We have recently discovered an alternative function of the putative metastasis suppressor protein Nm23, which is identical to nucleoside diphosphate kinase, as a protein phosphotransferase in vitro. While purified native Nm23 protein did not phosphorylate other proteins, we could purify a Nm23-associated protein that activates the protein phosphotransferase function; it was identified as a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isozyme. Co-expression and purification of (His)₆-tagged GAPDH in combination with either Nm23-H1 or Nm23-H2 in baculovirus-infected SF9 cells showed that only Nm23-H1, but not Nm23-H2, forms a stable complex with GAPDH. Protein phosphotransferase activity was confirmed for the recombinant GAPDH-Nm23-H1 complex but not for either of the enzymes alone, nor was this activity observed after simple mixing of the purified proteins in vitro. The molecular mass of the highly purified recombinant GAPDH-Nm23-H1 complex suggests that a dimer of GAPDH interacts with a dimer of Nm23-H1. In contrast to the complex with GAPDH, co-expression of Nm23-H1 with antioxidant protein (MER-5) or creatine kinase did not activate the protein phosphotransferase function, indicating that this activation may specifically require GAPDH as a binding partner.

The first member of the nm23 gene family has been isolated due to its reduced expression in highly metastatic clones of murine melanoma cells as compared with their nonmetastatic counterparts (1). The proposed metastasis suppressor activity of this gene, now termed nm23-H1, has subsequently been confirmed in numerous functional studies: transfection of nm23-H1 cDNA into highly metastatic melanoma or mammary carcinoma cell lines resulted in a significant reduction of metastatic potential in vivo (2–5). In many human tumors, such as mammary carcinomas, hepatocellular carcinomas, and malignant melanomas, reduced expression of nm23 was correlated with lymph node metastasis and decreased disease-free survival of the patients (reviewed in Ref. 6). In other tumor types, however, increased expression of nm23 was associated with cell proliferation and advanced stages of the disease (7, 8), suggesting complex biological functions of the gene product(s). The later cloned gene nm23-H2, although 81% identical to nm23-H1, provided little or no evidence for a metastasis suppressor activity in tumor expression studies and in a transfection assay (3, 9–12). Two further members of the nm23 gene family, DR-nm23 (13) and nm23-H4 (14), are more distantly related to the first discovered genes and no experimental data are known so far regarding their metastasis suppressor potential. The biochemical mechanism(s) underlying the antimitastatic properties of Nm23-H1 are unknown. All nm23 genes as known today have been demonstrated to encode for nucleoside diphosphate (NDP) kinases. These long known enzymes are ubiquitously expressed and remarkably conserved throughout evolution. For example, the human NDP kinase is 80, 62, and 40% identical to the NDP kinases from Drosophila, Dictyostelium, and Escherichia coli, respectively. The human genes nm23-H1 and nm23-H2 encode for NDP kinase A and B, respectively, which form mixed heterohexamers in vitro (15). However, it has been shown that this “housekeeping” enzymatic activity is not responsible for the observed metastasis suppressor function of Nm23-H1 (16). In Drosophila, the developmental effects of a specific point mutation in the NDP kinase (identical to the Awd protein) can be dissociated from its enzymatic activity, too. The so-called awd⁻⁻ mutation, which represents a substitution of proline 96 by serine (17), caused diverse developmental abnormalities when expressed in the context of pruned (pn) eye color mutations (reviewed in Ref. 18), although the Awd⁻⁻ protein retained significant NDP kinase activity (17, 19).

A distinct biochemical function as a transcription factor for c-myc was identified for Nm23-H2 but not for Nm23-H1 (20, 21). However, recent data suggest that the DNA binding activity is rather nonspecific, preferring single stranded polypyrrole sequences (21), and that the antimitastatic function of Nm23 is likely due to other biochemical activities (22). Three years ago, we discovered an alternative function of the Nm23 proteins from different species as serine/threonine-specific protein phosphotransferases (23), so called because, at least under in vitro assay conditions, phosphorylation of other proteins occurred stoichiometrically rather than catalytically. Using different mutants of Dictyostelium NDP kinase we could also show that the protein phosphotransferase activity depends on the same active site as the NDP kinase activity. Meanwhile the novel activity was confirmed by other groups (24–27). The possible phosphorylation reactions of Nm23/ NDP kinase can be summarized as in Equations 1–3

\[ NTP + Nm23 \rightleftharpoons NDP + Nm23-\text{His-P} \]  

(Eq. 1)

The abbreviations used are: NDP, nucleoside diphosphate; Awd⁻⁻, Awd killer of prune; (d)NTP, (deoxy)nucleoside triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
Protein Phosphotransferase Activity of Nm23-H1

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N.DP + Nm23-His-P → NTP + Nm23

(Eq. 2)

or

Prot-Ser/Thr + Nm23-His-P → Prot-Ser/Thr-P + Nm23

(Eq. 3)

The ability to phosphorylate serine/threonine residues of other proteins in addition to nucleoside diphosphates provides a promising hypothesis how Nm23 may participate in signal transduction, thus controlling tumor metastasis. Highly purified Nm23 proteins did not show any protein phosphotransferase activity unless nondenaturing amounts of urea were added which are suggested to induce conformation changes (23). Since these conditions were rather artificial, we investigated whether native Nm23 proteins could be isolated from human cells in a stable, protein phosphotransferase-active state. In the present study, we demonstrate that Nm23-H1 protein exists in a specific complex with glyceraldehyde-3-phosphate dehydrogenase in vivo that exhibits protein phosphotransferase activity.

EXPERIMENTAL PROCEDURES

Phosphorylation of Protein Phosphotransferase-activating Protein—Human erythrocyte concentrate (200 ml) was mixed with 800 ml of buffer A (10 mM Tris acetae, pH 7.5, 10% glycerol, 2 mM DTT, 2 mM (NH4)2SO4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. The lysate was centrifuged for 40 min at 14,000 × g and 4 °C. The supernatant was stirred gently overnight at 4 °C with 7 ml of cAMP-agarose (Sigma). Then the cAMP-agarose was filled into a column and washed with buffer A until absorption at 280 nm had decreased to zero. Washing was continued with two-column volumes of buffer A containing 1 mM cAMP, and bound proteins were eluted with 30 mM cAMP in buffer A. The cAMP eluate was precipitated overnight at 4 °C by dialysis against a saturated solution of (NH4)2SO4. The precipitate was redissolved in a small volume of buffer B (25 mM Tris/HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA) and applied to a Superdex-200 fast protein liquid chromatography column (Amer sham Pharmacia Biotech) previously equilibrated in buffer B containing 140 mM (NH4)2SO4. Gel filtration was carried out at a flow rate of 0.7 ml/min at 8 °C. The collected fractions were assayed for the presence of Nm23 proteins by incubation with [γ-32P]ATP in 20 mM Tris/HCl buffer (pH 8.0) also containing 2 mM EDTA, followed by SDS-PAGE and autoradiography. All fractions showing the characteristic Nm23-H1/H2 band pair around 18 kDa were then assayed for protein phosphotransferase activity as described previously (23), using heat-denatured CX-1 proteins as test substrate. Positive fractions were pooled and dialyzed against buffer C (20 mM Tris/HCl, pH 8.5, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM EDTA) and applied to a Mono-Q fast protein liquid chromatography column (Amersham Pharmacia Biotech) previously equilibrated with buffer C, and the bound proteins were eluted with a gradient of 0–1 M NaCl in buffer C at a flow rate of 0.5 ml/min. The collected fractions were analyzed for protein phosphotransferase activity, and proteins present in the fractions were visualized by SDS-PAGE silver staining.

Protein Sequencing—Proteins in the Mono-Q fraction containing the protein phosphotransferase-activating protein were precipitated by addition of 8 volumes of acetone, and the precipitate was washed with 80% ethanol, dried under vacuum, and dissolved in SDS-PAGE sample buffer without heating. SDS-PAGE was performed exactly as described by Dubarn and Wilson (28). The proteins were then transferred to an Immobilon-P© polyvinylidene difluoride membrane (Millipore) using a semidrying blotting apparatus (Trans-Blot SD; Bio-Rad). After staining the membrane with Coomassie Blue, the protein band of interest (~1 mg) was cut out and sent to a commercial sequencing service (ARIAD Pharmaceuticals, Inc., Cambridge, MA).

Plasmids—All cDNA fragments intended for cloning were generated by reverse transcriptase-PCR. RNA (10 mg) isolated from human colon tissue was reverse-transcribed using Moloney-murine leukemia virus reverse transcriptase, and aliquots of the obtained cDNA were used for PCR employing the following primer sequences: nm23-H1, H1–5′-Bam (5′-CGGATCATGAGGACATCTGCAGTAC-3′) and H1–3′ (5′-CCTCCTGTCATTCATAGTC-3′); and 3′-Eco (5′-GCGGATCCATTGCTGCTGCACTCC-3′) for cloning in pBac; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): GAP-5′-BaiHsi (5′-ATCTGATCATGCATCACTCCA-3′); and GAP-3′-Spe (5′-CGACTGTATTCTCTCCCAAGGCTATCGTTACGGAATTTCAGTAC-3′) for cloning in pBac; pyruvate kinase (PK): PK-5′-Bam (5′-GCGGATCCATGAGGACATCTGCAGTAC-3′) and PK-3′ (5′-GCGGATCCATTGCTGCTGCACTCC-3′) for cloning in pBac; MER5′-5′-Bam (5′-GCGGATCCATTGCTGCTGCACTCC-3′) and MER5′-3′ (5′-GAGAATTCGTCACCATGCTGCTGCACTCC-3′) for cloning in pQE-30. PCR conditions were 94 °C for 3 s, 57–62 °C (according to individual melting points of primers) for 30 s, 72 °C for 30 s (nm23), 1 min (GAPDH and MER-5) or 2 min (creatine kinase), 35 cycles; 72 °C, 1 min, 1 cycle. The PCR products were excised from agarose gel, purified with appropriate restriction enzymes, and ligated into the respective vectors that had also been cut appropriately and dephosphorylated. The correctness of the cDNA inserts was verified by double-stranded automatic sequencing.

Expression and Purification of Recombinant Proteins—The recombinant baculovirus pBac vector (4 mg) containing the GAPDH sequence and the nm23-H1 or -H2 sequence, respectively, was transfected in combination with 0.5 mg of Bac-N-Blue virus DNA (Invitrogen) in Sf9 insect cells (2 × 106). The transfection was carried out in 60-mm tissue culture dishes. 72 h after transfection a plaque assay was performed to purify recombinant virus from any uncut or nonrecombinant viral DNA according to the manufacturer’s protocol. Putative recombinant plaques were chosen, and viruses were amplified by infection of log-phase Sf9 cells with the respective recombinant stocks. Purified recombinant viruses were used to prepare high titer viral stocks (P-2) by re-infection. 1 ml of each of these P-2 stocks was added to 6 × 106 Sf9 cells grown in 75-cm2 flasks. 48 and 72 h after infection, respectively, the cells were harvested and checked for recombinant protein expression. Cells from 20 flasks were pooled and used for protein purification as described below.

pQ-30 vectors carrying GAPDH, MER-5, or creatine kinase inserts, respectively, and pALTER-Ex2-nm23-H1 were used to co-transform E. coli JM109 competent cells. Bacteria were plated on LB agar containing 13 µg/ml tetracycline, 100 µg/ml ampicillin, and 2% glucose. Single colonies were then picked, grown in 1-liter LB medium containing the same antibiotics to an A600 of 0.5 at 37 °C, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside to express recombinant proteins for 4 h at 30 °C.

After harvesting Sf9 cells (see above) or bacteria by centrifugation, cells were resuspended in binding buffer (25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10 mMimidazole, 2 mM β-mercaptoethanol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. Cell debris was removed by centrifugation for 20 min at 20,000 × g and 4 °C, and the supernatant was stirred in a Baker glass at 4 °C for 30 min with 0.1% deoxycholate and 1% Nonidet P-40. The Ni2+–agarose was filled into a column which was then connected to a fast protein liquid chromatography system. Washing was performed at room temperature with wash buffer (20 mM Tris/HCl, pH 7.0, 1% glycerol, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) until UV absorption had declined to almost zero, then proteins bound to the column were eluted with a flat gradient of 0–400 mM imidazole in wash buffer. Since this gradient is not compatible with 50% glycerol, the Ni2+–50% glycerol column is recommended for use with recombinant Nm23-H1 and the MER-5/nm23-H1 complexes were already about 85% pure at this stage (not shown) and no protein kinase activity was found (see “Results”), purification was stopped here.

The main peak fraction containing the recombinant GAPDH-Nm23-H1 complex was passed over a PD-10 desalting column (Amersham Pharmacia Biotech) at 4 °C to exchange buffer against RY buffer (20 mM Tris/HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 20% glycerol). The protein solution was then stirred gently for 1 h at 4 °C with Reactive Yellow agarose (Sigma) which had been equilibrated before in RY buffer. Then the agarose was added into a column, and the column was washed with RY buffer until the UV absorption had decreased to zero. The bound protein complex was eluted with 1 mM ATP in RY buffer and extensively dialyzed against 20 mM Tris/HCl, pH 7.5, 40 mM KCl, 1 mM DTT, and 0.5 mM EDTA at 4 °C. The buffer was changed three times over a period of 2 days and contained in addition a suspension of activated charcoal to remove most of the ATP from the solution. For size exclusion chromatography and stoichiometrical measurements, the buffer of the complex eluted from Reactive Yellow agarose was exchanged against buffer (20 mM HEPEs/NaOH, 10% glycerol, 1 mM DTT, pH 7) using a PD-10 column (Amersham Pharmacia Biotech). Then the solution was passed over a Source-30S cation exchange column (Amersham Pharmacia Biotech) at 4 °C at a flow rate of 0.2 ml/min. The complex was collected in the flow-through fraction.

When (His)6–GAPDH and (His)6–Nm23-H1 were purified as isolated proteins from bacteria transformed with the respective recombinant pQE-30 vector alone, Nm23-H1 agarose affinity chromatography was used to
as a single step method to purify the proteins. The procedure was essentially the same as described above, except that 1 mL NaCl was included in the binding and wash buffers in order to prevent nonspecific binding to the affinity material and to remove possible E. coli proteins interacting with the respective recombinant protein.

Analytical Size Exclusion Chromatography—GAPDH-Nm23-H1 complex (0.5 mg) was applied to a Superdex-200 column in a fast protein liquid chromatography system (Amersham Pharmacia Biotech) either alone or mixed together with standard proteins and chromographed in a buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM (NH₄)₂SO₄, 10% glycerol, 1 mM DTT, and 1 mM bovine serum albumin. The flow rate was 0.5 mL/min. Vₑ was determined using blue dextran. The elution volumes of the proteins as detected by UV absorbance that were used for the calibration curve were mean values from three consecutive runs.

NDP Kinase Activity Measurement—NDP kinase activity was determined using a coupled enzymatic assay. Nm23 samples were added to a reaction mixture containing 50 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM ATP, 2 mM dTDP, 1 mM phosphoenol pyruvate, 10 mM sodium phosphate, and 2 units of pyruvate kinase in a final volume of 100 μL. The reaction was performed at 30 °C for 15 min and stopped by the addition of 150 μL of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl and mixing. After addition of 10 N NaOH (200 μL) and ethanol (1 mL), absorption of the hydrazone was measured at 520 nm. A calibration curve was established by adding known amounts of pyruvate to the reaction mixture containing Nm23 and pyruvate kinase. Nm23 kinase activity was expressed as micromoles of ADP:min⁻¹·mg⁻¹ (units/mg).

GAPDH Activity Measurement—The activity of GAPDH was measured spectrophotometrically in the direction of NADH utilization. The reaction mixture contained 50 mM Tris/HCl, pH 7.5, 2 mM MgCl₂, 0.9 mM EDTA, 1 mM ATP, 0.2 mM NAD⁺, 15 units of phosphoglycerate kinase, 0.5 mg/ml bovine serum albumin, and the GAPDH samples in a final volume of 1 mL. Incubation was continued at room temperature until there was no change in UV absorbance. Then 3-phosphoglycerate was added to the reaction mixture (6 mM final concentration), and the change of UV absorbance was recorded at 340 nm. The enzymatic activity was expressed in micromoles of NADH oxidized per min per mg of GAPDH protein, units/mg (ε₅₆₅ = 6.22 cm²/mmol).

Densitometric Measurement—Samples intended for densitometry were electrophoresed on 12.5% SDS gels and stained with Coomassie Blue. The protein concentrations of noncomplexed GAPDH and Nm23-H1 were determined both by the method of Bradford and by comparing the values with a calibration curve obtained using the NIH Image program (version 1.60 for PowerPC). The amounts of GAPDH and Nm23-H1, respectively, in the complex were determined using the calibration curve.

RESULTS

Purification of an Nm23-associated, Protein Phosphotransferase-activating Protein—In order to isolate a protein phosphotransferase activity involving Nm23 from human erythrocytes we employed the following chromatographic steps: 1) cAMP-agarose affinity chromatography, 2) gel filtration on Superdex-200, and 3) anion exchange chromatography on Mono-Q. After each step, individual fractions were assayed for the presence of Nm23 protein and the positive fractions again for protein phosphotransferase activity. Fig. 1A shows the chromatogram of the last purification step. The peak fractions were analyzed by SDS-PAGE and silver staining and assayed for protein phosphotransferase activity using heat-denatured protein extract as a test substrate (Fig. 1B). The activity sought after was mainly found in the same fractions as the Nm23 hexamers containing equal proportions of Nm23-H1 and Nm23-H2 proteins (A and B). Silver staining of an analytical SDS-gel revealed that the protein phosphotransferase activity coincided with the presence of a 37-kDa protein in fractions 12, 18, and 20 (Fig. 1C). Other proteins co-purified with the Nm23-H1 and -H2 proteins were not consistently found in all fractions showing protein phosphotransferase activity. Moreover, the relative amount of 37-kDa protein correlated well with the strength of protein substrate phosphorylation, if a stoichiometric mode of phosphotransfer was assumed (23). The co-purified 37-kDa protein was N-terminally sequenced and surprisingly identified as a GAPDH isoenzyme (Table 1).

Confirmation of the Nm23-GAPDH Interaction by Co-expression Experiments—To examine the characteristics of the Nm23-GAPDH complex, we tried to reproduce the interaction in vitro by mixing purified (His)₆-GAPDH with both purified, non-(His)₆-tagged Nm23-H1 and Nm23-H2 protein, respectively. After incubation with Ni²⁺-chelate beads and washing of the beads, no binding of GAPDH to either of the Nm23 proteins was observed after elution of the bound fraction (data not shown). Since the purified proteins were all oligomers in solution, it was hypothesized that binding might occur only between monomers or lower order oligomeric forms. To further meet all possible needs for the action of chaperones or post-translational modifications to form a complex, we decided to reproduce the interaction in vivo by co-expressing the recombinant proteins in insect cells. Bicistronic vectors were constructed with both human GAPDH and Nm23-H1 or Nm23-H2 cDNA as inserts. After homologous recombination with baculovirus DNA the virus was used to infect Sf9 cells which simultaneously overexpressed the recombinant proteins in high quantity (Fig. 2A, lanes 1 and 6). GAPDH was fused to a (His)₆-tag which simplified purification and allowed to immediately check complex formation with non-(His)₆-tagged Nm23 proteins.

After purification of the recombinant proteins, it was observed that only Nm23-H1, but not Nm23-H2, protein forms a stable complex with GAPDH (Fig. 2A, lanes 3 and 4 versus lane 7). Protein phosphotransferase activity was confirmed for the GAPDH-Nm23-H1 complex (Fig. 2B). This complex was also produced by co-expression in E. coli and displayed the same protein phosphotransferase activity after purification (Fig. 3, lane 1). In contrast, the latter activity was not observed for either of the bacterially expressed enzymes alone or for a mixture of the purified components (Fig. 3, lanes 2–4).

Biochemical Characterization of the GAPDH-Nm23-H1 Complex—To determine the molecular mass of the GAPDH-Nm23-H1 complex, larger amounts of the recombinant complex were produced in E. coli. However, as observed before with the complex derived from insect cells, free GAPDH was in large excess over GAPDH actually bound to Nm23-H1. The reason was possibly that not every GAPDH molecule synthesized in vivo was captured in a complex, and part of the complexes may have dissociated during purification, resulting in loss of Nm23-H1. To free the protein complexes from unbound GAPDH, the mixture was passed over a Source30S cation exchange column. While noncomplexed GAPDH was retained by the column due to the high pl value (>8), the complex having an estimated pl value equal to or only slightly higher than the pH of the buffer (7.3) was collected in the flow-through. The pure complex thus obtained was separated by SDS-PAGE and stained with Coomassie Blue (Fig. 2A, lane 5). Relative amounts of GAPDH and Nm23-H1 were determined by densitometric measurement and by comparing the values with a calibration curve obtained by densitometry of known amounts of GAPDH and Nm23-H1, respectively (not shown). The stoichiometry of the binding partners in the complex was thus calculated to be 1:1 molar.

Next, the molecular mass of the complex was determined using size exclusion chromatography on a Superdex-200 column. Pure GAPDH-Nm23-H1 complex (500 μg) was injected together with known standard proteins and eluted at a volume corresponding to a calculated molecular mass of 107.4 kDa (Fig. 4). After gel filtration, the eluted fractions were reanalyzed by SDS-PAGE to ensure that the complex was still intact and had not dissociated during chromatography (data not shown).
shown). Considering the 1:1 molar ratio in the complex, the value of 107.4 kDa thus determined suggests that a dimer of GAPDH is associated with a dimer of Nm23-H1, which yields a calculated total molecular mass of 108 kDa. GAPDH has a basic pI very similar to Nm23-H2 (reported pI values for GAPDH range from 8.0 to 8.8), which explains the co-elution of the complex with the Nm23 hexamers containing equal proportions of Nm23-H1 and Nm23-H2 proteins (A_3B_3) during ion exchange chromatography (see above, Fig. 1B). Minor portions of protein phosphotransferase-active complex were found to co-elute with even more acidic peak fractions of the Nm23 hexamers (Fig. 1B, fractions 18 and 20). This observation might be explained by the involvement of more acidic GAPDH isoenzymes or protein kinase-phosphorylated GAPDH and/or Nm23-H1 species in complex formation. The main portion of GAPDH protein, which can be co-purified with the methods employed, was eluted in its isolated state at low salt conditions, as were the Nm23 complexes mainly consisting of Nm23-H2.

Since a novel function is activated by formation of the complex, it was interesting to see whether or not the “classical” enzymatic functions of the enzymes involved are influenced. The results of the activity measurements are summarized in

| Table 1 |
|---|

| N-terminal sequence of the Nm23-associated protein from fraction 12 (Fig. 1C) and homology with GAPDH |
|---|

| N-terminal amino acid sequence |
|---|

| Human GAPDH (muscle, liver) | VKVGVNGFGRIGRLVTRAAFN |
| Nm23-associated 37-kDa protein | VKVGVNGFGRIGRLVTXAFN |

Fig. 1. Chromatographic isolation of a Nm23-associated, protein phosphotransferase-activating protein. The chromatograph of the final purification step on Mono-Q is shown in A. Aliquots of the peak fractions were assayed for protein phosphotransferase activity by incubation with [γ-32P]ATP and denatured test substrate proteins. The reaction products were analyzed by SDS-PAGE and autoradiography of the dried gels, which is displayed in B. Protein phosphotransferase activity was correlated with the presence of a co-purified protein in column fractions 12, 18, and 20 (migration position indicated by an arrow). C. Silver-stained analytical SDS-gel loaded with the different Mono-Q fractions.
The autoradiograph of the dried gel is assayed radioactively for protein phosphotransferase activity using de-
from a Coomassie Blue-stained gel. The different column eluates were
panel A protein fraction shown in
lane 5 gels were silver-stained with the exception of
lanes 3 nating protein kinase activity visible in
M
agarose step (apparently bound to Nm23-H1 and removed after the Reactive Yellow-
lane 4, two different preparations).

Table II. The data show that neither GAPDH nor NDP kinase activity of the complex is significantly altered with the specific activity of the free enzymes. Similarly, the presence of the GAPDH substrate NADH in a concentration of 80 μM was without influence on the protein phosphotransferase activity (data not shown).

Investigation of a Possible Protein Phosphotransferase Activity of Nm23-H1 in Complex with Proteins Other Than GAPDH—To investigate if the protein phosphotransferase activity of Nm23-H1 is a general phenomenon when the protein is bound to other proteins or if it is a rather unique property of the Nm23-H1-GAPDH complex, Nm23-H1 was co-expressed in E. coli in combination with creatine kinase and antioxidant protein (MER-5/AOP1), respectively. Both proteins have been shown recently to co-purify with NDP kinase from frog heart (29). The recombinant complexes obtained were purified by

Ni²⁺–chelate chromatography as described. Fig. 5 shows the result of the protein phosphotransferase assay; although Nm23-H1 is obviously bound to the (His)_6-tagged creatine kinase and MER-5, as indicated by specific autophosphorylation signals (lanes 1 and 2), the complexes did not phosphorylate other proteins in contrast to the GAPDH/Nm23-H1 complex (lanes 3 and 4 versus lane 5).

DISCUSSION

In the protein phosphotransferase-active complex, a dimer of GAPDH is interacting with a dimer of Nm23-H1. This is the first report of a dimeric form of Nm23/NDP kinase existing in
with chromatography, the presence of Nm23-H1 was tested by incubation with (His)_6-tagged antioxidant protein (MER-5) and creatine kinase, respectively. After purification by Ni^{2+}-chelate-agarose chromatography, the presence of Nm23-H1 was tested by incubation with [γ-32P]ATP and analysis of the reaction products by SDS-PAGE and autoradiography. The autophosphorylated band of Nm23-H1 indicates complex formation with the respective binding partner (lanes 1 and 2). However, both MER-5/Nm23-H1 and CK/Nm23-H1 complexes did not show protein phosphotransferase activity (lanes 3 and 4) in contrast to the bacterially expressed GPDH-Nm23-H1 complex (lane 5).

**FIG. 5.** Investigation of a possible protein phosphotransferase activity of Nm23-H1 in complex with MER-5 and creatine kinase, respectively. Non-(His)_6-tagged Nm23-H1 was co-expressed in *E. coli* with (His)_6-tagged antioxidant protein (MER-5) and creatine kinase (CK), respectively. After purification by Ni^{2+}-chelate-agarose chromatography, the presence of Nm23-H1 was tested by incubation with [γ-32P]ATP and analysis of the reaction products by SDS-PAGE and autoradiography. The autophosphorylated band of Nm23-H1 indicates complex formation with the respective binding partner (lanes 1 and 2). However, both MER-5/Nm23-H1 and CK/Nm23-H1 complexes did not show protein phosphotransferase activity (lanes 3 and 4) in contrast to the bacterially expressed GPDH-Nm23-H1 complex (lane 5).

**TABLE II**

|          | GDPH | Nm23-H1 | Nm23-H1 | Nm23-H1 | Nm23-H1 | Nm23-H1 |
|----------|------|---------|---------|---------|---------|---------|
|          | units/mg |         |         |         |         |         |
| Free enzyme | 204 ± 3.5 | 1398 ± 54.5 | 1385 ± 47.5 | 198 ± 6 |         |         |
| Complex-bound enzyme | 198 ± 6 | 1398 ± 54.5 | 1385 ± 47.5 |         |         |         |

**vivo.** All eukaryotic NDP kinases are known to form very stable hexamers as free enzymes (30–32). GPDH seems to act in a way similar to a molecular chaperone, in that a normally transient, short lived oligomeric state of Nm23-H1 is stabilized and maintained after translation. According to the symmetry of the crystallized enzyme, the hexameric Nm23/NDP kinase can be viewed as a trimer of dimers (30). Presumably, GPDH preserves the dimeric form of Nm23-H1 by binding to and masking the region involved in trimer contacts. The *in vivo* occurrence of dimers was also discussed as a possible consequence of the Awd^k-mu mutation in *Drosophila* NDP kinase (17, 31, 33), which could explain the gain of function characteristics of this mutation.

GPDH is usually a homotetrameric enzyme, but the occurrence of dimeric species has been described in literature (34, 35). Furthermore, an isolated dimer of GPDH has been shown to act as an independent structural and functional unit of the enzyme, displaying full catalytic activity (36, 37). This is in agreement with our activity measurement of complex-bound, dimeric GPDH. The fact that the enzymatic activity of GPDH is not compromised in the complex suggests that the substrate binding sites of GPDH are not involved in binding of Nm23-H1. Furthermore, the presence of NADH did not affect protein phosphotransferase activity. These observations indicate that there is no allosteric interaction between GPDH and Nm23-H1 and that the conformational changes of GPDH induced by the co-factor NADH do not entail dissociation of the complex. The latter finding seems reasonable since any dissociation would lead to free dimers of Nm23-H1 which would readily form stable hexamers, resulting in a rapid and irreversible loss of all protein phosphotransferase-active Nm23 complexes. Therefore, the existence of the complex under *in vivo* conditions is only guaranteed if binding of the substrates to GPDH does not compete with the binding of Nm23-H1.

The shape of the Nm23 hexamer is very compact and moveable elements around the active site are absent (38), thus preventing any molecules bigger than (d)NTP from entering the small cleft that harbors the active site. For this reason, phosphorylation of other proteins by the Nm23 hexamer is impossible. However, in the dimeric state, where the forcing, close contacts to the other two dimer partners are absent, helical structures near the active site may render more flexible, thus enabling protein binding and phosphorylation in addition to (d)NTPs.

The finding that the NDP kinase activity of complex-bound Nm23-H1 was not altered is not unexpected considering the catalytic mechanism of the enzyme. The first step common to both NDP kinase and protein phosphorylation activity (23) requires binding of a nucleotide and autophosphorylation of the active site histidine residue (Equation 1) (39). This is only possible in an intact steric surrounding of the active site, which is, due to the Ping-Pong mechanism, as well efficient in catalyzing the back-phosphorylation of a nucleoside diphosphate. However, at least one difference between complexed and free Nm23-H1 regarding the active site is apparent: after the reactions shown in Fig. 3, there is virtually no autophosphorylation left at the complex-bound Nm23-H1 (lane 1), whereas the free protein displays a strong autophosphorylation signal. Since only part of the labeled phosphate was transferred to the substrate protein, the only explanation for this finding is that the phosphohistidine bond in the reactive intermediate of complexed Nm23-H1 must be prone to hydrolysis, yielding inorganic phosphate which is unavailable for rephosphorylation of ADP. This can be interpreted as an indirect evidence for a conformation change in the active site, since in the free Nm23-H1 hexamer, water is sterically hindered from attacking the Nδ atom of the imidazol where the phosphate is bound.

The results presented herein are a paradigm for the qualitative change of the activity of an enzyme within a multienzyme complex, representing an expansion of the biochemical function encoded by the primary structure. This may be one of the reasons for the existence of oligomeric multi-enzyme complexes in general (40).

An interesting analogy was observed recently with a double mutant of *Dictyostelium* NDP kinase, being a dimer in solution as opposed to the wild type hexamer. This dimeric form is able to bind to DNA, whereas the hexameric enzyme is not (41). Hence it can be speculated that the hexameric Nm23 protein is nothing but a NDP kinase, whereas all alternative functions are carried out by lower order oligomeric forms.

Only Nm23-H1 but not Nm23-H2 can form a complex with GPDH and thus carry out protein phosphorylations. This finding argues for a possible relevance of the novel Nm23 activity in the tumor metastatic process, since functional studies and expression analysis in tumors suggest that only the Nm23-H1 isoform has metastasis suppressor activity in humans (3, 9–12). Interestingly, a possible implication of GPDH in tumor metastasis was already suggested by Epner *et al.* (42). In their study, increased GPDH expression correlated with cell motility and metastatic potential of rat prostate adenocarcinomas.

GPDH itself is a multifunctional protein that can no longer be considered as a mere housekeeping enzyme. The additional
functions identified so far include binding of cytoskeleton organizing proteins such as tubulin (43), actin (44), and ICAM-1 (45), regulation of gene expression by binding of AU-rich 3'-untranslated regions of mRNA (46) and specific interaction with paraflavin and hepatitis A virus RNA (47, 48), participation in DNA replication as DNA polymerase α-associated, P1,P4-di(adenosine-5')-tetraphosphate-binding protein (49), catalysis of membrane vesicle fusion in endocytosis (50), binding of pathologic neurodegenerative proteins possessing poly-glutamine domains such as Huntington and DRPLA (51), participation in DNA repair as uracil DNA glycosylase (52), and modulation of phosphoribulokinase activity in *Chlamydomonas* chloroplasts (53, 54). In the last mentioned, complex formation with GAPDH alters the conformation and catalytic properties of phosphoribulokinase. This GAPDH activity might be comparable to the effect exerted on Nm23-H1 as described in the present study.

Our data suggest that the Nm23 protein phosphotransferase activity may be specific for GAPDH as a binding partner. The functions of other complexes involving Nm23/NDP kinase are unknown. However, it can be speculated that one possible effect may be the modulation of NDP kinase specificity for nucleoside diphosphates as phosphoacceptors, as it was shown recently for certain complexes of prokaryotic NDP kinase (55, 56).

Considering the highly abundant expression of Nm23-H1 in tumor and even normal tissue (57), a significant portion of this protein may be bound to GAPDH, which is also expressed usually in high levels. Thus a remarkable amount of protein phosphotransferase-active complex may be present in the cells without affecting total NDP kinase activity. This may explain why no correlation was found between NDP kinase activity of tumor and even normal tissue (57), a significant portion of this activity may be specific for GAPDH as a binding partner. The present study.

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