 ABROGATES ITS MOTILITY INHIBITORY ACTIVITY UPON TRANSFECION INTO HUMAN BREAST CARCINOMA CELLS*

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We report the first correlation of Nm23 sequence and its tumor metastasis-suppressive capacity using site-directed mutagenesis and an in vitro tumor cell motility assay. MDA-MB-435 human breast carcinoma cells were transfected with a control expression vector (pCMVβ-ampneo), the vector containing the wild type nm23-H1, or the nm23-H1 vector encoding mutations at the following amino acids: serine 44, a phosphorylation site; proline 96, the k-pn mutation in the Drosophila nm23 homolog that causes developmental defects; histidine 118, involved in Nm23’s nucleoside diphosphate kinase activity; and serine 120, a site of mutation in human neuroblastomas and phosphorylation. The wild type nm23-H1 transfectants were 44–98% less motile to serum and 86–99% less motile to autotaxin than control vector transfected. The proline 96 k-pn, serine 120 to glycine, and to a lesser extent serine 120 to alanine mutant nm23-H1-transfected cell lines exhibited motility levels at or above the control transfectants, indicating that these mutations can abrogate the motility-suppressive phenotype of nm23-H1. No effect was observed on cellular proliferation, nor were the serine 44 to alanine nm23-H1 mutant transfectants motile, demonstrating the specificity of the data. The data identify the first structural motifs of nm23-H1 that influence its metastasis suppressive effect and suggest complex biochemical associations or activities in the Nm23 suppressive pathway.

The metastatic spread of tumor cells and complications arising from the treatment of established metastases are major contributors to cancer patient mortality. As a result of differential colony hybridization between related high and low metastatic potential murine K-1735 melanoma cell lines, we identified nm23 on the basis of reduced mRNA (1) and protein (2) levels in highly metastatic tumor cells. Three human nm23 genes have been identified, nm23-H1, nm23-H2, and nm23-DR (2–4). Homologs of nm23 have been cloned in other species as follows: murine nm23-M1 and nm23-M2 (1, 5), rat NDPKb and NDPKa (6, 7), Drosophila awd (8, 9), Myxococcus ndk (10), Escherichia coli ndk (11), and Dictyostelium gip17 and guk7.2 (12, 13).

The correlation of reduced nm23 expression and high tumor metastatic potential has been confirmed in additional metastasis model systems (1, 14–17) and in human tumor cohort studies of breast (18–24), ovarian (25–27), cervical (28, 29), gastric (30), and hepatocellular (31–33) carcinomas and melanomas (35). In other cell types nm23 expression levels have been unrelated to or directly correlated with tumor metastatic potential, involved in cell growth, or altered in other ways, such as by mutation (reviewed in Ref. 36), suggesting multiple or complex biological functions of the gene product(s).

The functional involvement of nm23 gene expression in tumor metastatic potential was determined by its transfection into melanoma and breast carcinoma cell lines, which resulted in a 44–96% reduction in metastatic potential in vivo (37–41). In vitro characterization of control- and nm23-transfected cell lines indicated changes in motility in Boyden chamber assays (42), invasiveness through Matrigel-coated filters (39), immunonosensitivity to lymphokine-activated killer cells (39), and the colonization response in soft agar to tumor growth factor-β (37, 38). Cell proliferation rates were uniformly unchanged (37, 38, 40), providing evidence for specificity in biological function. Studies with homologs of nm23 in other species have provided evidence for a role in development and differentiation. Mutation or reduced expression of Drosophila abnormal wing discs (awd) resulted in abnorlal cell morphology, aberrant differentiation, and cell necrosis postmetamorphosis (8, 9). In the human MDA-MB-435 breast carcinoma model system, cells transfected with nm23-H1 exhibited both morphological and biosynthetic aspects of mammary differentiation upon three-dimensional culture in a basement membrane extract (43).

The biochemical mechanism(s) mediating nm23 modulation of tumor metastatic potential and differentiation are unknown. A plethora of biochemical activities have been confirmed or proposed for Nm23 proteins. Nm23 proteins exhibit a nonspecific nucleoside diphosphate kinase (NDPK) activity (6, 13, 44, 45). NDPK (EC 2.7.4.6) catalyzes the transfer of a terminal phosphate of 5’-triphosphate nucleotides to 5’-diphosphate nucleotides via a high energy NDPK-phosphohistidine intermediate (46–48). The proposed biological functions of the NDPK activity include modulation of nucleotide pools, G-protein function, microtubule polymerization, etc. and have been subject to debate (reviewed in Ref. 36). However, nm23 transfectants exhibiting suppressed metastatic potential did not have in:

1. J. Roys, E. Barrett, R. Rees, S. Cross, and T. Stephenson, submitted for publication.
2. The abbreviations used are: NDPK, nucleoside diphosphate kinase; ATX, autotaxin; awd, abnormal wing discs; awdEVe, awd killer of pru; PAGE, polyacrylamide gel electrophoresis.
Site-directed Mutagenesis of nm23-H1

Site-directed Mutagenesis of nm23-H1

creased total or subcellular NDPK activity (49). Additionally, the killer of pru ne mutation of Drosophila aud (aud-GG) (50) retained NDPK activity, yet caused all of the developmental abnormalities associated with null-aud when co-expressed with a pru ne (pn) mutation (51). Direct evidence indicating the necessity of the conserved histidine residue, but not for elevated NDPK activity, in Drosophila development was provided by transformation studies using nm23-H1, nm23-H2, and both wild type and histidine-mutated aud into the null-aud germ line (52). A second biochemical activity for Nm23 proteins utilizing histidine phosphorylation as a histidine protein kinase was recently reported (53).

Lower energy serine phosphorylation of Nm23, not directly involved in the phosphate transfers of the NDPK or histidine protein kinase activity, have also been described (54–57). For Nm23-H1, two proteolytic fragments containing serines 44 and 120, 122 and 125 exhibited serine autophosphorylation at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 1 cycle; The PCR products were excised from agarose gels and subcloned into the null-aud germ line (52). A second biochemical activity for Nm23 proteins utilizing histidine phosphorylation as a histidine protein kinase was recently reported (53).

Other proposed biochemical activities for Nm23 proteins have been reported. Nm23-H2 has been identified as a transcription factor for the PuF site found near the c-myc and other promoters (58, 59), but recent data suggest that the DNA binding activity is nonspecific, including single- or double-stranded pyrimidines (60). A phospha tase specific for Nm23 proteins, with homology to the Bax proteins involved in apoptosis, was recently described (61), suggesting complex regulatory interactions. Interaction of Nm23 proteins with cAMP have been described in vitro (54, 62).

We report herein site-directed mutagenesis of nm23-H1, assayed for effects on tumor cell motility in vitro. Our data provide the first link of Nm23 biochemical structure with its suppression of one component of the metastatic phenotype.

EXPERIMENTAL PROCEDURES

Plasmids—The nm23-H1 mutant cDNAs were generated as described (63). Briefly, 100 ng of pCMV Bamneo nm23-H1 (pCMV nm23-H1) (GenBank accession number X17620) (59) was used as the template for PCR amplification, using either the CMV5′-GGG GGC ATG CTG GA A GC ACC ATG GCC AAG TGT GAG CCG-3′ or PET3′-5′-GGG GGC ATC CTC CTC TCA TTC ATA GAT CC-3′) oligonucleotides, in conjunction with oligonucleotides listed below, containing alterations in the nm23-H1 nucleotide sequence. PCR conditions were 94°C for 5 min, 1 cycle; 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 15 cycles; 72°C for 15 min, 1 cycle. The PCR products were excised from agarose gels and used as a primer for a second round of pCMV nm23-H1 PCR amplification, with the CMV5′ or PET3′ oligonucleotides as the second primer. The 497-base pair PCR fragment containing the nm23-H1 translated sequence was digested with BamHI, and a 483-base pair fragment was excised from agarose gels and subcloned into the pCMV Bamneo (pCMV) expression vector. The integrity of the mutant cDNAs was verified by double-stranded sequencing. The following oligonucleotides were used to generate site-directed mutations, together with the PET3′ oligonucleotide in the second PCR amplification.

Mutated nucleotides are listed in boldface type, and the altered codon is underlined: serine 44 to alanine (Nm23-H114A), 5′-GGG AAG ATC TTC GCC GCC ATG TAT GAT C-3′; proline 96 to serine (Nm23-H196S), 5′-GGG GGC ATC TGG GAG TGC TGC G-3′; serine 120 to alanine (Nm23-H112A), 5′-GGG AAG ATC TTC GCC GCC ATG TAT GAT C-3′; and serine 120 to glycine (Nm23-H112G), 5′-GGG AAG ATC TTC GCC GCC ATG TAT GGG G-3′.

Transfections—MDA-MB-435 human breast carcinoma cells (64) were transfected with 10 μg of pCMV, pCMV nm23-H1, pCMV nm23-H114A, pCMV nm23-H196S, pCMV nm23-H112A or pCMV nm23-H112G expression vectors in a single side-by-side experiment, using a calcium phosphate transfection system (Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s instructions. Transfectants were selected in Dulbecco’s medium supplemented with 10% fetal bovine serum, 10 μg/ml gentamycin, 100 units/ml penicillin, 100 μg/ml streptomycin (medium), and 1.8 mg/ml G418 (Life Technologies, Inc.). Individual neomycin-resistant clones were isolated and stable high nm23-H1 expressing cell lines identified by northern and Western blots. Two neomycin-resistant pCMVBamneo (empty vector control) transfected clones were selected at random. For the E2 and E6 clones expressing the pCMV nm23-H114A construct, the expressed DNA sequence was verified using reverse transcription-PCR and double-stranded sequencing.

Expression—For Northern blot hybridization experiments, 5 μg of total cellular RNA from each neomycin-resistant transfec tant was re sorbed on a formaldehyde-containing 1.2% agarose gel, transferred to a nylon membrane (Schleicher and Schuell), hybridized with a full-length nm23-H1 cDNA, and washed to a final stringency of 60°C in 0.5× SSC, 0.2% SDS, 1 μm EDTA. After autoradiography, blots were stripped and rehybridized to a glyceraldehyde-3-phosphate dehydrogenase probe as a loading control.

Lysates of each neomycin-resistant transfec tant were prepared for Western blot analysis by washing a confluent monolayer of phosphatebuffered saline and lysis in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 100 μm Mn2+, 2 μm NaV04, and 10 μm NaH2PO4. Lysates were clarified by centrifugation and stored at −70°C. Lysates were resolved on a 14% SDS-PAGE, the proteins were transferred to Immobil P membrane (Millipore Corp., Bedford, MA), and the membranes were incubated with the rabbit anti-Nm23 peptide affinity-purified antibody (2) followed by HRP-rec-Protein G (Zymed, South San Francisco, CA). The Nm23-antibody-protein G complex was detected by autoradiography after incubating the membrane in chemiluminescence reagent (RenatauranTM, Du Pont NEN) as recommended by the manufacturer. Western blots were normalized by two methods: total cellular protein detected by a BCA kit (Pierce) and cell number.

Cell Motility Assays—Cell motility was determined using 48-well Boyden chemotaxis chambers (Neuro Probe, Cabin John, MD) (65). Two chemotactic attractants were utilized, fetal calf serum (Atlanta Biologicals, Norcross, GA) and Autotaxin (ATX) (66, 67). Human teratocarcinoma-derived recombinant ATX was partially purified from vaccinia virus-infected culture supernatants by sequential gel filtration and con canavalin A-agarose chromatography (68). Chemotactic attractants were diluted in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin, 10 μm HEPES, 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B, and serial dilutions were placed in the lower wells of the chamber. An 8-μm pore size polycarbonate filter (Nunc, Naperville, IL) or nitrocellulose filter (Hybond CF, Schleicher and Schuell, Dassel, West Germany) were preincubated at 4°C for 1 h in 0.1 μg/ml acetic acid containing 40 μg/ml type IV collagen (Collaborative Biomedical Research, Bedford, MA), and were wound with the upper and lower wells of the chamber. Cells from each transfectant were passaged 48–72 h prior to use at a density to obtain confluent cultures on the day of the motility assay. Cells were trypsinized, rinsed twice in phosphate-buffered saline, and reseeded in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin, 10 μm HEPES, 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B, at a density of 1.5 × 105 cells/ml, and 7.5 × 105 added to the upper wells of the chamber. The chambers were incubated for 8 h at 37°C in a humidified 5% CO2 incubator, the filters were stained with Diff Quick (Baxter Healthcare Corp., McGaw Park, IL), and the migrating cells were counted by light microscopy in six to nine fields at ×40. Each transfectant was tested at multiple concentrations of chemotacticant, and data for a particular concentration represent the mean of three replicate wells. The number of transfectants analyzed side by side in a single experiment was limited by the availability of Boyden chambers and the strict requirement for 2–3-day-old subconfluent cultures but always contained a vector control transfec tant and a wild type nm23-H1 transfectant for normalization purposes. Motility data are representative of at least three experiments conducted.

In Vitro Autophosphorylation—Nm23 proteins were immunoprecipitated from lysates of each transfectant as described previously (54), using anti-Nm23-H1 monoclonal (MAP301) antibody. Immune complexes of Nm23 proteins, antibody and GammaBind Glue gel (Pharma cia Biotech Inc.) were washed twice in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.05% (v/v) Brij 35 and twice in TMD buffer (20 mM Tris-HCl, pH 8, 5 mM MgCl2, 1 mM dithiotreitol). The beads were resuspended in 51 μl of TMD containing 20 μg/ml (-P)ATP (7000 Ci/mmol), incubated 10 min on ice, and washed
Site-directed Mutagenesis of nm23-H1

MDA-FB-435 human breast carcinoma cells were transfected side by side with the pCMVBamneo vector (control), pCMVBamneo-nm23-H1, or site-directed mutants of the latter construct. Clones resistant to G418 were harvested and tested for overexpression of nm23-H1 mRNA on Northern blots.

| Construct | Neomycin-resistant | Overexpressing nm23-H1 mRNA | Clones investigated for motility |
|-----------|-------------------|-----------------------------|---------------------------------|
| Control   | 14                | 0/14                        | K10, K27                         |
| nm23-H1   | 8                 | 5/8                         | P52, P57                         |
| nm23-H1F196-S | 41          | 7/41                        | S16, S22                         |
| nm23-H1F120-G | 32          | 7/32                        | I108, I205                      |
| nm23-H1F120-A | 8           | 1/8                         | E4                              |
| nm23-H1F144-A | 29          | 11/29                       | J20                             |
| nm23-H1F118-F | 6           | 2/6                         | E2, E6                           |

*Indicates total number of G418 colonies produced in transfection experiment. For other constructs, numerous colonies were produced, and approximately 30 were harvested for Northern analysis.

Twice in TMD (54). To detect phosphohistidine, autophosphorylated Nm23 was dissolved in a basic sample buffer (0.25 M Tris, pH 8.8, 8% SDS, 2.5% β-mercaptoethanol, 35% glycerol, 0.02% bromphenol blue) and resolved on a basic (pH 8.8) 14% SDS-PAGE run at 6 °C, and the gel was exposed while wet to film. Acid-stable autophosphorylation was detected by dissolving samples in 50 mM of 2× SB and boiling for 5 min; alternatively, samples were incubated for 75 s in 0.3M trichloroacetic acid (pH 1.7) at 100 °C, neutralized with 0.5 N NaOH, and acetone-exposed. Samples were resolved on 14% SDS-PAGE, which was fixed in 10% methanol, 10% acetic acid, dried at 80 °C, and exposed to film (54).

NDPK Assays—NDPK activity of cell lysate was assayed by thin layer chromatography (54). Lysate from 4×10^6 cells of each transfectant was incubated with 0.14 μM [γ-32P]ATP (10 μCi, 7000 Ci/mmol) and 14 μM GDP in TMD buffer at room temperature for 30 min. The reaction was stopped by the addition of 1 volume of 50 mM EDTA. One μl of the reaction products was spotted onto 20× 20-cm polyethyleneimine-cellulose thin layer chromatography plates (J.T. Baker Inc., Phillipsburg, NJ) and resolved by capillary action in a saturated tank of 0.75 mM KH2PO4, pH 3.65. The dried TLC plate was exposed to film, and the formation of [γ-32P]GTP was visualized by autoradiography.

Serine alteration of amino acid 96, the killer of prune mutation in the Drosophila Ast homolog of Nm23 (50); (c) nm23-H1F120-C, a serine to glycine alteration at amino acid 120, the most evolutionary conserved serine present on an Nm23-H1 acid-stable phosphorylated peptide (54), and a mutation found in 6 of 28 high grade human neuroblastomas (69); (d) nm23-H1F120-A, a serine to alanine alteration at amino acid 120; (e) nm23-H1H118-F, a histidine to phenylalanine alteration at amino acid 118, the phosphorylated intermediate for the NDPK and possibly the histidine protein kinase reactions (48, 53). Approximately 30 colonies/transfection construct were harvested if available, with the exception of the control (empty vector) construct. Clones were initially screened for the overexpression of a nm23-H1 transcript on Northern blots. From these blots, 2–3 clones/transfection construct were selected as available for further expression, biologic, and biochemical analyses.

A Northern blot showing nm23-H1 mRNA levels in the clones selected from the transfection experiment is shown in Fig. 1A. Two control-transfected clones, K10 and K27, were randomly selected and exhibited uniformly low nm23-H1 mRNA expression. Three wild type nm23-H1-transfected clones (P52, P57, and P62) were selected with varying degrees of mRNA overexpression. At least two clones overexpressing nm23-H1 mRNA on the Northern blots were identified for each mutant construct, with the exception of nm23-H1F118-F, for which only one expressing clone was identifiable. Additionally, levels of nm23-H1 mRNA just above those of the control transfecants were observed in one of the only two expressing nm23-H1F118-F clones. Rehybridization of the Northern blot to a glyceraldehyde-3-phosphate dehydrogenase probe demonstrated equivalent loading of RNA (Fig. 1B). Further biochemical and biologic analyses of the transfecants proceeded in parallel after Northern blot hybridization experiments.

**Fig. 1.** Northern blot of nm23-H1 mRNA levels of control-, wild type nm23-H1-, and site-directed nm23-H1-mutant-transfected clones. MDA-FB-435 human breast carcinoma cells were transfected with each construct, and clones were harvested as indicated in Table I. 5 μg of total RNA was electrophoresed in a 1.2% (w/v) formaldehyde-containing agarose gel, and a Northern blot was processed and hybridized to the cDNA insert of_nm23-H1_(A). The blot was subsequently stripped and rehybridized to a control glyceraldehyde-3-phosphate dehydrogenase probe (B).
level of the control (K) transfectants, as determined by densitometry of chemiluminescence exposures. At amino acid 96, two of the Nm23-H1S196-F (S) clones expressed significantly greater Nm23-H1 than the control (K) clones. The S29 clone, which exhibited Nm23-H1 RNA overexpression, expressed little additional Nm23-H1 protein when blots were normalized by cell number (Fig. 2) or by micrograms of protein in cell lysates (data not shown). At amino acid 120, two Nm23-H1S120-G (I) clones as well as the single Nm23-H1S120-A (R) clone expressed 1.6–3.1-fold greater Nm23-H1 than the control (K) transfectants. Interpretation of the J58 transfectant data at the same amino acid position is difficult. Whereas this clone overexpressed nm23-H1 mRNA, it exhibited little additional Nm23-H1 protein in Western blots normalized by cell number (Fig. 2). When normalized by micrograms of protein in the cell lysate, the J58 clone exhibited 2.1-fold Nm23-H1 overexpression relative to the control (K) transfectants (data not shown). The data suggest that expression of the transfected cDNA was obtained in this clone but was accompanied by additional changes in cell size, protein synthetic rates, etc. For the histidine 118 position, the E6 clone, which expressed little additional S29 17.50
6
K27 17.84
6
Nm23-H1K27 11.17
6
J20 11.17
6
E2 44.42
7.6 114.3
6
I108 42.17
6.6 88.28
6
K10 23.36
6.6 3.2
6
K27 17.84
6
Expt. 481 Expt. 482 Expt. 483
Transfection construct
Mean cells migrating to 0.5% serum ± S.E. a
Expt. 481 Expt. 482 Expt. 483
Control
K10 23.36 ± 0.3 39.78 ± 3.9 20.34 ± 2.8
K27 17.84 ± 0.4
Nm23-H1
P52 11.42 ± 5.5
P57 1.00 ± 0.7 1.54 ± 0.6 1.54 ± 0.4
P62 0.42 ± 0.3
Nm23-H1K96-F
S16 10.50 ± 1.2 162.0 ± 1.2
S22 41.84 ± 6.5 23.33 ± 3.6
S29 17.50 ± 3.7 57.56 ± 4.2
Nm23-H1S120-G
I108 42.17 ± 6.6 114.3 ± 8.5
I205 43.42 ± 3.2 62.75 ± 3.2
Nm23-H1S120-A
R4 1.08 ± 0.8 2.00 ± 1.7
Nm23-H1S144-A
J20 11.17 ± 0.6 0.6
J58 1.00 ± 0.6 15.11 ± 1.5
Nm23-H1S118-F
E2 44.42 ± 7.6 88.28 ± 3.2
E6 0
a Cells were tested in Boyden chambers for in vitro motility to 0.25,
0.50, 0.75, and 1.0% (v/v) fetal calf serum, in 8-h assays. The mean ± S.E. cells migrating through a type IV collagen-coated 8-µm filter toward serum, determined from counting six to nine × 40 microscopic fields per Boyden chamber, three chambers per data point, were calculated.

In Vitro Motility of Site-directed Mutants

**Fetal Calf Serum**—Motility was determined in Boyden chamber assays using fetal calf serum or partially purified ATX as a chemoattractant. Serum was selected for two reasons: (a) clones of murine K-1735 TK melanoma transfected with murine nm23-1 or human MDA-MB-435 breast carcinoma transfected with human nm23-H1 exhibited reduced motility responses to serum (42), and (b) serum contains a variety of motility stimulating and inhibiting factors, which may better reflect the milieu to which tumor cells are exposed in vivo. Each cell line was assayed in triplicate with 0, 0.25, 0.5, and 1.0% (v/v) serum, for which peak responses were typically obtained at 0.5%. Most experiments tested a subset of the total number of transfected cell lines, due to strict culture requirements for reproducible assays, the number of Boyden chambers available, and potential deleterious effects of long times involved in handling and counting multiple cell lines. All experiments included at least one control transfectant (K) and one wild type nm23-H1 transfectant (P). Table II lists data from three successive experiments, showing motility of cells from the various clones to 0.5% serum. For experiment 481, both control-transfected (K) clones migrated toward serum, with 17–23 cells/high power field. Expression of wild type nm23-H1 by the P52, P57, and P62 clones resulted in a 44–98% reduction in migration, calculated on the basis of the mean of the K clones. The migration of P52 exhibited variability between experiments, whereas that of the P57 and P62 clones was uniform. Reduced motility was exhibited by the P57 clone in experiments 482 and 483, expression of the awd killer of prune homolog of nm23-H1, a proline to serine alteration at amino acid 96, resulted in generally enhanced motility responses. The S16 and S22 clones exhibited motility levels varying between the upper limit of the

![Western blot of Nm23-H1 protein levels of control-, wild type nm23-H1-, and site-directed nm23-H1 mutant-transfected clones. MDA-MB-435 human breast carcinoma cells were transfected with each construct, and clones were harvested as indicated in Table I. Lysate from 3 × 10⁶ cells of each transfectant was electrophoresed in a 14% SDS-PAGE, which was probed as a Western blot using affinity-purified anti-nm23 peptide 11 antibody.](image)
Site-directed Mutagenesis of nm23-H1

wild type clones to levels above the vector (K) controls in experiment 481. Motility levels at or above the vector (K) controls were demonstrated for these clones in seven of seven additional experiments conducted (Table I and data not shown). The S29 clone, for which overexpression of an Nm23-H1 protein could not be confirmed in all assays conducted, was motile.

Two site-directed mutants at serine 120 were tested. Both the I108 and I205 clones of the Nm23-H1I205S transfection were motile above levels of the vector (K) controls in seven of seven experiments conducted (Table I and data not shown). In apparent contrast, mutation of this residue to alanine in the single Nm23-H1I205A R4 transfectant showed inhibited motility in three of four assays conducted (Table I and data not shown). Motility experiments using the same concentrations of serum in 18-h assays, as compared with the 8-h assays shown in Table I, indicated motility in the R4 clone comparable with that of the control (K) clones in six of seven experiments conducted (data not shown). This is the only instance in which the motility response of a clone varied significantly by the length of the in vitro assay. Thus, this single clone in which the serine 120 was substituted by an alanine exhibited some increase in motility in vitro, although the magnitude was not as great as replacement of the serine by a glycine.

The effect of serine 44 mutation was studied. For the nm23-H1P96S-A mutants, both the J20 and J58 clones exhibited motility within the general range of the P clones for experiment 481. Higher levels of motility were observed in one of the transfected cell lines in three of five experiments conducted (Table II and data not shown).

The single expressing nm23-H1I118F transfectant, E6, exhibited suppressed motility in experiment 481 (Table I), which was indicative of four experiments conducted. The E2 clone, for which protein overexpression could not be detected, was motile in five of six assays conducted (Table II and data not shown). In summary, for in vitro motility to serum, the killer of prune and serine 120 to glycine mutations yields a demonstrable reversal of the wild type nm23-H1 inhibition.

Partially Purified ATX—Fig. 3 shows representative dose response motility data to partially purified ATX for a subset of the transfected cell lines, determined side by side in a single experiment. In agreement with serum motility data, both vector control (K) clones exhibited dose-dependent motility, with a plateau at higher ATX concentrations (Fig. 3A). All three wild type nm23-H1 transfectants exhibited decreased motility to ATX at all concentrations tested (Fig. 3B).

Mutation of proline 96 to serine, the awwd killer of prune homolog, resulted in pronounced motility in the S22 clone (Fig. 3C). The S29 clone, for which Nm23-H1 overexpression could not be confirmed, exhibited comparable motility. In experiments not shown, the S16 clone exhibited motility to ATX at or above that of the vector (K) controls in four of four experiments conducted. Thus, two transfectants expressing the nm23-H1 killer of prune mutation, exhibited motile behavior to either serum or ATX.

Representative motility data for serine 120 mutants are shown in Fig. 3D. The nm23-H1I118F transfectant I205 exhibited motility above the vector (K) controls in five of five experiments conducted. In data not shown, the I108 transfectant was also motile above the K clones in four of four experiments conducted. The single nm23-H1I118F R4 transfectant was motile in this assay at levels comparable with the vector (K) controls (Fig. 3D) but less than that of the I clones in five of five experiments conducted (data not shown). These data confirm an abrogation of nm23-H1 inhibition of motility by serine 120 mutation, a loss more pronounced with a serine-glycine mutation found in high grade human neuroblastomas than with a more conservative serine to alanine substitution.

The motility of the J20 transfectant, which expressed the nm23-H1I118F construct altering serine 44 to alanine, was intermediate between that of the vector control (K) and wild type (P) transfectants (Fig. 3E). Of five experiments conducted, the J20 transfectant exhibited equivalent or greater motility than the control transfectants in three experiments. The J58 clone exhibited the same variable pattern of motility. The data suggest a potential weak modulation of nm23-H1 inhibitory activity using a defined chemoattractant.

The single expressing nm23-H1I118F E6 transfectant, exhibiting an alteration of histidine 118 to phenylalanine, remained nonmotile to ATX in four of four experiments conducted (Fig. 3F and experiments not shown). In contrast, the E2 clone, for which Nm23-H1 overexpression could not be detected, was motile in four of four experiments conducted (data not shown). These data compare closely for serum motility data and, within the limitations created by the availability of only a single expressing clone, indicate the wild type nm23-H1 phenotype of a histidine mutant in this assay.

In Vitro Proliferation—Transfection of wild type nm23 cDNA has not exerted a significant effect on tumor cell proliferation in multiple studies (37, 38, 40). To determine whether specific site-directed mutations of nm23-H1 altered cellular functions previously unrelated to nm23, the proliferative capacity of each transfectant in vitro was determined. Fig. 4 shows the in vitro proliferative capacity of each transfectant over 6 days of culture by use of a tetrazolium dye spectrophotometric assay. No consistent effects were discerned with regard to MDA-MB-435 cell proliferation and transfection with nm23-H1 molecules altered at specific residues.

In Vitro Biochemical Correlates—Lysates from each of the transfectants were tested for several in vitro biochemical activities described for Nm23-H1, to identify any correlates of its motility suppressive/nonsuppressive behavior upon transfection. Endogenous and transfected Nm23 proteins were immunoprecipitated from lysates of each transfectant, autophosphorylated with [γ-32P]ATP, and resolved under different electrophoretic conditions to detect various autophosphorylated amino acids. Histidine autophosphorylation was detected by a modification of Wagner and Vu (55); critical features of the procedure to preserve the labile phosphohistidine include electrophoresis of the sample without prior boiling in a basic gel and autoradiography of the gel without acid fixation or drying (see “Experimental Procedures”).Histidine autophosphorylation levels of Nm23 proteins immunoprecipitated from each transfectant are shown in Fig. 5A. The nm23-H1I118F (E2) clone, which overexpressed a Nm23-H1 protein lacking histidine 118, exhibited levels of histidine autophosphorylation comparable with those of the endogenous Nm23 proteins in the control (K) cell lines. Of the two site-directed mutations exhibiting the most potent reversal of wild type nm23-H1 inhibition of motility, the nm23-H1I118F (S) cell lines exhibited relatively high levels of histidine autophosphorylation, while the nm23-H1I118F (S) (I) cell lines exhibited levels of histidine autophosphorylation varying between those of the K and P cell lines. Levels of endogenous Nm23-H2 histidine autophosphorylation varied significantly.

Fig. 5B shows a side by side analysis of nonhistidine Nm23 autophosphorylation, in which immunoprecipitated Nm23 proteins from the same lysates were incubated with [γ-32P]ATP and subjected to electrophoresis in 14% SDS-PAGE, and the gel acid was fixed, dried, and subjected to autoradiography. Overall levels of autophosphorylation were much weaker than those presented in Fig. 5A, as indicated by the longer autoradi-
graphic exposure times. Each of the wild type (P) clones exhibited greater autophosphorylation than the control (K) clones. None of the individual serine mutant constructs (I, R, or J cell lines) exhibited autophosphorylation levels as low as those of the control (K) transfectants, suggesting that multiple serine autophosphorylation events occur. No uniform trend was apparent in the autophosphorylation levels of the most highly motile \( \text{nm23-H1}\) \(\text{P96-S} \) and \( \text{nm23-H1}\) \(\text{S120-G} \) clones. Similar results were obtained when data were normalized according to micrograms of protein in the starting lysate (data not shown) or when autophosphorylated Nm23-H1 protein was boiled in acid and neutralized prior to electrophoresis (see "Experimental

**Fig. 3.** Motility of control-, wild type \( \text{nm23-H1}\)-, and site-directed \( \text{nm23-H1} \) mutant-transfected cell lines to partially purified ATX in vitro. MDA-MB-435 human breast carcinoma cells were transfected with each construct, and clones were harvested as indicated in Table I. 7.5 \times 10^6 cells from 2-day semiconfluent cultures of the cell lines were incubated in the upper wells of Boyden chambers, with ATX as the motility stimulant in the bottom well. After 8 h, cells traversing a collagen-coated 8-\(\mu\)m porous filter toward the ATX were stained and counted microscopically. Data represent the mean \pm S.E. of triplicate Boyden chambers at each ATX dilution and were assayed side by side in a single experiment. A, control vector-expressing cell lines (K); B, wild type \( \text{nm23-H1}\)-expressing cell lines (P); C, \( \text{nm23-H1}\) \(\text{P96-S} \)-transfected cell lines, the killer of prune mutation homolog (S); D, serine 120 mutant \( \text{nm23-H1}\) \(\text{S120-G} \) (1205) and \( \text{nm23-H1}\) \(\text{S120-A} \) (R4) cell lines; E, serine 44 mutant \( \text{nm23-H1}\) \(\text{S44-A} \) (J) cell lines; F, histidine mutant \( \text{nm23-H1}\) \(\text{H118-F} \) cell line (E6).
Site-directed Mutagenesis of nm23-H1

Procedures). Nm23-H2 autophosphorylation levels varied independently of those of Nm23-H1. The data fail to establish any autophosphorylation event as a correlate of nm23-H1 motility-suppressive activity among all of the site-directed mutants.

The best established enzymatic activity for Nm23 proteins is its NDPK activity, using a Nm23-phosphohistidine intermediate. Fig. 6 shows an autoradiograph of TLC, measuring the NDPK activity of lysates from each of the transfected cell lines. Formation of $[^{32}P]GTP$ from $[^{32}P]ATP$ and cold GDP, was measured. All lysates tested exhibited detectable NDPK activity. No trend in NDPK activity was observed that correlated with nm23-H1 modulation of tumor cell motility.

**DISCUSSION**

Whereas five studies have demonstrated a suppressive effect of nm23 cDNA transfection on the *in vivo* metastatic aggressiveness of melanoma and breast carcinoma cells (37–41), the mechanism of this effect is unknown. Based on its inhibition of stimulated responses in transfection experiments, such as motility and colonization, we have postulated that Nm23 may participate in a signal transduction pathway to limit aberrant cellular responsiveness (36). An alternate hypothesis, taking into account the instability of *Drosophila* developmental abnormalities caused by *auw* mutations as well as the instability inherent in the metastatic process, proposes that Nm23 functions to stabilize phenotypic expression by an unknown mechanism. We have used site-directed mutagenesis of nm23-H1 and an *in vitro* assay of one component of the tumor metastatic process, cell motility, to provide the first direct association of nm23 structure and function in metastasis. We report that alterations to proline 96 and to serine 120 abrogated the motility-suppressive effect of wild type nm23-H1.

The biochemical function of Nm23 serine phosphorylation is unknown. Thermodynamically, phosphoserine cannot directly participate in the NDPK or histidine protein kinase activities of Nm23 because of its low bond energy. Nm23-phosphoserine could participate in a serine protein kinase activity or could indirectly influence other biochemical activities of Nm23 by modifying its three-dimensional structure. Four studies have reported serine phosphorylation of Nm23 proteins (54–57) at relatively low levels (57, 70). For autophosphorylated rNm23-H1, phosphorylation was observed at serine 44 and on a fragment containing serines 120, 122, and 125, of which only serine 120 was conserved in all species reported (54). Further evidence for the potential importance of serine 120 was its reported mutation in 6 of 28 stage IV human neuroblastoma tumors (69). We hypothesized that Nm23 serine phosphorylation may be involved in its suppressive mechanism on the basis of the direct correlation of Nm23 expression levels, metastasis-suppressive activity, and *in vivo* serine phosphorylation levels among control- and nm23-1-transfected melanoma cell lines (54). In the present study the wild type nm23-H1 transfecants again exhibited greater levels of serine autophosphorylation that control transfecants. Mutation of either serine 44 or 120 failed to abrogate the increased serine autophosphorylation levels, indicating that multiple residues are likely involved.

The motility data presented herein indicate the importance of serine 120 to the Nm23-H1 suppressive mechanism, but suggest the potential importance of both phosphorylation and active pocket structural modifications in this effect. Two transfecants expressing the serine 120 to glycine alterations, I108 and I205, exhibited motility at or above the control (K) transfecants. Serine 120 is positioned in an active site "pocket," two amino acids carboxyl to the histidine 118 that mediates the NDPK and possibly histidine protein kinase activities of Nm23. Studies in Dictyostelium indicate that serine 120 hydrogen bonds to a Glu in the pocket but does not significantly influence NDPK activity, the latter based on analysis of recombinant proteins mutated to Ala, Cys, or Gly amino acids at the corresponding position (71). In our studies a single R4 transfecant expressing a more conservative serine 120 to alanine substitution was analyzed. This transfecant retains a larger "R" group than the glycine substitution and permits hydrogen bonding to Glu, and thus it may be more specific for phosphorylation differences. This line remained nonmotile to serum in the 8-h assay but was motile at the level of the control (K) cell lines in a longer 17-h motility assay. Motility levels of the R4 cell line to ATX were at the level of control (K) cell lines. We conclude that phosphorylation at serine 120 may contribute to inhibition of motility. Additionally, it can be hypothesized that serine 120 influences the structure of the pocket, potentially influencing other biochemical activities.

In contrast, alterations to serine 44 of Nm23-H1 exerted little effect on its inhibitory activity. Serine 44 was a site of phosphorylation observed on rNm23-H1 (54). Since it is localized to the surface of the Nm23 hexamer (62), the mechanism

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5 J. Janin and M. Veron, personal communication.
of serine phosphorylation is unknown, but it could include transphosphorylation by Nm23 or another kinase. The J20 and J58 lines exhibited somewhat heterogeneous motility patterns to ATX or serum, ranging from levels as low as the most inhibited wild type (P) cell lines to the lowest of the vector control (K) cell lines. In no single experiment did the motility of both serine 44 mutant cell lines equal that of the vector (K) controls. While we cannot rule out a relatively minor functional effect of serine 44 on nm23-H1 suppression of tumor cell motility, the generation of convincing data to support this hypothesis would require a biological assay with improved sensitivity and accuracy. The fact that tumor cell motility by these mutants remained at or near wild type levels also provides support for the specificity of the serine 120 and proline 96 mutation data.

Data from the proline 96 to serine transfectants suggest the importance of protein-protein interaction in the suppressive function of Nm23. Mutation of proline 96 to serine resulted in pronounced motility to either serum or ATX in two stable overexpressing lines, S16 and S22. The S29 cell line failed to overexpress Nm23-H1 in all assays tested but was also motile. This mutation is homologous to the killer of prune mutation in the Drosophila Awd homolog of Nm23 (50). A conditional dominant lethal mutation, awd k-pn, alone has no obvious phenotype, but it exhibits all of the characteristic developmental abnormalities of null-awd mutations when expressed either heterozygously or homozygously in combination with the prune (pn) eye color mutation. Purified Drosophila Awd k-pn and the corresponding Dictyostelium 100P-S recombinant protein have been characterized (51, 72) and retain NDPK catalytic activity. X-ray crystallographic studies of Nm23 (34, 62, 73–76) suggest additional hypotheses concerning the possible function of the killer of prune mutation. Nm23 monomers exhibit a major structural motif containing four antiparallel b-sheets with two connecting a-helices (34). The proline involved in the k-pn mutation is localized to a killer of prune loop. This loop is distinct from the antiparallel b-sheet/a-helix motif and nonconserved among other proteins exhibiting this motif, suggestive of a novel function. A role for the killer of prune loop in Nm23 oligomerization has been proposed. Of the Nm23 family proteins studied by x-ray crystallography, the human, Drosophila, and Dictyostelium forms are hexamers, whereas the Myxococcus protein is a tetramer. In the hexamer, the killer of prune loop is buried, forming extensive contacts between two trimers and contributing to nucleotide binding (34). The proline-serine mutation has been proposed to increase the flexibility of the loop (51). However, in Myxococcus, the killer of prune loop is at the outer extremity of the tetramer and appears to be important for oligomerization of the enzyme (76). It is not known what proportion of Nm23 proteins are monomeric, dimeric, tetrameric, or hexameric within cells, or if they specifically bind other proteins. Taken together, the data suggest the hypothesis that (a) the killer of prune loop may serve a novel function; (b) it may be differently exposed in different forms of Nm23; and (c) the k-pn mutation may alter the flexibility of this loop, possibly affecting Nm23 interaction homotypically or with other cellular proteins.

Histidine 118, the phosphorylated intermediate in the NDPK activity of Nm23, likely serves a similar function in its recently described histidine protein kinase activity (53) and may influence serine phosphorylation through a transphosphorylation mechanism. Whereas the single histidine 118 to phenylalanine transfectant E6 exhibited a motility suppressed phenotype to fetal calf serum or ATX, we suggest a more cautious interpretation of the data. In three transfection experiments
using histidine-mutated murine nm23-1 or human nm23-H1, we have obtained a total of one stable expressing clone (Ref. 54). Inability to generate stable histidine-mutated nm23 transfectants has also been independently reported (40). It is therefore possible that additional events have occurred in this single cell line that render it viable and nonmotile, and conclusions concerning the relevance of the Nm23-H1 phosphohistidine on this one cell line may be premature. Of the biochemical activities mediated by phosphohistidine, our data indicate no difference in NDPK activity between lyses of various transfectants and continue to provide little support for the direct participation of the NDPK activity of Nm23 in motility suppression. For the histidine kinase activity of Nm23 (53), the data do not rule out its participation in the motility-suppressive mechanism. Nm23-H1 histidine autophosphorylation (53), the data do not rule out its participation in the motility-suppressive mechanism.

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