Phosphorylation of ATP-Citrate Lyase by Nucleoside Diphosphate Kinase*

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Rat liver nucleoside diphosphate kinase (NDPK) and PC12 cell cytosol were used to determine whether NDPK could function as a protein kinase. NDPK was phosphorylated on its catalytic histidine using [γ-32P]ATP, and the phosphorylated NDPK separated from [γ-32P]ATP. The addition of phosphorylated NDPK to dialyzed PC12 cell cytosol resulted in the phosphorylation of a protein with a subunit molecular mass of about 120 kDa. This phosphorylation occurred by a direct transfer of a phosphoryl group from the catalytic histidine of NDPK to a histidine on the 120-kDa protein. The 120-kDa protein was partially purified and shown by peptide sequencing to be ATP-citrate lyase. ATP-citrate lyase is the primary source of cytosolic acetyl-CoA. NDPK phosphorylated the histidine at the catalytic site of ATP-citrate lyase. This histidine can also be phosphorylated by ATP, and its phosphorylation is the first step in the conversion of citrate and CoA to oxaloacetate and acetyl-CoA by ATP-citrate lyase. The level of phosphorylation of PC12 cell ATP-citrate lyase by phosphorylated NDPK was comparable with that by ATP. Thus, in addition to its nucleoside diphosphate kinase activity, NDPK can function as a protein kinase.

Nucleoside diphosphate kinase (NDPK),1 catalyzes the phosphorylation of nucleoside 5′-diphosphates to triphosphates by a ping pong mechanism involving a high energy phosphorylated enzyme intermediate (1).

\[ N_1TP + E \rightarrow N_1DP + E-P \]

**REACTION 1**

\[ N_2DP + E-P \rightarrow N_2TP + E \]

**REACTION 2**

The high energy phosphate is usually supplied by ATP, and NDPK is thought to be responsible for maintaining nucleoside triphosphate pools. Most NDPKs autophosphorylate on a histidine residue at their catalytic sites (2–6). NDPKs from Myxococcus xanthus (4) and humans (5) have been reported to autophosphorylate on both histidine and serine residues. NDPK from rat mast cells has been reported to contain a phosphorylated aspartic or glutamic acid at its catalytic site (7).

The Drosophila awd gene and the murine nm23 genes encode NDPKs (8). The awd gene is essential for normal Drosophila development. Mutations in this gene cause severe developmental defects and result in death of the larvae (9). nm23 genes have been implicated in control of tumor metastasis. For some, but not all types, of tumor, there is an inverse relationship between the level of nm23 expression and metastatic potential (10–19). nm23 genes are also thought to be involved in cellular proliferation (20) and differentiation (21).

The simple maintenance of nucleoside 5′-triphosphate pools does not appear to explain the involvement of NDPK in these various cellular processes, and it seems possible that NDPK might have other activities. NDPK has been reported to be associated with GTP-binding proteins (22–25), and it has been suggested that NDPK might be involved in regulating GTP binding to these proteins. NDPK has also been reported to bind to DNA and stimulate c-myc transcription in vitro (26, 27).

We have reported previously that a GTP-binding protein that regulates excocytosis in rat pheochromocytoma PC12 cells may interact with NDPK (25). While investigating this interaction, we observed what appeared to be phosphorylation of NDPK and became interested in both the autophosphorylation of NDPK and whether NDPK might function as a protein kinase. To examine the latter possibility, rat liver NDPK containing 32P at its catalytic histidine was used to phosphorylate an extract of PC12 cells. NDPK appeared to directly transfer a phosphate from its catalytic histidine to a histidine on another protein. This protein was isolated and shown to be ATP-citrate lyase. This enzyme is the primary source of cytosolic acetyl-CoA which is used in a number of biosynthetic pathways, including lipogenesis and cholesterol synthesis. The phosphorylation of ATP-citrate lyase by NDPK suggests that NDPK may have a role in the regulation of membrane biosynthesis. It also seems possible that NDPK can phosphorylate proteins other than ATP-citrate lyase.

**MATERIALS AND METHODS**

PC12 Cell Cytosol—PC12 cells were cultured as described previously (28). A cytosolic extract of PC12 cells was prepared by suspending 0.3 g of cells in 1.5 ml of 20 mM PIPES, pH 7.0, 140 mM potassium glutamate, 2 mM MgCl2, 5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, and 20 μg/ml soybean trypsin inhibitor on ice. The cells were lysed by 20 passages through a 25-gauge needle and centrifuged for 2 min at 12,000 × g to remove nuclei and intact cells. The supernatant was centrifuged for 1 h at 100,000 × g to give cytosolic and membrane fractions. The cytosolic fraction was dialyzed against 140 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, 5 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, and 1 mM PMSF. The cells were lysed with a glass Teflon homogenizer. The extract was clarified by centrifugation at 100,000 × g for 1 h and fractionated with (NH4)2SO4. The 25–45% (NH4)2SO4 pellet was dialyzed overnight against 50 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 20 mM Tris, pH 7.5 at 4 °C, for 3 days with two buffer changes.

Partial Purification of the 120-kDa Phosphoprotein—For isolation of the 120-kDa phosphoprotein 4–5 g of PC12 cells were suspended in 25 ml of cold 20 mM HEPES, pH 7.4, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, 5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 1 mM PMSF. The cells were lysed with a glass Teflon homogenizer. The extract was clarified by centrifugation at 100,000 × g for 1 h and fractionated with (NH4)2SO4. The 25–45% (NH4)2SO4 pellet was dialyzed overnight against 50 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 20 mM Tris, pH 7.5 at 4 °C, clarified by centrifugation at
Isolation and Characterization of Phosphorylated NDPK—Rat liver NDPK incubated with \([γ-32P]ATP\) incorporated a maximum of 0.5–0.6 mol of \(^{32}\text{P}/\text{mol of NDPK} 18\text{-kDa subunit}.

When 4 \(μ\text{M} \text{NDPK} \) was used, 5 \(μ\text{M} \ [γ-32P]ATP \) gave half-maximum phosphorylation, and 50 \(μ\text{M} \ [γ-32P]ATP \) gave maximum phosphorylation. The time course of phosphorylation of NDPK was rapid with maximum phosphorylation occurring in less than 5 s at room temperature. When 100 \(μ\text{M} \ [γ-32P]ATP \) was used, there was no incorporation of \(^{32}\text{P} \) into NDPK. When the phosphorylation reaction was carried out with 50 \(μ\text{M} \ [γ-32P]ATP \) in 10 mM EDTA instead of 5 mM MgCl\(_2\), NDPK still incorporated approximately 0.6 mol of \(^{32}\text{P}/\text{mol of NDPK} 18\text{-kDa subunit}.

Gel filtration was used to separate phosphorylated NDPK, \([γ-32P]NDPK\), from \([γ-32P]ATP\). The concentrations of \([γ-32P]NDPK\) given in this paper refer to the concentration of \(^{32}\text{P} \) bound to NDPK after gel filtration. Thin layer chromatography showed that 1 \(μ\text{M} \ [γ-32P]NDPK (0.4–0.5 \text{ mol of } [γ-32P]NDPK 18\text{-kDa subunit}) \) contained less than 25 \(μ\text{M} \ [γ-32P]ATP \) of \([γ-32P]NDPK \) for 30 min at 37°C in 1 \(M \) KOH resulted in no loss of bound \(^{32}\text{P}\), but incubation of \([γ-32P]NDPK \) for 30 min at 37°C in 1 \(M \) HCl resulted in loss of 98% of the bound \(^{32}\text{P}\). This indicates that most of the \(^{32}\text{P} \) bound to NDPK was on a histidine as phosphohistidines are acid-labile and base-stable (33, 34). In contrast, phosphoserine, phosphothreonine, and phosphotyrosine are acid-stable, and phosphoglutamic acid and phosphoaspartic acid are both acid- and base-labile. Hydroxylamine hydrolyzes phosphohistidines, phosphoglutamates, and phosphoaspartates (33). Incubation of \([γ-32P]NDPK \) in hydroxylamine resulted in the loss of 98% of the bound \(^{32}\text{P}\). Phosphoglutamic and phosphoaspartic acids, but not phosphohistidine, can be readily cleaved by treatment with sodium borohydride (32, 33). Incubation of \([γ-32P]NDPK \) with 25 \(m\text{M} \) sodium borohydride did not cause any release of \(^{32}\text{P}\). Phosphohistidines are thermolabile (6). Incubation of \([γ-32P]NDPK \) for 30 min at 37°C in a neutral buffer resulted in a loss of about 30% of the bound \(^{32}\text{P}\), and a 2-min incubation in boiling water resulted in the loss of more than 95% of the bound \(^{32}\text{P}\).

The addition of GDP, UDP, or ADP to \([γ-32P]NDPK \) resulted in the \(^{32}\text{P} \) being transferred to the nucleoside diphosphates to give \([γ-32P]triphosphates \) (data not shown). After incubation with 50 \(μ\text{M} \) GDP, UDP, or ADP, less than 2% of the \(^{32}\text{P} \) remained bound to NDPK, indicating that most of the \(^{32}\text{P} \) bound to NDPK was at the catalytic site.

Phosphorylation of Cytosolic Proteins by \([γ-32P]NDPK\)—Initially the phosphorylation of cytosolic PC12 cell proteins by \([γ-32P]NDPK \) was analyzed using Laemmli SDS-PAGE and fixing the gel in 10% acetic acid. Protein phosphorylation was detected by autoradiography. Incubation of dialyzed PC12 cell cytosol with 1 \(μ\text{M} \ [γ-32P]ATP \) resulted in a very low level of protein phosphorylation compared to that obtained with 1 \(μ\text{M} \ [γ-32P]ATP \) (data not shown), and there was no convincing evidence for a direct transfer of \(^{32}\text{P} \) from \([γ-32P]NDPK \) to other proteins. However, phosphohistidine, phosphoarginine, and phosphoseryllysine are unstable under conditions used for Laemmli SDS-PAGE. For example, less than 1% of \(^{32}\text{P} \) bound to NDPK remained after Laemmli SDS-PAGE and acid fixing. Conditions used for SDS-PAGE were modified as described under "Materials and Methods" to stabilize phosphohistidines, phosphoserines, and phosphoarginines. This method is referred to as basic SDS-PAGE.

PC12 cell cytosol was incubated in MgCl\(_2\) with 1 \(μ\text{M} \ [γ-32P]ATP \) or 1 \(μ\text{M} \ [γ-32P]NDPK \) and the products analyzed by basic SDS-PAGE and autoradiography (Fig. 1). While the sam-
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partially purified 120-kDa protein with \([\gamma-^{32}P]ATP\) in either MgCl_{2} or EDTA resulted in the incorporation of 0.4–0.5 mol of \(^{32}P/mol\) of 120-kDa protein. There was no detectable \(^{32}P\) incorporation into other proteins. The \(^{32}P\) incorporated into the 120-kDa protein was acid-labile and base-stable, which is consistent with a histidine residue being phosphorylated in both MgCl_{2} and in EDTA. A comparison of the autoradiograms of the 120-kDa protein phosphorylated with \([\gamma-^{32}P]ATP\) with those of the 120-kDa protein phosphorylated with \([32P]\)NDPK (Fig. 3) indicate that the 120-kDa protein phosphorylated by \([32P]\)NDPK also contained about 0.4 mol of \(^{32}P/mol\) of 120-kDa protein.

Thin layer chromatography was used to directly show the presence of phosphohistidine. \(^{32}P\) Labeled 120-kDa protein was separated from free \([\gamma-^{32}P]ATP\) and hydrolyzed in KOH. The resulting products were analyzed by thin layer chromatography. About half of the \(^{32}P\) remained at the origin as free phosphate, and the other half co-migrated with the N\(^{1}\)-phosphohistidine standard (Fig. 4, lanes 1 and 2). An alkali digest of \([^{32}P]\)NDPK gave a \(^{32}P\) labeled product that migrated at the solvent front (Fig. 4, lane 3). This is where N\(^{1}\)-phosphohistidine should migrate. NDPK is thought to contain an N\(^{1}\)-phosphohistidine (35). An alkali digest of a mixture of \([^{32}P]\)NDPK and the 120-kDa protein phosphorylated by \([^{32}P]\)NDPK gave both N\(^{1}\) and N\(^{3}\)-phosphohistidines.

Direct Transfer of \(^{32}P\) from \([^{32}P]\)NDPK to the 120-kDa Protein—Glucose and hexokinase were used to examine the possibility that the phosphorylation of the 120-kDa protein by \([^{32}P]\)NDPK was due to a small amount of \([\gamma-^{32}P]ATP\) which was either present in the \([^{32}P]\)NDPK or formed from contaminating ADP. The addition of 1.7 mM glucose and 1.5 units/ml hexokinase completely prevented the phosphorylation of the partially purified 120-kDa protein in MgCl_{2} by 1 \(\mu\)M \([\gamma-^{32}P]ATP\), but not its phosphorylation by 1 \(\mu\)M \([^{32}P]\)NDPK (Fig. 5). When five times as much glucose and hexokinase were used, there was a decrease in the phosphorylation of the 120-kDa protein by \([^{32}P]\)NDPK.

Nondenaturing gel electrophoresis was also used to look for a direct transfer of \(^{32}P\) from \([^{32}P]\)NDPK to the 120-kDa protein. NDPK and partially purified 120-kDa protein were electro-
The blotted proteins were then incubated either with 200 nM [\(^{32}\)P]NDPK or 200 nM [\(^{32}\)P]GTP for 1 h at room temperature. The blots were washed and analyzed by autoradiography. The positions of NDPK and 120-kDa proteins are indicated. [\(^{32}\)P]NDPK used in this experiment was made by phosphorylating NDPK with [\(^{32}\)P]GTP and separating [\(^{32}\)P]NDPK from [\(^{32}\)P]GTP by gel filtration.

**Identification of 120-kDa Phosphoprotein as ATP-Citrate Lyase**

The partially purified 120-kDa protein (50 \(\mu\)g/ml) was incubated in Buffer A containing 5 mM MgCl\(_2\) and either 1 \(\mu\)M [\(^{32}\)P]ATP (lanes 1-4) or 1 \(\mu\)M [\(^{32}\)P]NDPK (lanes 5-8). After 10 min at room temperature, the samples in lanes 1 and 5 were frozen. The following additions were made to the other samples: none (lanes 2 and 6), 10 mM citrate (lanes 3 and 7), 25 \(\mu\)M ADP (lanes 4 and 8). After an additional 15 min at room temperature, the samples were analyzed by basic SDS-PAGE and autoradiography.

**Fig. 7. Citrate and ADP remove \(^{32}\)P from the 120-kDa protein.**

Partially purified 120-kDa protein (50 \(\mu\)g/ml) was incubated in Buffer A containing 5 mM MgCl\(_2\) and either 1 \(\mu\)M [\(^{32}\)P]ATP (lanes 1-4) or 1 \(\mu\)M [\(^{32}\)P]NDPK (lanes 5-8). After 10 min at room temperature, the samples in lanes 1 and 5 were frozen. The following additions were made to the other samples: none (lanes 2 and 6), 10 mM citrate (lanes 3 and 7), 25 \(\mu\)M ADP (lanes 4 and 8). After an additional 15 min at room temperature, the samples were analyzed by basic SDS-PAGE and autoradiography.

The rate of transfer of phosphate from NDPK to ATP-citrate lyase was determined using 0.1–0.5 \(\mu\)M [\(^{32}\)P]NDPK and 5 \(\mu\)M ATP-citrate lyase 120-kDa subunit. After 2 min, about half of the [\(^{32}\)P] was transferred to ATP-citrate lyase. When [\(^{32}\)P]ATP

The phosphorylation of NDPK and the 120-kDa protein after native gel electrophoresis. The following were electrophoresed on nondenaturing polyacrylamide gels and transferred to nitrocellulose: lanes 1 and 4, 25 \(\mu\)g of NDPK; lanes 2 and 5, 7 \(\mu\)g of protein from Superose 12 column fractions containing the 120-kDa protein; lanes 3 and 6, 140 \(\mu\)g of protein from a Mono Q column fraction containing the 120-kDa protein. The blots were washed and incubated with either 200 nM [\(^{32}\)P]NDPK or 200 nM [\(^{32}\)P]GTP for 1 h at room temperature. The blots were washed and analyzed by autoradiography. The positions of NDPK and 120-kDa proteins are indicated. [\(^{32}\)P]NDPK used in this experiment was made by phosphorylating NDPK with [\(^{32}\)P]ATP and separating [\(^{32}\)P]NDPK from [\(^{32}\)P]GTP by gel filtration.
was used, ATP-citrate lyase was maximally phosphorylated in less than 10 s.

**DISCUSSION**

Rat liver NDPK incorporated 0.5–0.6 mol of $^{32}$P/mol of 18-kDa subunit. In agreement with most of the data in the literature (2, 3, 6), the phosphorylated residue was primarily histidine. As only 2% of the $^{32}$P incorporated into rat liver NDPK was acid-stable, autophosphorylated rat liver NDPK appeared to contain less than 0.01 mol of phosphoserine or phosphothreonine per 18 kDa subunit. As reported for NDPK in extracts from human colon cancer tissues (45) and for NDPKs from Xenopus oocytes (46) and Myxococcus xanthus (4), rat liver NDPK autophosphorylated in EDTA. Oocyte NDPK and a commercial bovine liver NDPK preparation have low levels of nucleoside diphosphate kinase activity in EDTA (46). Rat liver NDPK had nucleoside diphosphate kinase activity in EDTA, but the rate at which it formed UTP from UDP and ATP in EDTA was only about 1% the rate in MgCl$_2$ (data not shown).

Incubation of PC12 cell cytosol with $^{32}$P NDPK resulted mainly in the phosphorylation of a single protein with a mobility on SDS gels of about 120 kDa. This phosphorylation appears to result from a direct transfer of $^{32}$P from the histidine at the catalytic site of NDPK to a histidine on the 120-kDa protein. Sequencing of peptides from the 120-kDa protein showed that it was ATP-citrate lyase. The only difference in the phosphorylation of ATP-citrate lyase and that of the 120-kDa protein is that the phosphorylation of ATP-citrate lyase is reported to require a divalent cation (44). The 120-kDa protein was phosphorylated in EDTA, but the rate of phosphorylation was slower in EDTA than in MgCl$_2$ and higher concentrations of ATP were required for phosphorylation in EDTA (data not shown). $^{32}$P NDPK appeared to phosphorylate the histidine at the active site of ATP-citrate lyase.

The amino acid sequence of residues 560–800 of rat liver ATP-citrate lyase has 33% sequence identity with residues 60–290 of the $\alpha$ chain of E. coli succinyl-CoA synthetase (38). These enzymes catalyze similar reactions, and both autophosphorylate on a catalytic histidine (47). The sequence around the catalytic histidine of succinyl-CoA synthetase, GHAGA, is the same as that around the catalytic histidine of ATP-citrate lyase. NDPK from Pseudomonas aeruginosa associates and copurifies with succinyl-CoA synthetase (48). It was suggested that NDPK might either phosphorylate this succinyl-CoA synthetase or funnel ATP to its active site. It has also been suggested that in the mitochondrial matrix NDPK interacts with succinyl-CoA synthetase (49).

NDPK has been reported to co-purify with microtubules, and immunofluorescence suggests some NDPK is bound to microtubules in the cell (8). However, this interaction is not very strong as NDPK does not appear to bind to purified microtubules (50). Most tubulins contain the sequence FGGGQA, which is similar to the amino acid sequence FHAGA around the catalytic histidine of ATP-citrate lyase. (Q for H and S for A are considered conservative replacements). This sequence similarity suggests that these residues might be the site where NDPK binds to microtubules.

While histidine kinases in eukaryotes are only just beginning to be identified, a number of bacterial histidine kinases have been extensively studied (51). Most of these kinases autophosphorylate on a histidine and then usually transfer the phosphate to an acyl group either on the same or different protein. One of the most extensively characterized processes in bacteria that involves histidine phosphorylation is the phosphoenolpyruvate-sugar phosphotransferase system (PTS). PTS transfers a phosphate from phosphoenolpyruvate to a sugar hydroxyl via a series of transfer proteins (52). A phosphoryl group is transferred sequentially from phosphoenolpyruvate to enzyme I, from enzyme I to the protein HPr, from HPr to enzyme IIA, from enzyme IIA to enzyme IIB, and from enzyme IIB to a sugar. Enzyme I, HPr, and enzyme IIA are all phosphorylated on histidines.

There are two examples of PTS proteins being phosphorylated on their active site histidines by kinases other than PTS transfer proteins (52). Enzyme I can be phosphorylated on its active site histidine by phosphoenolpyruvate and by acetate kinase, and HPr can be phosphorylated on its active site histidine by phosphoenolpyruvate and by a glycerol kinase. These reactions are analogous to that reported here for the phosphorylation of ATP-citrate lyase by ATP and by phosphorylated NDPK.

The physiological significance of the phosphorylation of ATP-citrate lyase by NDPK is unclear as it is phosphorylated much more rapidly by ATP than by NDPK. However, in addition to binding ATP, ATP-citrate lyase binds citrate, CoA, ADP, oxaloacetate, and acetyl-CoA, and it is possible that the binding of one of these substrates or products may inhibit the phosphorylation of ATP-citrate lyase by ATP but not by phosphorylated NDPK. Alternatively, the binding of NDPK to the catalytic site ATP-citrate lyase might inhibit the phosphorylation of ATP-citrate lyase by ATP and, thereby, reduce the rate of acetyl-CoA formation.

ATP-citrate lyase is the primary source of cytosolic acetyl-CoA, which is used in a number of biosynthetic pathways, including fatty acid, cholesterol, and ganglioside biosynthesis. A change in the rate of acetyl-CoA production could affect the synthesis of one or more of these molecules all of which have been implicated in tumorigenesis and/or cell growth (53-55). Recently, a prognostic molecule in tumor cells of breast cancer, OA-519, has been shown to be fatty acid synthetase (53). Tumors marked by OA-519 were nearly four times more likely to recur and metastasize as tumors not marked by this antigen. Inhibition of fatty acid synthesis inhibited the growth of tumor cells with high levels of fatty acid synthetase (53). An inhibition of ATP-citrate lyase could also result in a decrease in fatty acid synthesis.

The results presented here demonstrate that NDPK can phosphorylate ATP-citrate lyase. Whether this phosphorylation is relevant to the role of NDPK in differentiation and metastasis remains to be determined. While incubation of PC12 cell cytosol with $^{32}$P NDPK resulted mainly in the phosphorylation of ATP-citrate lyase, it is possible that NDPK can also phosphorylate other proteins, but these proteins are present in low amounts or they are nuclear or membrane proteins. The $t_0$ for transfer of phosphate from NDPK to ATP-citrate lyase at room temperature was about 2 min. This rate is comparable with that of some prokaryotic histidine kinases (56). Escherichia coli nitrogen regulator I protein transfers a phosphate from its catalytic histidine to nitrogen regulator I protein. In the presence of an excess of nitrogen regulator I, about 80% of the phosphate bound to nitrogen regulator I is transferred in 1 min at 37 °C (32).

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