Site-directed Mutation of Nm23-H1

MUTATIONS LACKING MOTILITY SUPPRESSIVE CAPACITY UPON TRANSFECTION ARE DEFICIENT IN HISTIDINE-DEPENDENT PROTEIN PHOSPHOTRANSFERASE PATHWAYS IN VITRO

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We previously compared the structure and motility suppressive capacity of nm23-H1 by transfection of wild type and site-directed mutant forms into breast carcinoma cells. Wild type nm23-H1 and an nm23-H1S44A (serine 44 to alanine) mutant suppressed motility, whereas the nm23-H1S120G, nm23-H1S120A, and to a lesser extent, nm23-H1S120A mutant forms failed to do so. In the present study wild type and mutant recombinant Nm23-H1 proteins have been produced, purified, and assayed for phosphorylation and phosphotransfer activities. We report the first association of Nm23-H1 mutations lacking motility suppressive capacity with decreased in vitro activity in histidine-dependent protein phosphotransferase assays. Nm23-H1S120G, a Drosophila developmental mutation homolog, exhibited normal autophosphorylation and nucleoside-di-phosphate kinase (NDPK) characteristics but deficient phosphotransfer activity in three histidine protein kinase assays, using succinic thiokinase, Nm23-H2, and GST-Nm23-H1 as substrates. Nm23-H1S120G, found in advanced human neuroblastomas, exhibited deficient activity in several histidine-dependent protein phosphotransfer reactions, including histidine autophosphorylation, downstream phosphorylation on serines, and slightly decreased histidine protein kinase activity; significant NDPK activity was observed. The Nm23-H1S120A mutant was deficient in only histidine-dependent serine autophosphorylation. Nm23-H1 and Nm23-H1S44A exhibited normal activity in all assays conducted. Based on this correlation, we hypothesize that a histidine-dependent protein phosphotransfer activity of Nm23-H1 may be responsible for its biological suppressive effects.

The nm23 family of genes has been characterized on the basis of its reduced expression in certain highly metastatic cell lines (1) and tumors (reviewed in Ref. 2). In human tissues, three members have been identified, nm23-H1 (3), nm23-H2 (4), and nm23-DR (5). Transfection of nm23 cDNAs into murine K-1735 TK (6, 7), B16F10 (8), or B16FET7 (9) melanoma cells, rat MTLn3 mammary adenocarcinoma cells (10), or human MDA-MB-435 breast carcinoma cells (7, 11, 12) resulted in a significant reduction in tumor metastatic potential in vivo, providing evidence that nm23 can have a metastasis suppressor function.

Nm23 proteins also participate in the development and differentiation process. The most thoroughly studied model system is Drosophila, in which reduced expression of the abnormal wing discs (awd) gene, which encodes a protein approximately 77% identical to Nm23, causes widespread aberrant differentiation post-metamorphosis leading to lethality (3, 13, 14). Additionally, mutation of proline 96, the killer-of-prune mutation (awd k-prn), causes developmental abnormalities when expressed in the context of prune (pn) eye color mutations (reviewed in Ref. 15). Associations of nm23 and development have been reported in higher organisms. In murine embryogenesis, elevated Nm23 expression has been correlated with the functional differentiation of most epithelial tissues (16). Human MDA-MB 435 breast carcinoma cells transfected with nm23-H1 showed morphological and biosynthetic evidence of differentiation in vitro (12). The phenotypic similarities of the tumor metastatic and developmental processes have been long recognized, leading to the hypothesis that they may involve similar regulatory factors, such as nm23.

A long list of known and postulated biochemical activities and protein:protein associations have been reported for Nm23, which could serve as candidate biochemical mechanisms for its metastasis suppressive effects. Nm23 proteins exhibit nucleotide-di-phosphate kinase (NDPK) activity (EC 2.7.4.6), a non-specific phosphotransferase that mediates the transfer of a terminal phosphate from a NTP to a NDP via a Nm23-phosphate intermediate (17–23). Nm23 proteins have been reported to associate with microtubules (17, 24–26) and small or heterotrimeric G proteins (27–33), and its NDPK activity could serve as a candidate biochemical mechanism for its metastasis suppressive effects. Nm23 proteins exhibit nucleotide-di-phosphate kinase (NDPK) activity (EC 2.7.4.6), a non-specific phosphotransferase that mediates the transfer of a terminal phosphate from a NTP to a NDP via a Nm23-phosphate intermediate (17–23). Nm23 proteins have been reported to associate with microtubules (17, 24–26) and small or heterotrimeric G proteins (27–33), and its NDPK activity was postulated to directly transphosphorylate or indirectly modulate nucleotide pool sizes to affect their activity; however, each of these hypotheses remains controversial. In Drosophila, the developmental effects of awd can be dissociated from its NDPK activity: (a) Awd k-prn protein retained significant NDPK activity (34); (b) transformation of the null germ line with human nm23 cDNAs restored NDPK activity but not the fully differentiated phenotype (35). In transfection model systems, neither total nor subcellular NDPK activities correlated with Nm23 expression and suppression of metastasis (36).

Nm23 proteins have been recently reported to exhibit histi-
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dine protein kinase activity (37), presumably using the same histidine 118 phosphorylated intermediate involved in the NDKP activity. Histidine protein kinases form the “two component” signal transduction system in bacteria (reviewed in Ref. 38) and have recently been described in eukaryotic systems, including human platelet activation (39, 40).

A lower energy serine autophosphorylation of Nm23 was described in vitro and in vivo (41–44); levels of Nm23-phosphoserine were directly correlated with Nm23 expression and metastasis suppression in model systems (41). Recombinant Nm23-H1 exhibited autophosphorylation on two proteolytic fragments containing serine 44 and serines 120, 122, and 125, of which position 120 remains the most conserved evolutionarily (41).

Nm23-H2 has been identified as a transcription factor for the PuF site/CT element found in the c-myc promoter (45–48). Other groups have found nonspecific binding of Nm23-H2 to single-stranded polypyrimidines and RNA (49), and we have found no evidence in vivo for a direct transcriptional activity.

We recently reported a different strategy to investigate the functional biochemical activity of Nm23 in metastasis suppression using site-directed mutagenesis (51). Human MDA-MB-435 breast carcinoma cells were transfected with wild type or mutant forms of nm23-H1, and their phenotype was examined using an in vitro assay for one component of the metastatic process, motility in Boyden chamber assays. Tumor cell motility to either fetal calf serum or partially purified autotaxin (ATX) was reduced by transfection with wild type or serine 44 mutated forms of nm23-H1 but not by transfected nm23-H1 cDNAs mutated at either serine 120 or at the homolog of the killer-of-prune position (proline 96).

The present report describes in vitro biochemical activities of recombinant Nm23-H1 wild type and site-directed mutated proteins with and without motility suppressive capacity. We report a stunning correlation in that those mutant proteins incapable of motility suppression exhibited reduced activity in biochemical assays of histidine-dependent protein phosphotransferases in vitro. The data permit the development of the hypothesis that a histidine-dependent protein phosphotransferase activity of Nm23-H1 may mediate its biological motility suppressive activity.

EXPERIMENTAL PROCEDURES

Expression of Site-directed Mutants—Site-directed mutagenesis of nm23-H1 was carried out by PCR as described previously (51), using the oligonucleotide pET5′ (5′-GGGGGATCCATATGGCCAACTGTGAG-3′) instead of CMV5. The PCR products were double-digested with NdeI and BamHI, gel-purified, and subcloned into the pET3c expression vector (52). The resulting plasmids were analyzed by double-stranded sequencing and transformed into Escherichia coli strain BL21(DE3). Transformed BL21(DE3) bacteria were grown overnight in LB broth containing 100 µg/ml AMP at 37°C, diluted 1/20 in the same medium, and grown under the same conditions until the culture reached an A600 of 1.0. Expression of the nm23 cDNAs was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. After a 2-h induction, bacteria were collected by centrifugation, washed twice with 1/5 vol. of TBS (20 mM Tris-HCl, pH 8.0, 150 mM NaCl), resuspended in 1/50 vol. of the same buffer, and disrupted by sonication. The bacterial lysates were centrifuged at 35,000 × g for 20 min at 4°C, and the soluble extracts were stored at −20°C. Alternatively, in the preparation of bacterial lysates for HPLC purification, the above buffer was replaced with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl (rNm23-H1) or 20 mM Tris-HCl, pH 8.3 (rNm23-H2).

HPLC Purification of Recombinant Nm23 Proteins—Lysates from bacteria expressing wild type or site-directed mutant rNm23-H1 proteins were loaded in a DEAE-Sepharose CL-6B column (Pharmacia Biotech Inc.), equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl (0.5-ml bed volume/ml lysate). The column was washed with 20 column volumes of the same buffer and finally eluted with 20 mM Tris-HCl, pH 8.0, 200 mM NaCl. Nm23-H1 was eluted with 250 mM NaCl instead of 200 mM. The fractions from the eluate containing significant amounts of protein were pooled and dialyzed against 50 mM sodium acetate buffer, pH 5.0, and 1 m sodium dithiothreitol (Pharmacia), equilibrated with the same buffer. After washing the column with 10 volumes of the starting buffer, proteins were eluted with a linear gradient (20 ml) of 0–1 M NaCl in the same buffer at a flow rate of 1 ml/min. 0.5-ml fractions were collected, analyzed by SDS-PAGE, and those containing rNm23 proteins were pooled, concentrated, and applied to a Superdex 75 HR 10/30 size exclusion column (Pharmacia), equilibrated, and eluted with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected, and those containing pure recombinant proteins, as judged by silver-stained SDS-PAGE, were stored at 4°C or at −20°C for long-term storage. HPLC purification of rNm23-H2 was carried out as described previously (53).

Both cation exchange and size exclusion chromatography were performed using a Dionex DX 500 chromatography system, equipped with a GP40 gradient pump and an AD20 absorbance detector (Dionex Corp., Sunnyvale, CA). The absorbance of the eluate was monitored at 280 nm in the sensitivity range of 2.0.

Purification of Recombinant Nm23 Proteins by Affinity Chromatography—Bacterial lysates containing wild type or mutant rNm23 proteins were loaded into Cleaved Blue 3G-agarose columns, type 1000 (Sigma) (0.25-ml column volume/ml lysate), equilibrated with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. After washing the column with 20 volumes of the same buffer, Nm23 proteins were eluted with 20 mM Tris-HCl, pH 8.0, 10 mM ADP, 1 mM NaCl. Eluate containing affinity purified rNm23-H1 proteins was diluted 10-fold with 20 mM Tris-HCl, pH 8.0, and applied to a DEAE-Sepharose CL-6B column (Pharmacia), equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl (0.1-ml bed volume/ml lysate), washed, and eluted as indicated above. Fractions containing purified recombinant Nm23-H1 proteins were stored at 4°C or at −20°C. Affinity purified rNm23-H2 was dialyzed against 20 mM Tris-HCl, pH 8.3, and loaded onto a DEAE-Sepharose column equilibrated with the same buffer. Fractions of the flow-through containing purified Nm23-H2 protein were concentrated and stored as indicated above.

Expression and Purification of Glutathione S-Transferase-Nm23 Proteins—Two oligonucleotides containing EcoRI restriction sites were synthesized (5′-Nm23-H1+RI, 5′-CCCCGAAATCCGAAAACCTGTTGACCG-3′; 3′-Nm23-H1+RI, 5′-GCGCAATCCGAAATCCGAAAACCTGTTGACCG-3′). These two primers were used to amplify the Nm23-H1 coding sequence, using rNm23-H1 as a template. The PCR reaction mixture, overlaid with 75 µl of mineral oil to prevent evaporation, was heated at 94°C for 3 min and then 35 cycles were run as follows: 94°C, 1 min; 55°C, 1 min, 72°C, 3 min. The PCR product was digested with EcoRI, and the resulting 459-base pair fragment was subcloned into the EcoRI site of pGEX-2T (Amersham, Little Chalfont, UK). The recombinant plasmid containing the insert in the correct orientation, named pGEX-H1, was analyzed by double-stranded sequencing and transformed into E. coli DH5α bacteria. Lysates from pGEX-H1-transformed bacteria were prepared as indicated above for BL21(DE3) cells. Recombinant GST-Nm23-H1 fusion protein was purified from the lysates by affinity chromatography on glutathione-Sepharose 4B columns (Pharmacia Biotech, Uppsala, Sweden), following the manufacturer’s instructions. The glutathione used in the elution of the fusion protein was removed by gel filtration on PD-10 columns (Pharmacia) equilibrated and eluted with 20 mM Tris, pH 8.0, 100 mM NaCl.

Autophosphorylation of Nm23 Proteins—20 µg of purified rNm23 proteins were incubated with 100 µM [γ-32P]ATP (300 µCi) for 15 min at room temperature in 100 µl of TMD buffer (20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol). Free [γ-32P]ATP was removed by successive rounds of dialution with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and concentration with Centricon-30 tubes (Amicon Inc., Beverly, MA 01915) until concentration of [γ-32P]ATP, estimated by counting of the filtrate, was less than 5% (approximately 0.1% of the total counts in the phosphorylated proteins). The absence of detectable amounts of free [γ-32P]ATP was checked by thin layer chromatography.

Nucleotide-Diphosphate Kinase Assays—NDPK activity of purified rNm23 proteins was assayed by thin layer chromatography (53). Briefly, 50 ng of purified rNm23 proteins were incubated with 1 µM [γ-32P]ATP (0.2 µCi/ml) and 100 µM GDP in TMD buffer (20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol) in a 10-µl total volume at room temperature for 10 min. The reaction was stopped by addition of 10 µl of 50 mM EDTA, and 2 µl were applied to a 20 × 20 cm

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TABLE I
Summary of in vitro motility of nm23-H1 wild type and site-directed mutant transfectants of human MDA-MB-435 breast carcinoma cells in Boyden chamber assays (51)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Reported significance</th>
<th>Range of cells migrating to</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0.5% serum 5 mM ATX</td>
</tr>
<tr>
<td>Nm23-H1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nm23-H1S44A</td>
<td>Phosphorylation site</td>
<td>0.4–11.4 a 0–3.8 c</td>
</tr>
<tr>
<td>Nm23-H1S120A</td>
<td>Phosphorylation site</td>
<td>1.0–11.2 5.4–10.1</td>
</tr>
<tr>
<td>Nm23-H1S118F</td>
<td>Killer of prune mutation</td>
<td>10.5–41.9 28.2–33.1</td>
</tr>
<tr>
<td>Nm23-H1S120G</td>
<td>Mutation in neuroblastoma</td>
<td>42.2–43.4 29.4</td>
</tr>
<tr>
<td>Nm23-H1S44A</td>
<td>Phosphorylation site</td>
<td>1.1 17.3</td>
</tr>
<tr>
<td>Nm23-H1H118F</td>
<td>Histidine kinases</td>
<td>0 0</td>
</tr>
</tbody>
</table>

Nm23-H2

- Cells migrating through a type IV collagen-coated 8 μm filter toward the chemoattractant were determined by counting 6–9 40 × microscopic fields per Boyden chamber, three chambers per data point. Data show the range of 2–3 independent transfectants of each construct, with Nm23-H1 overexpression verified by Northern and Western blots, with the exception of a single clone obtainable for the S120-A and H118-F constructs.

- Control transfectants (empty construct), 17.8–23.4.

- Control transfectants (empty construct), 9.0–26.7.

Expression and Purification of Recombinant Nm23 Proteins—Wild type nm23-H1 and nm23-H2 cDNAs were cloned into a pET3c expression vector. Site-directed mutants of nm23-H1, previously described (51) and listed in Table I, were cloned into the same vector. Recombinant wild type and mutated Nm23 proteins produced by isopropyl-1-thio-β-D-galactopyranoside-induced BL21(DE3) E. coli transformed with the pET3c constructs were purified by two protocols. Protocol 1 involved sequential DEAE-Sepharose, Mono S cationic exchange, and Superdex 75 gel filtration chromatography steps, resulting in >95% purity. Protocol 2 used a 3G Avidin Blue agarose column and DEAE-Sepharose chromatography, with essentially equivalent results. Fig. 1A shows a Coomassie Blue-stained gel of representative preparations. As shown, rNm23-H1 and its site-directed mutant forms migrated as a single band of approximately 20,000, while rNm23-H2 exhibited a molecular weight of approximately 18,000. Silver-stained gels of each protein purification were devoid of detectable contaminating bands (data not shown).

Also listed in Table I is a synopsis of the biological relevance of each nm23-H1 site-directed mutant to motility suppression upon transfection into human MDA-MB-435 breast carcinoma cells, previously reported (51). Representative motility experiments using fetal calf serum or partially purified autotaxin (ATX) as chemoattractants are shown as indicators of relative motile behavior. Wild type nm23-H1 consistently inhibited motility, as compared with control transfectants (empty constructs); the nm23-H1S44A (serine 44 to alanine) transfectants exhibited variable levels of motility that were never significantly different from those of wild type. Both the nm23-H1P96S (proline 96 to serine, the k-pan homolog) and nm23-H1S120G (serine 120 to glycine, neuroblastoma mutation) transfectants consistently moved toward chemoattractants at levels or higher than the control constructs. Another mutation at serine 120, nm23-H1S120A (serine 120 to alanine) which maintained...
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Fig. 2. Dose-response characteristics of histidine autophosphorylation by wild type and S120G mutant rNm23-H1 proteins. 0.5 μg of purified wild type and S120G mutant rNm23-H1 proteins were incubated with the indicated concentrations of [γ-32P]ATP for 10 min. Basic SDS-PAGE sample buffer was then added, and the samples were loaded in 13% gels without boiling. After electrophoresis, the gels were directly exposed to autoradiography. The amount of phosphorylated protein was estimated by densitometry of the X-ray film. Data are representative of three independent experiments conducted.

the position’s “R” group and hydrogen bonding characteristics, exhibited deficient motility to serum in the 8-h assays shown in Table I but was motile at or above the levels of the control transfectants in both longer assays to serum and in all ATX assays. The motility suppressive characteristics of the nm23-H1H118F (histidine 118 to phenylalanine) construct could not be determined with confidence due to poor expression characteristics.

Autophosphorylation Characteristics—Approximately 2 μg of each rNm23 protein was incubated with [γ-32P]ATP for 10 min. Half of the sample was electrophoresed in a basic sample buffer without boiling, and the gel was subsequently exposed for autoradiography without further fixation to observe histidine autophosphorylation levels. The remaining half of the sample was boiled in traditional sample buffer and electrophoresed, and the gel was subsequently fixed in 20% (w/v) trichloroacetic acid, Coomassie-stained and destained in methanol/acetic acid to permit autoradiography of serine autophosphorylation. A radioautograph of histidine autophosphorylation of the rNm23 proteins is shown in Fig. 1B. The Nm23-H1H118F site-directed mutant protein, lacking the histidine previously shown to form the phosphorylated intermediate of the Nm23 NDPK activity, remained unphosphorylated. This protein thus provided a control for the absence of significant contamination by wild type bacterial Nm23, which should autophosphorylate. Histidine autophosphorylation levels exhibited by the other rNm23 proteins were relatively uniform, with the exception of Nm23-H1S120G. Experiments using three preparations showed a 2–5-fold decrease in histidine autophosphorylation levels, as compared with wild type, determined by densitometry of autoradiographs. We have noted that, upon storage at 4 °C, the S120G mutant rNm23-H1 lost histidine autophosphorylation capacity at a greater rate than wild type Nm23 (data not shown), suggesting a defect in stability. Fig. 2 shows a comparison of wild type and freshly purified rNm23-H1S120G histidine autophosphorylation at varying concentrations of [γ-32P]ATP. Autophosphorylation levels for the S120G mutant failed to equal those of the wild type protein at any [γ-32P]ATP concentration tested.

Acid-stable serine autophosphorylation of Nm23 proteins was demonstrated in vitro (41–44) and upon [32P]orthophosphate labeling of MCF-7 breast carcinoma cells (41), although it represents only a minor proportion of total phosphorylation (44, 56). Serine autophosphorylation levels among the rNm23 proteins are shown on Fig. 1C. While we previously reported that purified, recombinant murine Nm23–1 protein, in which the histidine 118 was mutated to a glycine, was capable of serine autophosphorylation, the corresponding human protein, Nm23-H1H118F is not. Thus, serine phosphorylation in this system likely represents a transfer from phosphohistidine. Among the other wild type and mutant rNm23 proteins, serine autophosphorylation levels were quantitatively decreased in the S120G (1.5–2-fold) and S120A (2–3-fold) mutants, determined by densitometry of radioautographs representing three independent experiments. That serine autophosphorylation remained on both of these proteins confirms the existence of multiple serine autophosphorylation sites.

NDPK Activity—Nm23 proteins and homologs identified in many species possess a nonspecific NDP phosphotransferase activity, in which the terminal phosphate of a NTP is removed, transferred to Nm23-histidine 118, and subsequently transferred to a NDP (reviewed in Ref. 2). The NDPK activity of the rNm23 proteins is shown in Fig. 3, in which the formation of [γ-32P]GTP from [γ-32P]ATP and cold GDP is determined by autoradiography of TLC separations. With the exception of Nm23-H1H118F, all of the rNm23 proteins demonstrated significant NDPK activity. The reduced amount of [γ-32P]GTP product formed by the S44A and P96S mutants represented slightly decreased rNm23-H1 protein input into the reaction, as all of the available [γ-32P]ATP was consumed. Under conditions where [γ-32P]ATP was not rate-limiting, significant NDPK activity was also exhibited by each of the site-directed mutant Nm23-H1 proteins, with the exception of H118F (data not shown).

Histidine Protein Kinase Activity—Nm23 protein was recently reported to exhibit histidine protein kinase activity in vitro (37), in which the terminal phosphate of [γ-32P]ATP was transferred to a histidine residue of Nm23, which then kinased ATP citrate lyase on a histidine residue. Fig. 4A presents a radioautograph of the histidine protein kinase activity of the wild type and mutant rNm23 proteins in vitro. Briefly, Nm23 proteins were autophosphorylated with [γ-32P]ATP, and the unbound label was removed; each recombinant protein, normalized by cpm of autophosphorylation, was subsequently incubated with a novel histidine protein kinase substrate, porcine succinyl thiokinase (STK, also known as succinyl-CoA synthetase, EC 6.2.1.4), selected on the basis of its phosphorylatable histidine residue. Reaction substrates and products were visualized by electrophoresis and autoradiography. Both wild type rNm23-H1 and rNm23-H2 kinased the α-subunit of
STK; the lack of STK phosphorylation by incubation with identically treated Nm23-H1H118F protein confirms the lack of \[^{\gamma-32}P\]ATP contamination in this series of experiments and indicates histidine 118 as the Nm23-phosphorylated intermediate. Histidine kinase activities of the rNm23 proteins were relatively equivalent with the notable exception of the rNm23-H1P96S protein, representing the *Drosophila* k-pn developmental mutation. By densitometry of radioautographs, the STK kinase level was 6–10-fold lower when incubated with the k-pn, as compared with wild type Nm23-H1 protein, over three independent experiments. A corresponding greater percentage of histidine phosphorylation remained on the Nm23-H1P96S, indicating that the deficit was due to lack of transfer to the STK protein. Fig. 5 presents time course data for a STK histidine kinase reaction using autophosphorylated wild type and P96S mutant rNm23-H1 proteins. As shown, the killer of prune rNm23-H1 failed to histidine kinase STK over an extended period of reaction times.

Phosphoamino acid analysis of the wild type rNm23-H1 and STK were performed to verify that histidines were involved in the reactions previously described. Autophosphorylated rNm23-H1 was incubated with STK, the two proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Phosphorylated rNm23-H1 and STK were excised and subjected to alkaline hydrolysis in 3 M KOH. Phosphoamino acids were separated by thin layer chromatography. As shown in Fig. 6, N^2-phosphohistidine was detected in the Nm23-H1 hydrolysate, as expected (37), whereas the product of STK alkaline hydrolysis was N^3-phosphohistidine.

To determine the generality of these data, additional histidine protein kinase substrates were tested, including cold rNm23-H2 protein. Fig. 4B shows a radioautograph of an experiment in which rNm23 proteins were autophosphorylated with \[^{\gamma-32}P\]ATP, and excess label was removed; the autophosphorylated proteins were subsequently incubated with cold, purified rNm23-H2 and transphosphorylation of rNm23-H2 detected by electrophoresis and autoradiography. Histidine autophosphorylation of Nm23-H2 occurred in the presence of autophosphorylated wild type rNm23-H1 but not rNm23-H1H118F. The histidine kinase activity of the various
rNm23-H1 proteins were approximately equivalent, with the exception of decreased phosphotransferase activity of rNm23-H1P96S. A corresponding increase in the amount of residual autophosphorylated rNm23-H1 protein was observed. The histidine protein kinase activity of the k-pn rNm23-H1 was 4–6 times lower than that of wild type protein, determined from densitometry of autoradiographs representing three independent experiments. A slight but reproducible decrease in rNm23-H2 histidine phosphorylation was observed using rNm23-H1S120G as the kinase.

Fig. 4C provides a third test of the in vitro histidine protein kinase activity of the rNm23 proteins, using the experimental design previously described and a GST-Nm23-H1 fusion protein as a substrate. Transphosphorylation of GST-Nm23-H1 by autophosphorylated wild type and mutated rNm23 proteins was dependent on the presence of a histidine 118 in the rNm23-H1, in agreement with previous results. Transphosphorylation was negligible using rNm23-H1P96S as the kinase and slightly but reproducibly reduced using rNm23-H1S120G.

Additional aliquots of the histidine protein kinase reactions were electrophoresed under conditions to permit visualization of acid-stable phosphorylation. For GST-Nm23-H1, this phosphorylation is expected to be serine and represent downstream transfer from phosphohistidine; it is unclear if acid-stable phosphorylation of the remaining proteins represents independent serine or other kinase events or a downstream transfer from phosphohistidine. A very minor proportion of acid-resistant protein phosphorylation was observed using each of the rNm23 substrates described above and exhibited autoradiographic patterns among the various mutant donor proteins similar to that of histidine phosphorylation (data not shown).

DISCUSSION

The long-term goal of the research described herein is to identify the biochemical pathway whereby increased Nm23 expression suppresses tumor metastatic potential in vivo in melanoma and breast carcinoma cells (6, 8–11). Previous approaches have identified numerous biochemical activities and/or associations for Nm23 proteins. However, many of these activities remain debated, and few have been linked to biological changes in development or tumor metastasis. An alternate approach using site-directed mutagenesis was therefore adopted, in order to more closely link biology and biochemistry.

We have previously reported the transfection of wild type nm23-H1 and several site-directed mutant constructs into human MDA-MB-435 breast carcinoma cells, using in vitro motility in Boyden chamber assays as an indicator of aggressiveness (51). Mutation of proline 96 to serine (P96S), the killer or prune (k-pn) developmental mutation in the Drosophila homolog of nm23, awd, or mutation of serine 120 to glycine (S120G), a mutation of a phosphorylatable serine residue (41) found in human stage IV neuroblastomas (57), failed to inhibit the motility of MDA-MB-435 breast carcinoma cells to either serum or partially purified ATX. A serine 120 to alanine (S120A) substitution, which preserved "R" group and hydrogen bonding characteristics at this position, lacked motility inhibitory activity in some, but not all assays performed. In contrast, wild type Nm23-H1 protein and a serine 44 to alanine (S44A) mutation produced transfectants with suppressed motility characteristics. In the present report purified rNm23-H1 and its site-directed mutants have been surveyed for activity in several assays involving phosphorylation and phosphotransfer, the results of which are summarized on Fig. 7. The question asked by these experiments is whether those mutant proteins lacking motility suppressive capacity in vivo exhibit altered activity in a particular biochemical pathway.

The most striking observation reported herein concerns Nm23-H1P96S. This mutation represents the homolog of a P97S mutation in Drosophila awd, which causes no phenotype alone but widespread aberrant development leading to lethality in the third instar stage when coexpressed with a pn mutation (58), most likely the result of an almost complete loss of Pn product (reviewed in Ref. 15). X-ray crystallography studies in several species have localized the k-pn mutation to a k-pn loop, whose function changes depending on the oligomeric structure of Nm23/Awd (59). We report that purified rNm23-H1P96S deficient in the phosphotransferase portion of a protein histidine kinase reaction in vitro. While the autophosphorylation portion of this pathway remained functional, transfer of phosphate from rNm23-H1 to a histidine residue on three substrates, STK, Nm23-H2 and GST-Nm23-H1, was significantly reduced. In contrast, no significant diminution of Nm23-H1P96S histidine phosphotransfer was noted to NDPs in the NDPK reaction, or internally to Nm23-H1 serine residues.

The coordinate abrogation of motility suppressive capacity in vivo and histidine protein kinase activity in vitro by Nm23-H1P96S suggests the intriguing hypothesis that the two may be functionally linked. If Nm23 functions biologically via a protein histidine phosphotransferase pathway, it can be hypothesized that this activity normally favors the nonmetastatic cellular state. Reduced expression of Nm23 or its k-pn mutation could diminish levels of the kinased product, resulting in increased metastatic potential. Further experimentation to test this hypothesis will necessarily address the following: (a) the contribution of small amounts of wild type Nm23-H1 and Nm23-H2,
as found in transfection systems; (b) possible contributions by relevant Nm23 binding proteins; (c) the histidine protein kinase activity of these wild type and mutant rNm23 proteins using as yet unidentified physiologically relevant substrates; (d) the potential contribution of pn and other interacting genes that may be discovered. It also remains possible that loss of Nm23 histidine protein kinase activity and/or mutation of Nm23 at the k-pn or other sites exerts effects on protein kinase activity not observed in the present series of analytical reactions, such as alterations in substrate specificity.

Histidine protein kinases have been thoroughly characterized in prokaryotes, where they form the “two-component” regulatory system, (reviewed in Ref. 38). This is signal transduction system, linking extracellular changes in chemotactrant, nutrient, osmotic, or other gradients to transcriptional alterations. The two-component system has also been linked to differentiation in Caulobacter crescentus (60). The prototypic elements of this system include a protein with a periplasmic sensor; when an external event is detected it modulates the activity of a protein kinase catalytic domain, which in turn phosphorylates a histidine residue in the substrate domain. The phosphate on the histidine is typically transferred to an aspartate on a second protein, which activates a coupled effector domain. Phosphohistidine is undetected in many studies due to its lability, but recent studies in eukaryotes have estimated that 6% of protein phosphorylation is histidine, a level higher than phosophotyrosine (50). Two-component systems have been described in yeast, where they are thought to regulate mitogen-activated protein kinase activity (62). Recently histidine phosphorylation of P-selectin was reported upon activation of human platelets, providing a first example of histidine phosphate involvement in mammalian signaling (39). Comparison to Nm23 function is noteworthy on several points. First, transfection of nm23 cDNAs has been reported to inhibit tumor cell signal responsiveness in colonization, motility, invasion, etc., and a role in limiting the signal transduction process has been previously proposed (6). Second, in prokaryotic histidine protein kinase systems phosphate is transferred from histidine to another amino acid residue, whereas in the assays reported herein transfer is from one histidine to another. This activity may be better termed a histidine protein phosphotransferase. However, using GST-Nm23-H1 and rNm23-H2 as a substrate we noted subsequent transfer to serine residues in vitro, which would represent an example of a downstream phosphorylation. The identification of physiologically relevant substrates will enable further definition of this activity.

The serine 120 to glycine mutation of Nm23-H1 demonstrated altered activity in multiple phosphorylation and phosphotransferase assays. In transfection experiments, nm23-H1S120G failed to inhibit tumor cell motility (51); cohort sequence data in childhood neuroblastoma identified this mutation in 6/28 aggressive (stage IV) tumors (57). Serine 120 is a potential site of phosphorylation (41), and is exhibited reduced serine autophosphorylation levels. However, this represents another histidine-dependent protein phosphotransferase activity, consistent with the hypotheses previously developed. Mutation of histidine 118 abrogated activity in all of the biochemical phosphorylation assays conducted; comparison to transfection data, however, is prevented by its poor expression characteristics.

In conclusion, assays of phosphorylation and phosphotransferase activity indicate a correlation of Nm23-H1 mutations lacking motility suppressive capacity upon transfection and exhibiting deficient activity in histidine-dependent protein phosphotransferase pathways. It is recognized that the mutant proteins may also differ in additional biochemical activities and associations untested in the present series of experiments, which could be biologically relevant. However, the data permit the development of the hypothesis that a histidine-dependent protein phosphotransferase pathway is functionally involved in the biological metastasis suppressive effect of Nm23.

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Site-directed Mutation of Nm23-H1: MUTATIONS LACKING MOTILITY SUPPRESSIVE CAPACITY UPON TRANSFECTION ARE DEFICIENT IN HISTIDINE-DEPENDENT PROTEIN PHOSPHOTRANSFERASE PATHWAYS IN VITRO

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