Histidine to aspartate phosphotransferase activity of nm23 proteins: phosphorylation of aldolase C on Asp-319

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nm23 genes have been implicated in the suppression of tumour metastasis and cell motility; however, the biochemical mechanisms for these suppressions are not known. We have previously described the transfer of phosphate from the catalytic histidine residues of nm23 proteins to an aspartic or a glutamic residue on one or more 43 kDa proteins in detergent extracts of bovine brain membranes. To gain a better understanding of this transferase activity, we partly purified this 43 kDa protein and identified aldolases A and C as the major 43 kDa proteins present in the preparation. Aldolase was purified from brain cytosol; its phosphorylation by rat liver nm23 proteins and by recombinant human nm23-H1 was examined. The site of phosphorylation was identified as Asp-319 on aldolase C. The

equivalent residue on aldolase A, a glutamic residue, was not phosphorylated. Aldolase C was rapidly phosphorylated by wild-type nm23-H1 but was not phosphorylated, or was phosphorylated very slowly, by either nm23-H1^{P96S} or nm23-H1^{S120G}, mutants of nm23-H1 that do not suppress cell motility. This is the first identification of a protein that is phosphorylated on an aspartic residue by nm23 proteins. The sequence around Asp-319 of aldolase C has some similarities to those around the histidine residues on ATP-citrate lyase and succinic thiokinase that are phosphorylated by nm23 proteins.

Key words: cell motility, metastasis, nucleoside diphosphate kinases.

INTRODUCTION

nm23 genes have been implicated in the control of tumour metastasis [1]. For many, but not all, types of tumour there is an inverse relationship between the level of *nm23* expression and metastatic potential; overexpression of *nm23* in highly metastatic cell lines decreases their metastatic potentials (reviewed in [2,3]). *nm23* genes are also thought to be involved in cellular proliferation [4] and differentiation [5]. In human tissues the two major forms of *nm23* are *nm23-H1* [6] and *nm23-H2* [7]. The expression of *nm23-H1* has been correlated with the suppression of metastasis [2]; overexpression of human *nm23-H1* suppresses cell motility [8]. A third member of this family is *nm23-DR*, a gene that seems to have a role in early stages of haemopoietic differentiation [9].

The biochemical mechanisms by which nm23 proteins exert their effects on metastasis, motility and differentiation are not known. nm23 proteins are nucleoside diphosphate kinases (NDP kinases) [10] and catalyse the phosphorylation of nucleoside 5'-diphosphates to triphosphates by a Ping Pong mechanism involving a high-energy phosphohistidine intermediate [11]. However, this activity does not account for the effects of nm23 proteins on tumour metastasis [2] or cell motility [8] or for the effect of Awd protein, an NDP kinase, on *Drosophila* development [12].

nm23 proteins have been reported to have other biochemical activities. For example, nm23-H2 has been identified as PuF, a transcription factor of *c-myc* [13]. nm23-H2 also binds to a variety of single-stranded polypyrimidine-rich DNA and RNA sequences [14], and there are conflicting results about its ability to stimulate *myc*-CAT expression *in vivo* [15–17]. nm23-H1, the isoform that has been correlated with suppression of metastasis, does not bind to the *c-myc* promoter.

nm23 proteins have protein kinase or phosphotransferase activities that could provide an alternative mechanism by which

they might affect motility and/or metastasis. Mammalian NDP kinases transfer phosphates from their catalytic histidine residues to histidines at the catalytic sites of ATP-citrate lyase [18] and succinic thiokinase [19]. *Escherichia coli* NDP kinase transfers a phosphate to histidine residues on the bacterial histidine kinases EnvZ and CheA *in vitro* and to a Tar/EnvZ chimera *in vivo* [20]. NDP kinases also transfer phosphates to serine and threonine residues on several unidentified proteins in heat-inactivated cell extracts [21,22].

We have examined several cell and tissue extracts for proteins that can be phosphorylated by NDP kinases. Although most of these experiments have met with limited success, we have identified ATP-citrate lyase in PC12 cell extracts [18] and one or more 43 kDa proteins in bovine brain membrane extracts [23] as substrates for NDP kinases. NDP kinases phosphorylate the 43 kDa bovine brain membrane protein on either a glutamic or an aspartic residue. This 43 kDa protein is rapidly phosphorylated by wild-type nm23-H1 but is not phosphorylated, or is phosphorylated very slowly, by nm23-H1^{P96S} or nm23-H1^{S120G} [23], mutants of nm23-H1 that do not suppress cell motility [8]. In contrast, the rates of phosphorylation of ATP-citrate lyase and succinic thiokinase on histidine residues by nm23-H1^{P96S} and nm23-H1^{S120G} are similar to those by wild-type nm23-H1 [23].

Here we show that aldolases A and C were the major proteins present in partly purified preparations of the 43 kDa bovine membrane protein. Like the 43 kDa membrane protein, bovine brain cytosolic aldolase was rapidly phosphorylated by wild-type nm23-H1 but was not phosphorylated, or was phosphorylated very poorly, by nm23-H1^{P96S} or by nm23-H1^{S120G}. Asp-319 of aldolase C was identified as the site of phosphorylation by nm23-H1. The equivalent residue on aldolase A, a glutamic residue, was not phosphorylated. The amino acid sequence around this aspartic residue is compared with those around the histidine residues on ATP-citrate lyase and succinic thiokinase that are phosphorylated by nm23 proteins.

Abbreviations used: DTT, dithiothreitol; NDP kinase, nucleoside diphosphate kinase.

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MATERIALS AND METHODS

Purification and phosphorylation of rat liver NDP kinase and nm23-H1

Rat liver NDP kinase and recombinant human nm23-H1, nm23-H1^{P96S} and nm23-H1^{S120G} were purified as described previously [18,23]. Phosphorylated NDP kinases or nm23 proteins were made by incubating rat liver NDP kinase or recombinant nm23-H1 proteins with 100 μ M [γ -³²P]ATP (2×10^{15} c.p.m./mol) for 5 min in 100 mM NaCl/5 mM MgCl₂/1 mM dithiothreitol (DTT)/20 mM Tris/HCl (pH 7.5). Phosphorylated NDP kinase or nm23 proteins, [³²P]NDP kinase or [³²P]nm23, were separated from [γ -³²P]ATP by gel filtration on a PD10 column (Pharmacia) equilibrated in 100 mM NaCl/1 mM DTT/20 mM Tris/HCl (pH 7.5) [18]. After gel filtration, phosphorylated NDP kinases contained 0.4–0.5 mol of ³²P/mol of 18 kDa subunit. The concentrations of [³²P]NDP kinase refer to the concentration of ³²P bound to the NDP kinase.

Isolation of aldolases from bovine brain, rat brain and rat muscle

Aldolases were purified from bovine brain, rat brain and rat muscle cytosols by following the method described for the purification of aldolases from rabbit muscle and brain cytosols [24]. Rabbit muscle aldolase was purchased from Sigma.

Phosphorylation of proteins by [³²P]NDP kinase

Protein samples were incubated with either rat liver [³²P]NDP kinase or [³²P]nm23-H1 at room temperature in 100 mM NaCl/5 mM MgCl₂/1 mM DTT/20 mM Tris/HCl (pH 7.5) (some fractions also contained 0.25–0.5% sodium cholate). After 10 min or the duration indicated, phosphorylation was stopped by the addition of 0.5 vol. of SDS-sample buffer [5% (w/v) SDS/25% (v/v) glycerol/180 mM Tris/HCl (pH 8.8)/0.01% Bromophenol Blue/50 mM DTT]. Unless stated otherwise, the samples were frozen on solid CO₂, thawed and then loaded, without being boiled, on an SDS/polyacrylamide gel. After electrophoresis the gels were either dried without fixing or stained for 1 h with 0.20% Coomassie Brilliant Blue in 25% (v/v) propan-2-ol/10% (v/v) acetic acid, destained for 1.5 h in 10% (v/v) acetic acid/5% (v/v) methanol and then dried. Phosphorylated proteins were detected by autoradiography and quantified by phosphorimaging with a BAS2000 system (Fujix).

Purification of a 43 kDa protein from bovine brain membranes that is phosphorylated by NDP kinase

The bovine membrane 43 kDa proteins that are phosphorylated by [³²P]NDP kinase were partly purified as described previously [23]. In brief, membranes from the grey matter of bovine brains were extracted with 3% (w/v) sodium cholate and fractionated on a DEAE-Sephacel column. Fractions from the DEAE-Sephacel column enriched in 43 kDa proteins phosphorylated by [³²P]NDP kinase were pooled and fractionated on a Blue Sepharose column (Pharmacia). Fractions from this column enriched in these 43 kDa proteins were fractionated on a Sephacryl S200 column.

Fractions from the S200 Sephacryl column enriched in these 43 kDa proteins were dialysed against 25 mM NaCl/1 mM EGTA/1 mM DTT/0.25% CHAPS/20 mM Tris/HCl (pH 8.5) at 4 °C and loaded on a Mono Q HR 5/5 column (Pharmacia) equilibrated in this buffer. Proteins were eluted with a 24 ml linear gradient of 25–400 mM NaCl. A fraction eluting in 200 mM NaCl contained 43 kDa proteins that were phos-

phorylated by [³²P]NDP kinase. This fraction was applied to a 2.6 mm $\times$ 250 mm Vydac C₄ reverse-phase HPLC column equilibrated with 0.1% (v/v) trifluoroacetic acid/20% (v/v) acetonitrile; proteins were eluted with a gradient of 20–55% (v/v) acetonitrile at a flow rate of 1 ml/min. Fractions from this column were dried and resuspended in 100 μ l of 2 mM DTT/200 mM Tris/HCl (pH 9). Aliquots from these resuspended fractions were incubated with [³²P]NDP kinase in 150 mM NaCl/5 mM MgCl₂/1% (w/v) CHAPS and examined by SDS/PAGE and autoradiography. Fractions that contained 43 kDa proteins were digested for 20 h at 30 °C with 1 μ g of lysyl endopeptidase (Wako Bioproducts) in the presence of 2 M urea; the resulting peptides were separated on a Vydac C₁₈ reverse-phase HPLC column [18]. Peptides from this HPLC column were sequenced with an Applied Biosystems gas-phase sequencer.

Reduction of phosphorylated aldolase with [³H]NaBH₄

Aldolase (2.5 mg/ml) in 50 mM NaCl/5 mM MgCl₂/1 mM DTT/0.5% sodium cholate/20 mM Tris/HCl (pH 7.5) was incubated for 10 min in the presence or the absence of phosphorylated NDP kinase. The reactions were stopped by the addition of 1% (w/v) SDS; 200 μ l of 62.5 mM [³H]NaBH₄ dissolved in DMSO [25] was added to each 400 μ l sample. After 10 min at room temperature, an equal volume of 50% (v/v) trichloroacetic acid was added. The pellets obtained by centrifugation at 12000 *g* and 4 °C were washed twice with 1 ml of 440 mM HClO₄ [26]. The final pellets were dissolved in 400 μ l of 50% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid. The samples were diluted with 0.1% (v/v) trifluoroacetic acid to give 20% (v/v) acetonitrile, then clarified and applied to a 2.6 mm $\times$ 250 mm Vydac C₄ reverse-phase HPLC column. Proteins were eluted with a gradient of 20–55% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min. The absorbance was monitored at 214 nm; 1 ml fractions were collected. Aliquots from each fraction were counted; samples from those fractions that contained ³H were examined by SDS/PAGE and fluorography. Unphosphorylated aldolase reduced with [³H]NaBH₄ gave one peak of ³H. The ³H in this peak was bound to a polypeptide of approx. 36 kDa, which resulted from reductive cleavage of the aldolase [27]. Phosphorylated aldolase reduced with [³H]NaBH₄ gave two protein peaks that contained radioactivity. One was the same as that obtained with unphosphorylated aldolase. The other peak contained ³H-labelled intact aldolase. The ³H-containing peaks from the phosphorylated aldolase were dried, resuspended in 100 mM Tris/HCl (pH 9.0)/2 M urea and digested for 20 h at 30 °C with 1 μ g of lysyl endopeptidase. The resulting peptides were separated on a 2.1 mm $\times$ 150 mm Vydac C₁₈ reverse-phase HPLC column with a 5–65% (v/v) gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 150 μ l/min. The absorbance was monitored at 214 nm; 2 min fractions were collected. Aliquots from each fraction were counted. The major ³H-labelled peptide in the digest of phosphorylated aldolase was not present in the digest of the polypeptide generated by the reductive cleavage of unphosphorylated aldolase. The ³H-labelled peptide was sequenced with an Applied Biosystems gas-phase sequencer.

RESULTS

Purification of 43 kDa protein(s) phosphorylated by NDP kinase

Bovine brain membrane extracts contain one or more 43 kDa proteins that are phosphorylated by [³²P]NDP kinase [23]. This protein was partly purified by sequential chromatography on

Figure 1 Purification and phosphorylation of 43 kDa bovine brain membrane protein

(A) Proteins eluted from the Mono Q column in 200 mM NaCl were incubated with [32 P]NDP kinase and separated by SDS/PAGE. Lane A, gel stained with Coomassie Brilliant Blue; lane B, autoradiogram of this gel. The region containing the [32 P]NDP kinase was cut off. (B) C_4 reverse-phase HPLC column of proteins eluted from the Mono Q column in 200 mM NaCl. (C) Proteins eluted from the C_4 reverse-phase HPLC column between 16 and 17 min and between 20 and 21 min, and the proteins eluted from the Mono Q column in 200 mM NaCl (MQ) were separated by SDS/PAGE; the gel was stained with Coomassie Brilliant Blue. (D) Equal volumes of fractions from the C_4 column were dried, resuspended, incubated with [32 P]NDP kinase, separated by SDS/PAGE and examined by autoradiography. The positions of molecular-mass markers are indicated at the left of (A) and (C).

DEAE-Sephacel, Blue Sepharose and S200 Sephacryl columns as described previously [23]. The S200 fractions containing the 43 kDa protein were then fractionated on a Mono Q column as described in the Materials and methods section. A fraction that was eluted in 200 mM NaCl contained a 43 kDa protein that was phosphorylated by [32 P]NDP kinase (Figure 1A). This 32 P-labelled protein (Figure 1A, lane B) aligned with the upper portion of a 43 kDa doublet seen in the Coomassie Blue-stained gel (lane A). Proteins that were eluted from the Mono Q column in 200 mM NaCl were applied to a C_4 reverse-phase HPLC column equilibrated in 0.1% (v/v) trifluoroacetic acid/20% (v/v) acetonitrile and eluted with a gradient of 20–55% (v/v)

acetonitrile (Figure 1B). The Mono Q fraction shown in Figure 1(A) was from a different preparation from that shown in Figures 1(B) to 1(D). Fractions that were eluted from the C_4 column between 16 and 17 min and between 20 and 21 min contained proteins of approx. 43 kDa (Figure 1C). The 43 kDa protein that was eluted between 20 and 21 min aligned with the upper portion of the 43 kDa protein doublet in the Mono Q fraction (Figure 1C). Equal volumes of fractions from the C_4 column were incubated with [32 P]NDP kinase (Figure 1D). The 43 kDa protein that was eluted between 20 and 21 min incorporated more 32 P than did the 43 kDa protein from between 16 and 17 min.

Figure 2 Phosphorylation of bovine brain cytosolic aldolase by nm23-H1

(A) Aldolase (12 μ M) was incubated with 260 nM [32 P]nm23-H1 for 15 min in the indicated concentrations of sodium cholate. (B) Aldolase (10 μ M) was incubated in 0.5% sodium cholate for 10 min with 0.7 μ M [γ - 32 P]ATP, [γ - 32 P]GTP or [32 P]nm23-H1. (C) Aldolase (10 μ M) was incubated with 330 nM [32 P]nm23-H1 in 0.5% sodium cholate for the durations indicated. (D) Aldolase (12 μ M) in 0.5% sodium cholate was incubated for 10 min with the indicated concentrations of [32 P]nm23-H1. (E) The indicated concentrations of aldolase in 0.5% sodium cholate were incubated for 10 min with 330 nM [32 P]nm23-H1. (F) Aldolase (12 μ M) was incubated in 0.5% sodium cholate for 10 min with 330 nM [32 P]nm23-H1. After this incubation, the samples were treated as follows: lane 1, frozen immediately after the addition of SDS sample buffer; lane 2, incubated in SDS-sample buffer in boiling water for 2 min; lane 3, incubated for 10 min at 37 $^{\circ}$ C in SDS-sample buffer; lane 4, incubated for 10 min at 37 $^{\circ}$ C in SDS-sample buffer containing 0.5 M HCl; lane 5, incubated for 10 min at 37 $^{\circ}$ C in SDS-sample buffer containing 0.5 M NaOH; lane 6, incubated for 10 min at 37 $^{\circ}$ C in 0.8 M hydroxylamine at pH 5.4; lane 7, incubated for 10 min at 37 $^{\circ}$ C in 0.8 M NaCl at pH 5.4; lanes 8 and 9, samples were precipitated on ice in 20% (w/v) trichloroacetic acid, rinsed with cold 10 mM HCl, dried under vacuum, dissolved in DMSO and incubated at room temperature for 30 min in the absence (lane 8) or presence (lane 9) of 50 mM NaBH $_4$. After these various incubations, the samples were neutralized or SDS-sample buffer was added; they were then analysed by SDS/PAGE and autoradiography. The top panel shows the part of the gel that contained aldolase and the bottom panel the part that contained nm23-H1. The top panel was exposed for longer than the bottom panel.

Fractions that were eluted between 16 and 17 min and between 20 and 21 min were digested with lysyl endopeptidase; the resulting peptides were separated on a C $_{18}$ reverse-phase HPLC column. Sequencing of seven of these peptides showed that the major 43 kDa protein that was eluted between 16 and 17 min was aldolase A and that the major 43 kDa protein that was eluted between 20 and 21 min was aldolase C. Rabbit muscle aldolase A was eluted between 16 and 17 min.

Aldolase is a ubiquitous glycolytic enzyme [24,28]. There are three different isoforms of aldolase; A is present in all tissues, B

is specific to liver and C is found mostly in the brain. Aldolase forms both homotetramers and heterotetramers. Although aldolase is predominately cytosolic, a fraction is bound to membranes [29,30].

Phosphorylation of bovine brain aldolase by NDP kinases

Aldolase was isolated from a soluble extract of bovine brains by using a standard isolation procedure [24] that included the selective elution of aldolase from a phosphocellulose column

with the substrate fructose 1,6-bisphosphate followed by fractionation on DEAE-cellulose. This aldolase contained both aldolases A and C.

Because the phosphorylation of the 43 kDa proteins from bovine brain membranes by NDP kinases was performed in the presence of 0.25–0.5% sodium cholate or CHAPS [23], bovine brain aldolase was phosphorylated by [32 P]NDP kinase in both the presence and the absence of detergent (Figure 2A). The level of aldolase phosphorylation by NDP kinase in the absence of detergent was about one-quarter of that obtained in the presence of 0.5% sodium cholate.

The 43 kDa bovine brain membrane protein was phosphorylated when incubated with [32 P]NDP kinase but not when incubated with either [γ - 32 P]ATP or [γ - 32 P]GTP in the absence of added NDP kinase [23]. Similarly, bovine brain aldolase was phosphorylated when incubated with [32 P]NDP kinase but not when incubated with either [γ - 32 P]ATP or [γ - 32 P]GTP in the absence of added NDP kinase (Figure 2B). Aldolase was also phosphorylated when incubated with [γ - 32 P]ATP and added NDP kinase (results not shown). The time course of aldolase phosphorylation by 70 nM [32 P]nm23-H1 is shown in Figure 2(C). Maximum phosphorylation occurred within 5 min. The amount of 32 P incorporated into aldolase depended on the concentrations of both aldolase and [32 P]NDP kinase (Figures 2D and 2E).

The phosphate incorporated into the brain membrane 43 kDa proteins by incubation with [32 P]NDP kinase is acid-labile and base-labile, is lost on boiling and is removed by hydroxylamine [23]. The phosphate incorporated into brain aldolase by incubation with [32 P]nm23-H1 in the presence of sodium cholate (Figure 2F, top panel) was also acid-labile (lane 4) and base-labile (lane 5), was lost on boiling (lane 2) and was removed by hydroxylamine (lane 6). Simply incubating the phosphorylated aldolase in SDS-sample buffer for 10 min at 37 °C resulted in a substantial loss of 32 P (Figure 2F, top panel, lane 3). These stability measurements indicate that the phosphorylated residue on aldolase was either an aspartic or a glutamic residue because acylphosphates are unstable at both high and low pH [26,27,31]. In contrast, the phosphohistidine in nm23-H1 (Figure 2F, bottom panel) was base-stable (lane 5).

Phosphoaspartic and phosphoglutamic residues can be reduced with NaBH₄ to give free phosphate and either a homoserine or a δ -hydroxy- α -aminovaleric residue [26,27]. Phosphohistidine residues are not reduced by NaBH₄. The phosphate incorporated into aldolase by incubation with [32 P]nm23-H1 in the presence of sodium cholate was lost after treatment with NaBH₄ (Figure 2F, lane 9), but the phosphate incorporated into nm23-H1 was not removed by NaBH₄. This removal of 32 P from the aldolase by NaBH₄ indicates that it was an acylphosphate.

The stoichiometry of aldolase phosphorylation by [32 P]nm23-H1 was estimated from the amount of 32 P bound to aldolase compared with that bound to nm23 after SDS/PAGE. A maximum of 0.01 mol of 32 P was incorporated per mol of aldolase monomer. Because this acylphosphate was very labile, the true stoichiometry might be higher.

As noted above, the level of aldolase phosphorylation by NDP kinase in the absence of detergent was less than that obtained in the presence of sodium cholate (Figure 2A). When high concentrations of bovine brain aldolase (more than 10 μ M) were incubated with [32 P]nm23-H1 in the absence of detergent, only approximately half of the 32 P incorporated into aldolase was acid-labile and base-labile or was removed by boiling (results not shown), indicating that a significant fraction of the phosphate was on residues other than aspartic or glutamic residues. Phosphoamino acid analysis showed that aldolase phosphorylated

Figure 3 Phosphorylation of bovine brain aldolase by nm23-H1, nm23-H1^{P96S} and nm23-H1^{S120G}

(A) Aldolase (12 μ M) was incubated in 0.5% sodium cholate for the durations indicated in the presence of 200 nM [32 P]nm23-H1, [32 P]nm23-H1^{P96S} or [32 P]nm23-H1^{S120G}. (B) Aldolase (0.5 μ M) was incubated in 0.5% sodium cholate for the durations indicated in the presence of 200 nM [32 P]nm23-H1, [32 P]nm23-H1^{P96S} or [32 P]nm23-H1^{S120G}. (C) Aldolase (0.5 μ M) was incubated for the durations indicated in the presence of 200 nM [32 P]nm23-H1, [32 P]nm23-H1^{P96S} or [32 P]nm23-H1^{S120G}. After these incubations the samples were analysed by SDS/PAGE and autoradiography.

by [32 P]nm23-H1 under these conditions contained a very small amount of phosphoserine, less than 0.001 mol of [32 P]-phosphoserine per mol of aldolase monomer.

Bovine brain aldolase phosphorylation by nm23-H1, nm23-H1^{P96S} and nm23-H1^{S120G}

The phosphorylation of aldolase by wild-type [32 P]nm23-H1 was compared with that by two mutant forms of nm23-H1, [32 P]nm23-H1^{P96S} and [32 P]nm23-H1^{S120G} (Figure 3). When a high concentration of aldolase (more than 10 μ M) was used in the presence of sodium cholate, the rate of phosphorylation of aldolase by 200 nM [32 P]nm23-H1 was rapid: maximum phosphorylation occurred within 1 min (Figure 3A). Under these conditions, there was much less phosphorylation of aldolase by either [32 P]nm23-H1^{P96S} or [32 P]nm23-H1^{S120G} (Figure 3A). Similar results were obtained with low concentrations of aldolase (0.5 μ M) in the presence (Figure 3B) and in the absence (Figure 3C) of sodium cholate.

When high concentrations of aldolase (more than 10 μ M) were used in the absence of detergent, the level of aldolase phos-

Figure 4 Amino acid sequence of the site of aldolase phosphorylation by NDP kinase

(A) Sequence of ³H-labelled peptide isolated from a lysyl endopeptidase digestion of phosphorylated aldolase reduced with [³H]NaBH₄, aligned with the corresponding regions of aldolases C and A [32,33,40–42]. Broken lines indicate amino acid residues identical with those in the observed sequence. (B) Comparison of the sequence around the site of aldolase C phosphorylation (Asp-319) by NDP kinase with the corresponding region on aldolase A. Broken lines indicate amino acid residues identical with those in rat aldolase C. The phosphorylated aspartic residue is shown in bold. (C) Comparison of the sites of NDP kinase phosphorylation of aldolase C, ATP-citrate lyase [43], succinyl thiokinase [44], CheA [45] and EnvZ [46]. The phosphorylated residues are shown in bold.

phorylation by [³²P]nm23-H1 was much less than that observed in the presence of detergent; the levels of aldolase phosphorylation by [³²P]nm23-H1^{P96S} and [³²P]nm23-H1^{S120G} were about the same as that by [³²P]nm23-H1 (results not shown). Under these conditions, high aldolase and no detergent, only half of the ³²P incorporated into aldolase seemed to be on an aspartic or a glutamic residue.

Effect of sodium cholate on NDP kinase and aldolase activities

The phosphorylation of aldolase was performed with concentrations of nm23-H1 of more than 70 nM. The NDP kinase activity, conversion of ATP and TDP into ADP and TTP [11], of 30–70 nM wild-type nm23-H1 was compared with those of nm23-H1^{S120G} and nm23-H1^{P96S}. In the absence of detergent, nm23-H1 and nm23-H1^{S120G} had the same NDP kinase activities, to within $\pm 2\%$ S. D. The addition of 0.5% sodium cholate caused a 15–20% decrease in their NDP kinase activities. In the absence of detergent, the NDP kinase activity of nm23-H1^{P96S} was $91 \pm 9\%$ that of wild-type nm23-H1. However, in the presence of 0.5% sodium cholate, the NDP kinase activity of nm23-H1^{P96S} was only $54 \pm 3\%$ that of wild-type nm23-H1. The addition of 0.5% sodium cholate caused a 5% decrease in aldolase enzymic activity.

Identification of aldolase residue phosphorylated by NDP kinase

To determine the site of aldolase phosphorylation by NDP kinase it was necessary to reduce the phosphorylated aldolase with [³H]NaBH₄. Phosphoaspartic and phosphoglutamic residues are reduced with [³H]NaBH₄ to give [³H]homoserine or δ -hydroxy- α -amino[³H]valeric residues respectively [25–27].

Aldolase was phosphorylated by NDP kinase in the pres-

Figure 5 Phosphorylation of rabbit muscle and rat brain aldolases by NDP kinase

(A) Rat brain aldolase (2 or 20 μ M) was incubated with various concentrations of [³²P]NDP in the presence or absence of 0.5% sodium cholate for 10 min. (B, C) Rat brain aldolase (10 μ M) was incubated in the presence (B) or the absence (C) of 0.5% sodium cholate for 10 min with 500 nM [³²P]NDP kinase. After this incubation, the samples were treated as follows: lanes 1, frozen immediately after adding SDS sample buffer; lanes 2, incubated in SDS-sample buffer in boiling water for 2 min; lanes 3, incubated for 10 min at 37 $^{\circ}$ C in SDS-sample buffer; lanes 4, incubated for 10 min at 37 $^{\circ}$ C in SDS-sample buffer containing 0.5 M HCl; lanes 5, incubated for 10 min at 37 $^{\circ}$ C in SDS-sample buffer containing 0.5 M NaOH. (D) Bovine brain, rabbit muscle and rat brain aldolases (each 10 μ M) were incubated in 0.5% sodium cholate for 2 or 10 min in the presence of 500 nM [³²P]nm23-H1.

ence of 0.5% sodium cholate, reduced with [³H]NaBH₄ and fractionated on a C₄ column as described in the Materials and methods section. Fractions containing ³H-labelled aldolase were digested with lysyl endopeptidase; the resulting peptides were separated on a C₁₈ reverse-phase HPLC column. The major ³H-labelled peptide from this digest was not present in digests of unphosphorylated aldolase reduced with [³H]NaBH₄. This ³H-labelled peptide was sequenced; aliquots from 46 cycles were counted. The sequence of the first 29 residues of this peptide was YSPEEIAMATVTALRRTVAPPVXPVTF. After this cycle, the sequence was unreadable. This sequence (Figure 4A) is identical with that of residues 244–271 of rat aldolase C [32] and differs by one residue from that of human aldolase C [33]. No ³H was released during the 46 cycles of sequencing. The filter containing the remaining peptide was counted and found to contain the ³H, indicating that the ³H-labelled amino acid is between residue 290 and Lys-330, the next lysine residue in aldolase C. Between residues 290 and 330 of human and rat aldolases C there are two glutamic residues (Glu-326 and Glu-327) and one aspartic residue (Asp-319).

The above result indicates that the site of aldolase C phosphorylation by NDP kinase is between residues 244 and 330. Between these two residues in both rat and human aldolases C [32,33] there is only one aspartic residue (Asp-319). To determine whether NDP kinase phosphorylated aldolase on an aspartic residue, unphosphorylated bovine brain aldolase and aldolase

phosphorylated by NDP kinase were reduced with $[^3\text{H}]\text{NaBH}_4$ and hydrolysed with 6 M HCl for 20 h at 110 °C [25,27]. This treatment converts phosphoglutamate into δ -hydroxyl- α -amino- $[^3\text{H}]$ valeric acid and phosphoaspartate into $[^3\text{H}]$ homoserine lactone. These hydrolysates were subjected to high-voltage paper electrophoresis at pH 1.9 [25,27]. The hydrolysate from phosphorylated aldolase contained a peak of radioactivity that co-migrated with the homoserine lactone standard. The hydrolysate from unphosphorylated aldolase did not contain this peak of radioactivity. There was no peak of radioactivity that co-migrated with the δ -hydroxyl- α -aminovaleic acid standard. Brain aldolase phosphorylated by NDP kinase therefore contained a phosphoaspartic residue, indicating that the site of phosphorylation on aldolase C is Asp-319.

Phosphorylation of aldolases from rabbit muscle and rat brain

The phosphorylation of rat brain aldolase by NDP kinase was similar to that of bovine brain aldolase except that it was less sensitive to sodium cholate. The level of phosphorylation of rat brain aldolase in the absence of detergent was more than half that observed in the presence of sodium cholate (Figure 5A), and the phosphates that were incorporated into rat brain aldolase by incubation with $[^{32}\text{P}]\text{NDP}$ kinase in the presence (Figure 5B) or absence (Figure 5C) of sodium cholate were acid-labile (lane 4) and base-labile (lane 5) and were lost on boiling (lane 2), indicating that the phosphorylated residue was either an aspartic or a glutamic residue.

Rat and rabbit muscle aldolases contain only aldolase A; they were phosphorylated by $[^{32}\text{P}]\text{NDP}$ kinase to a much smaller extent than were either bovine brain aldolase or rat brain aldolase, which contain both aldolases A and C (Figure 5D). The small amounts of phosphate incorporated into muscle aldolases, even in the presence of sodium cholate, were mostly stable to acid and base (results not shown).

DISCUSSION

The results presented here indicate that the 43 kDa bovine membrane protein phosphorylated by NDP kinase was aldolase. Although brain aldolase is mostly cytosolic, a fraction is associated with membranes [29,30]. Under the conditions used with the 43 kDa membrane protein, bovine brain cytosolic aldolase was phosphorylated by NDP kinase primarily on an aspartic residue. The site of phosphorylation seems to be Asp-319 on aldolase C. Aldolase C is found mostly in the brain but it is found in low levels in other tissues [28] and in some types of tumour [34–36].

The stoichiometry of the phosphorylation of brain aldolase by nm23-H1 on aspartic residue, as determined by the amount of ^{32}P bound to aldolase after SDS/PAGE, was only approx. 0.01 mol of ^{32}P /mol of aldolase monomer. However, this acylphosphate was very labile. Simply incubating the phosphorylated brain aldolases in SDS-sample buffer for 10 min at 37 °C resulted in substantial losses of ^{32}P (Figures 2F, 5B and 5C). The phosphoaspartic residues of CheY [27,37] and NR₁ [26], bacterial 'two-component' response regulator proteins, have hydrolysis half lives of approx. 6 s and 3.5 min respectively. It is therefore possible that the observed low stoichiometry of aldolase phosphorylation by nm23-H1 is due in part to the instability of the phosphoaspartic residue. The stoichiometry of the phosphorylation of ATP-citrate lyase by NDP kinase on a histidine residue is 0.4 mol of ^{32}P /mol of ATP-citrate lyase monomer [18].

In addition to this phosphorylation of an aspartic residue, NDP kinases phosphorylated bovine brain aldolase on serine

residues. The relative extents of these two phosphorylation reactions depended on the aldolase concentration and on whether the reaction contained sodium cholate; however, under all conditions the phosphorylation of serine residues was very low, less than 0.001 mol of ^{32}P /mol of aldolase monomer. The phosphorylation of bovine brain aldolase by nm23-H1 can be summarized as follows: (1) the apparent affinity of phosphorylated nm23-H1 for Asp-319 on aldolase C is greater than its apparent affinity for the serine residue(s) phosphorylated; (2) the phosphorylation of aspartic but not serine residues is increased by sodium cholate; and (3) wild-type nm23-H1 phosphorylates aspartic residues more rapidly than does nm23-H1^{S120G} or nm23-H1^{P96S}. At low concentrations of aldolase, the phosphorylation of aspartic residues predominates; there is more aldolase phosphorylation by wild-type nm23-H1 than by nm23-H1^{P96S} or nm23-H1^{S120G}. At high concentrations of aldolase in the absence of detergent, both aspartic and serine phosphorylations occur; there is little difference in the observed phosphorylation by wild-type nm23-H1 and those by nm23-H1^{P96S} or nm23-H1^{S120G}. In the presence of detergent and at high aldolase concentrations, aspartic phosphorylation predominates; the observed phosphorylation by nm23-H1 is greater than that by nm23-H1^{P96S} or nm23-H1^{S120G}.

The phosphorylation of rat brain aldolase by NDP kinase was similar to that of bovine brain aldolase except that it was less affected by sodium cholate. In both the presence and the absence of sodium cholate, NDP kinase seemed to phosphorylate rat brain aldolase predominantly on an aspartic residue. Muscle aldolases were phosphorylated by NDP kinases to a much smaller extent than were brain aldolases; the residues phosphorylated were neither aspartic nor glutamic residues. Muscle aldolases contain only aldolase A.

NDP kinases seem to phosphorylate aldolase C on Asp-319. The equivalent residue on aldolase A is a glutamic residue (Figure 4B). Another difference in the amino acid sequence in this region is that aldolase C contains a glycine residue at position 322. The equivalent position in aldolase A is a lysine residue. The crystal structure of rabbit aldolase A shows that Glu-318, equivalent to Asp-319 of aldolase C, is located on an exposed surface of the enzyme in a loop between two α -helices [38].

Rat liver and recombinant human NDP kinases can also transfer phosphates to histidine residues on ATP-citrate lyase [8] and succinic thiokinase [19]. The amino acid sequences around these histidine residues have some similarities to that around Asp-319 of aldolase C (Figure 4C). Most noticeably, they all contain the sequence AGA C-terminal to the site of phosphorylation. Therefore, although nm23 proteins phosphorylate an aspartic residue on aldolase C and histidine residues on ATP-citrate lyase and succinic thiokinase, nm23 proteins might be binding to similar amino acid sequences on all three proteins. *E. coli* NDP kinase phosphorylates histidine residues on the histidine kinases EnvZ and CheA [20]. The sequences around the phosphorylated histidine residues of EnvZ and CheA have no obvious similarity to the sites of phosphorylation on aldolase or ATP-citrate lyase (Figure 4C).

Even when very high concentrations of brain aldolase (20 μM) were used, only 2–4% of the ^{32}P that bound to NDP kinases was transferred to aldolase. This suggests that only a small fraction of the phosphorylated NDP kinase was capable of transferring a phosphate to aldolase. This is not unexpected because the catalytic histidine residue of NDP kinase is at the bottom of a cleft and the phosphate bound to this residue does not seem to be in a position favourable for transfer to another protein [39]. However, fully folded phosphorylated NDP kinase

might be in equilibrium with partly folded intermediates. These partly folded intermediates might be responsible for the protein phosphotransferase activity. Sodium cholate caused a decrease in NDP kinase activity, but it increased the phosphorylation of brain aldolase by NDP kinase on aspartic residues. This is consistent with partly unfolded NDP kinase's being responsible for this protein phosphotransferase activity.

A dimer of nm23-H1 complexed with a dimer of glyceraldehyde-3-phosphate dehydrogenase has been isolated from human erythrocytes [22]. This complex has a higher serine/threonine protein phosphotransferase activity than does the purified nm23-H1 hexamer; the phosphohistidine in this complex is more exposed to solvent. The small fraction of phosphorylated nm23 that transfers phosphate to an aspartic residue on aldolase C might have a conformation similar to that of the nm23 complexed with glyceraldehyde-3-phosphate dehydrogenase.

Wild-type nm23-H1 phosphorylated aldolase C on an aspartic residue much more rapidly than did either nm23-H1^{S120G} or nm23-H1^{P96S}. This histidine to aspartic phosphotransferase activity seems to be correlated with the inhibition of cell motility. Transfection with wild-type nm23-H1, but not with nm23-H1^{S120G} or nm23-H1^{P96S}, inhibits the motility of human breast carcinoma cells [8]. Other activities of nm23 proteins, such as NDP kinase activity, histidine to histidine phosphotransferase activity and binding to the *c-myc* promoter, are not correlated with the inhibition of cell motility. At present it is not known whether the phosphorylation by nm23 of an aspartic residue on aldolase C or on some other protein is involved in suppressing motility and/or metastasis. Nevertheless, the identification of aldolase C as a substrate for phosphorylation by nm23 proteins and the identification of the site of phosphorylation might help in the identification of more physiologically relevant substrates. Our inability to detect these substrates in cell extracts might reflect a combination of their being present in low concentrations and the instability of acylphosphates.

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