Nucleoside-diphosphate kinase (NDP kinase), a key enzyme in nucleotide metabolism, is also known to be involved in growth and developmental control and tumor metastasis suppression. Interestingly, we find that coexpression of NDP kinase with Taz1, a Tar/EnvZ chimera, in the absence of its native signal, can activate a porin gene ompC-lacZ expression in Escherichia coli. Further studies show that NDP kinase can act as a protein kinase to phosphorylate histidine protein kinases such as EnvZ and CheA which are members of the His-Asp phosphorelay signal transduction systems in E. coli. Instead of ATP, the exclusive phosphodonor for histidine kinases, GTP can be utilized in vitro in the presence of NDP kinase to phosphorylate EnvZ and CheA, which then transfer the phosphoryl group to OmpR and CheY, the respective response regulators. The direct involvement of GTP for the phosphorylation of EnvZ through NDP kinase was further demonstrated by the use of a mutant EnvZ, which lost ability to be autophosphorylated with ATP. Phospho-OmpR thus formed can bind specifically to an ompF promoter sequence. These results suggest that NDP kinase may play a physiological role in signal transduction.

Nucleoside-diphosphate kinase (NDP kinase) is considered to play a key role in nucleotide metabolism to generate all nucleotide triphosphates from their corresponding nucleoside diphosphates using the γ-phosphate from ATP or other (d)NTPs via a phosphoenzyme intermediate (1). The genes and structures of NDP kinases are highly conserved from Escherichia coli to human (43% identity) (2, 3), and NDP kinase is believed to be a housekeeping enzyme essential for DNA and RNA synthesis (4).

Besides its role in nucleotide metabolism, NDP kinase is also involved in a number of cellular regulatory functions such as growth and developmental control and tumor metastasis suppression. Genetic analysis of NDP kinase from Myxococcus xanthus, a Gram-negative bacterium, has indicated that it may be essential for cell growth (5). The ndk gene from Schizosaccharomyces pombe was shown to regulate gene expression in sexual development in response to mating pheromone signaling (6). A null mutation of the awd gene, a Drosophila homologue of NDP kinase, results in abnormalities in larval development (7). A cAMP receptor-stimulated NDP kinase activity was found in the cytoplasmic membrane of Dictyostelium discoideum which mediates the hormone action for the activation of G proteins (8). In addition, the genes of human and mouse NDP kinases/Nm23 have been shown to be involved in tumor metastasis suppression (9–11). High metastatic potential of low nm23-expressing murine melanoma and human breast carcinoma cell lines was inhibited by transfection with nm23 cDNA. A human DNA-binding protein, PuF, identified as NDP kinase Nm23-H2, was shown to bind to the promoter region of c-myc in vitro and to activate c-myc transcription (12). Surprisingly, the ndk genes from E. coli, yeast, and S. pombe could be knocked out without affecting cell viability (6, 13, 14). However, the disruption of ndk, the only structural gene for this enzyme in E. coli, results in a mutator phenotype (13). It is highly interesting to investigate and better understand the precise molecular mechanisms of these diverse regulatory functions of NDP kinases in normal growth and development and tumor metastasis.

Recently, NDP kinase was found to exhibit a phosphotransferase activity by phosphorylating other cellular proteins. NDP kinase from rat liver was able to phosphorylate ATP-citrate lyase from PC12 cell cytosol on a histidine residue in vitro (15). Nm23/NDP kinase preparations from human, Drosophila, yeast, and Dictyostelium were also shown to exhibit a serine/threonine-specific protein phosphotransferase activity when incubated with colon carcinoma cell lysate in the presence of urea (16). Since NDP kinase can be autophosphorylated with (d)NTP at a specific histidine residue to form a high-energy phosphated enzyme intermediate, it is intriguing whether the high-energy phosphate on NDP kinase can be transferred to other regulatory proteins inside the cell and whether such a phosphorelay through NDP kinase may be a cause for a number of unexplained roles of the enzyme discussed above.

In prokaryotes, histidine protein kinases play the major role in signal transduction required for adaptive responses to numerous environmental stresses (17). Recently, histidine protein kinases were also found in yeast (18, 19) and in plants (20), suggesting that histidine protein kinases may prevail from prokaryotes to eukaryotes. During adaptation processes, the major players in E. coli are the sensor kinase (usually a transmembrane receptor) and the response regulator which mediates changes in gene expression and/or locomotion. The sensor kinase utilizes ATP to phosphorylate a histidine residue. Subsequently, the phosphoryl group is transferred to an aspartyl residue on the response regulator which causes a functional switch in the response regulator. Signal transduction is uniquely carried out by the reversible phosphorelay of a high energy phosphate between histidine and aspartate residues, which defines the histidyl-aspartyl signal transduction system (also known as the two-component signal transduction system, Ref. 21).

In the present study, we investigate whether NDP kinase can activate histidine protein kinases as an upstream phosphodonor in the signal transduction pathway. In E. coli, EnvZ...
is a trans-inner membrane histidine protein kinase in the EnvZ-OmpR phosphorelay signal transducing system that serves as an osmosensor in response to osmolality, and CheA is a cytoplasmic histidine kinase required for controlling bacterial chemotaxis. Conserved features that are shared by members of the histidine protein kinase family include: the conserved amino acid residues, His(H), the autophosphorylation site, Asn(N), and two glycine-rich segments (G1 and G2) which are involved in nucleotide binding (22–24). EnvZ consists of a periplasmic sensor domain, transmembrane domain, and a cytoplasmic signaling domain. Once activated, EnvZ becomes autophosphorylated at a histidine residue (His-245) in the cytoplasmic signaling domain (25). EnvZ has the ability to act as both kinase and phosphatase to regulate the level of phosphorylated OmpR. OmpR receives the phosphate from phosphorylated EnvZ onto a conserved aspartate residue (Asp-55) (26, 27). Following its activation, phospho-OmpR acts as a cytoplasmic transcription factor to bind upstream sites on porin promoters to downregulate expression of the outer membrane genes ompF and ompC (28, 29). Although both NDP kinase and histidine protein kinases are phosphorylated at a specific histidine residue to form a high-energy phosphoenzyme, histidine protein kinases can only use ATP as the phospho- donor in contrast to NDP kinase which can use all dNTPs. This ATP limitation of histidine protein kinases may be overcome if phosphorylated NDP kinase could serve as the phospho- donor for histidine kinase.

Here, we demonstrate that NDP kinase can indeed mediate bacterial signal transduction by activation of a histidine protein kinase in the E. coli EnvZ-OmpR system in vivo and in vitro. Phosphorylation of histidine protein kinases such as E. coli EnvZ and CheA by NDP kinase using GTP as a phos- phodonor can be observed, and the phosphorylated histidine protein kinases result in the transfer of the high-energy phos- phate to their cognate response regulators. The present finding raises an intriguing possibility that NDP kinase may play an important physiological role under certain stress conditions acting as a phospho- donor for the His-Asp phosphorelay signal transducing systems.

MATERIALS AND METHODS

Reagents—[γ-32P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq), [γ-32P]GTP (5000 Ci/mmol), and [α-32P]dGTP (5000 Ci/mmol) were obtained from Amersham. Bovine serum albumin (fraction V) was obtained from Sigma. ATP and GTP were purchased from Boehringer Mannheim.

E. coli Strains—BL21(DE3) strain was used to express EnvZ(C) and EnvZ(C)N347D proteins (24, 30). Strain AT142 (MC4100 (10–25

ompC::Kmr) was used for the purification from inner membranes (31). RU1012 (ΔompF ΔompI ΔompJ ΔompK) were used to express EnvZ(C) (24).

Plasmid pKT8P3 (ampicillin-resistant), a pUC9-derived plasmid contains its endogenous promotor, was used to express NDP kinase (2, 32). Plasmid pYYO401 (chloramphenicol-resistant), a pACYC184-derived plasmid (33), was used to express Tar1 which is a fusion between the N-terminal Tar residues 1–256 and the C-terminal EnvZ residues 223–450. pYYO401 was constructed from pYT0310 (34) by first excising an NdeI- and HindIII-digested fragment of EnvZ (encoding residues 223–450) from pYT0310. This fragment was then ligated to pYYO401 plasmid that had been digested with NdeI and HindIII, thus generating pYYO401.

Plasmid pPH001, a pET11a-EnvZ(C) derivative, containing His6- tagged EnvZ(C), was constructed and used for the expression of His6- tagged EnvZ(C)I. The 1.4-kilobase NdeI-BamH1 fragment from pYT0310 containing triple point mutations (G375A, G377A, and A379S) in the G1 domain of EnvZ(C) (23) was subcloned into pPH001 to construct plasmid pH001, which was used to express the His6-tagged EnvZ(C)G1 mutant protein.

In Vivo Activation of ompC-lacZ Expression—RU1012 cells were transformed with pKT8P3 and/or pYYO401 followed by plating onto lactose MacConkey agar plates (35) with or without the addition of 5 mM aspartate. Transformants were selected by using 50 μg/ml ampicillin for pKT8P3, 25 μg/ml chloramphenicol for pYYO401, or the addition of both ampicillin and chloramphenicol when plating cotransformants. Lac+ colonies were red, and Lac− colonies were white. Plates were incubated for 15 h at 37 °C, and only portions of the plates are shown.

Protein Expression and Purification—EnvZ(C), containing a C-ter- minal fragment of EnvZ from residues Ile-179 to Gly-450, was ex- pressed using a T7 expression system. BL21(DE3) strain was trans- formed with pET11a-EnvZ(C), and the production of EnvZ(C) was induced in the presence of 1 mM isopropyl-β-D-thiogalactoside. EnvZ(C) was purified to homogeneity by a modified procedure described previ- ously (24); the DE52 column was substituted with a Q-Sepharose ion exchange column (Bio-Rad), and the Green A affinity column was substi- tuted with a Blue Sepharose CL-6B chromatography column (Pharma- cia Biotech Inc.). Proteins were further purified by hydroxyapatite chromatography (Bio-Rad), and S-100 Sephadex gel filtration (Sigma). Similarly, His6-EnvZ(C) and His6-EnvZ(C)G1 proteins were purified through Ni2+-affinity chromatography performed on a Bio-Rad Econo system. The purity of the purified proteins was >95% as judged by Coomassie Brilliant Blue staining.

EnvZ(M) was expressed using pDR200 containing envZ under the control of the Ipp promoter and purified as associated with the inner membrane (34). OmpR was purified to homogeneity according to the previously published method (36). Purified CheA and CheY were ob- tained from Dr. A. Stock (Robert Wood Johnson Medical School).

In Vitro Phosphorylation Assays—Autophosphorylation of EnvZ with [γ-32P]ATP, phosphorylation of OmpR, and dephosphorylation of phos- pho-OmpR were carried out as described previously (34).

Phosphorylation of E. coli NDP Kinase—E. coli NDP kinase was purified as described in Ref. 32. The phosphorylated form of NDP kinase was generated by incubating 2 μM of NDP kinase with 40 μM of [γ-32P]GTP for 15 min at 30 °C in 20 μL mixture containing 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 50 mM KCl, 5% glycerol. Phospho-NDP kinase was separated from free [γ-32P]GTP by gel filtration on a Sephadex G-50 column (Pharmacia) equilibrated in 50 mM KCl and 20 mM Tris-HCl, pH 8.0. Phosphoenzyme was collected at 50 μL of each fraction from the column. Phosphoenzyme concentration was determined by the method of Bradford using a reagent purchased from Bio-Rad.

Gel Mobility Shift Assay—Oligonucleotides used for the assay con- tain the ompF −100 to −64 regulatory sequences (29). Oligonucleotides (5′-GATCCCTTTACATTGGTTACATATTTTTTCTTTCGGACAC-3′ and 5′-GATCGTTCATCTAAGAAAAAGAAAAAGATAGCACCATT-3′) were annealed and labeled by incubation with the Klenow frag- ment in the presence of [α-32P]dGTP. The probe was purified using a Nuctrap (Stratagene) column to remove unincorporated nucleotides followed by ammonium acetate and ethanol precipitation. Binding rea- ctions were carried out in binding buffer (50 mM Tris-HCl, pH 8.0 (50 mM Tris-HCl, 5 mM CaCl2, 5% glycerol, 1 mM DTT, and 0.2 μg/reaction poly(dIdC)). Purified proteins were combined (0.48 μg of NDP kinase, 1.8 μg of EnvZ(C), and 0.25 μg of OmpR) using a 2:1 molar ratio of EnvZ(C):NDP kinase and incubated for 20 min at 37 °C. ATP or GTP was added to a final concentration of 0.3 mM, and reactions were carried out for 50 min at 37 °C. Labeled DNA was then added to each reaction using 5000 cpm/reaction, and samples were incubated for another 20 min at 25 °C. The final reaction volume was 15 μL. Samples were immediately loaded onto a 5% acrylamide/bisacrylamide (40:1.2) gel which was run in 1 × TBE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer with recirculation at 25 °C using 120 V. The dried gel was exposed to autoradiography overnight at ~80 °C.

RESULTS

In Vivo Activation of Signal Transduction through NDP ki- nase—To demonstrate whether NDP kinase can function as a protein kinase in E. coli, we examined whether coexpression of the ndk gene together with the tarI gene can activate an E. coli EnvZ-OmpR signal transduction pathway in vivo. Tar1, a hybrid chimeric kinase consisting of the N-terminal Tar chemo- receptor domain and the C-terminal EnvZ signaling domain had been previously constructed to examine activation of the EnvZ signaling pathway because the natural ligand for EnvZ has not been identified (31). Taz1 has been shown to activate ompC-lacZ expression in response to aspartate which is the natural ligand for Tar (31). Interestingly, transformation of strain RU1012 (ompC-lacZ, ΔenvZ::Kmr) with both plasmid
pYY0401, harboring the taz1 gene, and plasmid pKTSP3, harboring the wild type ndk gene as shown in Fig. 1, can significantly activate ompC-lacZ expression in the absence of 5 mM aspartate, as evident from the red colony formation on lactose MacConkey agar plates (35). In contrast, cells with pYY0401(taz1) alone formed white colonies in the absence of aspartate and formed red colonies only in the presence of aspartate, and cells with pKTSP3(ndk) alone were unable to form red colonies either in the presence or in the absence of aspartate (Fig. 1). These results demonstrated that Taz1 can be activated by another mechanism rather than by transmembrane signaling with its natural ligand. Clearly, NDP kinase is responsible for this activation and the ompC expression observed above was through the EnvZ(C) signaling domain of Taz1.

Phosphorylation of OmpR by NDP Kinase and EnvZ(C) in the Presence of GTP—We next examined in vitro whether NDP kinase can directly phosphorylate the EnvZ(C) signaling domain of Taz1, which results in the concomitant phosphorylation of OmpR and activation of the ompC transcription in vivo. To avoid autophosphorylation of EnvZ(C) by [γ-32P]ATP, [γ-32P]GTP was used as the phosphate donor for the phosphorylation of NDP kinase in reaction mixtures. To assess the kinase activity of NDP kinase in the presence of [γ-32P]GTP toward histidine protein kinases, the purified C-terminal domain of EnvZ, EnvZ(C), and a kinase ‘phosphatase’ EnvZ(C) mutant, EnvZ(N347D(C)) (24), were examined. As shown in Fig. 2A, EnvZ(C) (lane 3), EnvZ(N347D(C)) (lane 10), and OmpR (lane 4) cannot undergo direct autophosphorylation by [γ-32P]GTP. EnvZ(C), however, can be phosphorylated by NDP kinase in the presence of [γ-32P]GTP (lane 6). Phosphorylated EnvZ(C) can then serve as a phosphodonor for efficient transfer to OmpR (lane 7) which is similar to autophosphorylation of EnvZ(C) by [γ-32P]ATP (lane 8) and phosphoryl transfer to OmpR (lane 9). This result indicates that phosphorylation of EnvZ(C) by NDP kinase in the presence of [γ-32P]GTP occurs at the same site as the autophosphorylation of EnvZ(C) by [γ-32P]ATP. In contrast, the EnvZ-N347D(C), a kinase ‘phosphatase’ EnvZ(C) mutant but with an intact autophosphorylation site, was only weakly phosphorylated by NDP kinase in the presence of [γ-32P]GTP (lane 11). This may be due to non-specific phosphorylation since no phosphoryl transfer to OmpR can be observed (lane 12). An autophosphorylation site mutant protein EnvZ(H243V(C)) (34) cannot be phosphorylated by NDP kinase in the presence of [γ-32P]GTP (data not shown), indicating that the His residue at position 243 is responsible for phosphorylation of EnvZ(C) by NDP kinase. Although NDP kinase can phosphorylate EnvZ(C) in vitro, there is no phosphorylation of OmpR by NDP kinase in the presence of [γ-32P]GTP (lane 5), indicating that NDP kinase cannot directly phosphorylate OmpR to activate signal transduction in E. coli. Note that no direct phosphotransfer to bovine serum albumin (lane 2) from NDP kinase was observed.
In addition, the phosphorylated form of NDP kinase was isolated from the reaction mixture of NDP kinase and [γ-32P]GTP through gel filtration to remove free [γ-32P]GTP. When the isolated phospho-NDP kinase was incubated with EnvZ(C) in reaction buffer A at 30 °C for 30 min, EnvZ(C) was found to be phosphorylated (Fig. 2B, lane 2), indicating that phosphorylation occurred directly from phospho-NDP kinase to EnvZ(C).

To further test whether NDP kinase is able to phosphorylate EnvZ(C) mutant protein incapable of autophosphorylation, we used a nucleotide binding defective EnvZ(C) mutant protein, H6-EnvZ(C)G1 with triple point mutations (G375A, G377A, and A379S) in the G1 domain of EnvZ(C) (23). As shown in Fig. 3A, in the presence of 20 μM [γ-32P]ATP, H6-EnvZ(C)G1 mutant protein was found defective in autophosphorylation by [γ-32P]GTP (lane 1) and also could not be phosphorylated by [γ-32P]ATP when incubated with 20 μM [γ-32P]GTP (lane 2). In contrast, the H6-EnvZ(C)wt protein could be highly phosphorylated by [γ-32P]GTP (lane 6). However, when the H6-EnvZ(C)G1 mutant protein was incubated together with NDP kinase in the presence of 20 μM [γ-32P]GTP, a significant amount of phosphorylation of H6-EnvZ(C)G1 mutant protein was observed (lanes 3). This phosphorylated H6-EnvZ(C)G1 mutant protein was able to transfer the phosphate group to OmpR, although the efficiency of the phosphotransfer reaction was reduced (lane 4) when compared with phosphotransfer from H6-EnvZ(C)wt protein to OmpR (lane 5). This is probably due to the mutations in the G1 domain of EnvZ(C) affecting the phosphorylase efficiency. Note that the amounts of H6-EnvZ(C)G1 used for [γ-32P]ATP (Fig. 3B, lane 1), [γ-32P]GTP (lane 2) and [γ-32P]GTP plus NDP kinase (lane 3), and [γ-32P]GTP plus NDP kinase plus OmpR (lane 4) reactions were identical and approximately 8 times more than that of H6-EnvZ(C) (lanes 5 and 6). This result further excludes the possibility that EnvZ(C) was phosphorylated by [γ-32P]ATP which might have been generated through NDP kinase and thus clearly supports the phosphotransfer from NDP kinase to EnvZ(C) using [γ-32P]GTP. It should be noted that when taxl with the G1 mutations was coexpressed with ndk in pKT8I3, colonies on a MacConkey plate became reddish (not shown), indicating that a weak His-Asp phosphorylase can be established with EnvZ(C)G1 in the presence of NDP kinase. However, taxl with the G1 mutations when expressed alone gave white colonies on the plate.

To eliminate the possibility of any contaminating ADP which could be converted to [γ-32P]ATP that in turn phosphorylates EnvZ(C) by NDP kinase in the presence of [γ-32P]GTP, the purified NDP kinase was incubated in the presence of nonradioactive GTP to 25 μM containing 5 μCi of [γ-32P]GTP at 37 °C for 30 min. By thin layer chromatography (TLC), no [γ-32P]ATP formation was detected (Fig. 3C, lane 2), indicating that all the purified NDP kinase, carrier GTP, and radiolabel [γ-32P]GTP used in the present study were free of ADP contamination. Even in the reaction mixture including purified EnvZ(C), NDP kinase, and 25 μM [γ-32P]GTP, no [γ-32P]ATP was found to be generated (lane 3). In contrast, when exogenous 0.1 μM ADP was added to the above reaction mixture, the [γ-32P]ATP was clearly synthesized (lane 4). These results further demonstrated that the purified EnvZ(C), NDP kinase, and GTP used in the reaction mixture did not contain ADP. We further examined the phosphorylation of a full-length EnvZ membrane preparation, EnvZ(M), by NDP kinase in the presence of [γ-32P]GTP. As shown in Fig. 4A, in the presence of [γ-32P]GTP, EnvZ(M) can be phosphorylated by NDP kinase (lane 3), and subsequently the phosphate can be transferred to OmpR (lane 4), which is similar to the autophosphorylation of EnvZ(M) through ATP (lane 1) which then serves as a phosphodonor for OmpR (lane 2).

**Reversible Phosphotransfer between NDP Kinase and EnvZ(M)**—To investigate the reversibility of the phosphoryl

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**Fig. 3. Phosphorylation of an ATP-binding defective EnvZ(C) mutant EnvZ(C)G1 by NDP kinase in the presence of GTP.** A, histidine-tagged proteins H6-EnvZ(C)G1 and H6-EnvZ(C) were purified as described under “Materials and Methods.” H6-EnvZ(C)G1 (1.1 μg) was incubated in the reaction mixture with either 20 μM ATP containing 10 μCi of [γ-32P]ATP (lane 1), with 20 μM GTP containing 10 μCi of [γ-32P]GTP (lane 2), or with NDP kinase (0.26 μg) in the presence of 20 μM GTP containing 10 μCi of [γ-32P]GTP at 37 °C for 30 min (lanes 3 and 4). For the reaction in lane 4, OmpR (0.6 μg) was further added to the reaction mixture, and the reaction mixture was incubated at 37 °C another 30 min (lane 4). The autophosphorylation of H6-EnvZ(C) (0.13 μg) was performed (lanes 5 and 6) in the presence of 20 μM ATP containing 10 μCi of [γ-32P]ATP at 37 °C for 10 min, and, for the reaction in lane 6, OmpR (0.08 μg) was further added into the reaction mixture and the mixture was incubated at 37 °C for an additional 30 min. Reaction mixtures were subjected to 16% SDS-PAGE and the autoradiogram of phosphorylated proteins (H6-EnvZ(C)G1, H6-EnvZ(C)G1, OmpR, and NDP kinase (Ndk)) is shown. B, Coomassie Brilliant Blue staining of gel shown in A. The proteins of H6-EnvZ(C)G1, H6-EnvZ(C)G1, OmpR, and NDP kinase (Ndk) are shown as indicated. C, detection of possible formation of ATP from contaminating ADP in the reaction mixtures. Reaction mixtures (10 μl each for lanes 2, 3, and 4) were incubated at 37 °C for 30 min and after the reaction 2 μl of each reaction mixture was subjected to thin layer chromatography (TLC) on a polyethyleneimine-cellulose plate. Chromatography was carried out in 0.75 M KH2PO4 (pH 3.7). Lane 1, [γ-32P]ATP; lane 2, 0.2 μg of purified NDP kinase was incubated with 25 μM GTP containing 5 μCi of [γ-32P]GTP; lane 3, 0.45 μg of EnvZ(C) was incubated with purified NDP kinase (0.2 μg) in the presence of 25 μM GTP containing 5 μCi of [γ-32P]GTP; lane 4, addition of 0.1 μM ADP to the reaction mixture containing 0.45 μg of EnvZ(C) and 0.2 μg of purified NDP kinase in the presence of 25 μM GTP containing 5 μCi of [γ-32P]GTP; lane 5, [γ-32P]GTP. Note that no [γ-32P]ATP formation was detected with either the reaction mixture of NDP kinase with GTP (lane 2) or the reaction mixture of NDP kinase and EnvZ(C) in the presence of GTP (lane 3), indicating that all components used for the reaction were free of ADP contamination.
Transfer between NDP kinase and EnvZ, phospho-EnvZ(M) was generated by [γ-32P]ATP, and free [γ-32P]ATP was completely removed by extensive washing followed by centrifugation. As shown in Fig. 4B, when NDP kinase was added to the phosphorylated EnvZ(M) preparation, NDP kinase was subsequently phosphorylated (lane 2). NDP kinase could not be phosphorylated when incubated with the supernatant of the EnvZ(M) preparation (lane 1), confirming that the phosphorylated EnvZ membrane preparation did not contain free [γ-32P]ATP. These results indicate that the reversible transfer of a phosphoryl group between EnvZ and NDP kinase may occur through a direct protein-protein interaction. The complex formation between the purified H6-EnvZ(C) and NDP kinase was found by means of Ni2+-His6 tag affinity chromatography with the similar binding affinity of dimer formation of EnvZ(C) (data not shown).

Characterization of the Phosphorylation of EnvZ(C) by NDP Kinase—Divalent cations are generally required for bacterial histidine kinases involved in the His-Asp phosphorelay systems. In E. coli, there are at least 17 documented members of His-Asp phosphorelay signal transduction systems (40). In addition to the EnvZ-OmpR system, we also evaluated another system for its interaction with NDP kinase. CheA-CheY phosphorelay system is similar to the EnvZ-OmpR phosphorelay system. CheA is a cytoplasmic histidine kinase required for the chemotactic response (41). While NDP kinase cannot directly phosphorylate CheY (Fig. 7, lane 5), NDP kinase can phosphorylate CheA in the presence of [γ-32P]GTP (lane 4), and the phosphorylated CheA results in an efficient transfer of the phosphoryl group to its cognate response regulator CheY (lane 6). In the control reaction, CheA was phosphorylated in the presence of [γ-32P]ATP (lane 7) and the autophosphorylated CheA resulted in transfer of its phosphate to CheY (lane 8). Note that neither CheA (lane 2) nor CheY (lane 3) could be autophosphorylated by [γ-32P]GTP.

Phosphorylated Response Regulator OmpR Is Biologically Active—To demonstrate that phospho-OmpR generated via NDP kinase in the presence of GTP was biologically active and able to bind the ompF promoter region, a gel mobility shift assay was performed (Fig. 8). Phosphorylated OmpR formed by EnvZ(C) and NDP kinase in the presence of GTP can indeed bind the promoter sequence of ompF (lane 7), in a similar manner to the binding of phospho-OmpR generated by EnvZ(C) in the presence of ATP (lane 8). In contrast, when EnvZ(C), OmpR, or NDP kinase were individually incubated with GTP from NDP kinase to EnvZ(C) (data not shown). This result suggests that protein phosphotransfer activity is dependent on the phosphorylation of the catalytic site (His-117) of EnvZ(C) NDP kinase.

A time course of phosphorylation of EnvZ(C) is shown in Fig. 6. EnvZ(C) was incubated with NDP kinase prephosphorylated with 25 μM [γ-32P]GTP. Aliquots of the reactions were removed at the indicated time points and subjected to 16% SDS-PAGE. EnvZ(C) labeled with 32P was quantitated by phosphor Image analysis. EnvZ phosphorylation with [γ-32P]GTP by NDP kinase was linear for the first 30 min and reached a steady state. Phosphorylation of EnvZ(C) with 25 μM [γ-32P]ATP was about 3 times faster than that with NDP kinase and GTP under the condition used. Furthermore from the levels of the steady state phosphorylation, phosphorylation of EnvZ(C) with ATP was approximately 2.5-fold more effective than that with NDP kinase and GTP.

Phosphorylation of CheA by NDP Kinase in the Presence of GTP—In contrast, the activity was only slightly enhanced (Fig. 5, lane 3) compared to CheA phosphorylated in the presence of ATP (lane 4). The strong preference for manganese to stimulate phosphorylation has been demonstrated for other bacterial histidine kinases such as the FrzE chemotaxis sensor (37), FixL nitrogen fixation sensor (38), and EnvZ osmosensor (39). An active site mutation of NDP kinase with replacement of histidine 117 to glutamine resulted in defective autophosphorylation of NDP kinase and led to a loss of phosphotransfer activity.
NDP Kinase-mediated Signal Transduction in E. coli

**FIG. 6.** Time course of the phosphorylation of EnvZ(C) by NDP kinase in the presence of GTP compared with that of the autophosphorylation of EnvZ(C) in the presence of ATP. The purified NDP kinase (0.8 μM) was preincubated with 25 μM GTP containing 8 μCi of [γ-32P]GTP in buffer A at 37 °C for 15 min. Then, the phosphorylation of EnvZ(C) was initiated by the addition of EnvZ(C) to a final concentration of 1.6 μM. Aliquots of the reaction were taken at the indicated time points, and the reactions were stopped by the addition of 5 × SDS loading buffer. The final mixtures were subjected to 16% SDS-PAGE. For the autophosphorylation of EnvZ(C), purified EnvZ(C) (0.4 μM) was incubated with 25 μM ATP containing 8 μCi of [γ-32P]ATP in buffer A at 37 °C. Aliquots of reaction mixture were taken at each time point as indicated. The level of phosphorylation of EnvZ(C) was determined by densitometry analysis (Molecular Imager GS-250, Bio-Rad). The level of phosphorylation of EnvZ(C) per μmol by NDP kinase in the presence of GTP ($) was expressed as a percentage relative to the maximum amount of autophosphorylation of EnvZ(C) per μmol in the presence of ATP ($).

**FIG. 7.** CheA–CheY phosphorelay mediated via NDP kinase. Proteins indicated in the table were incubated in 15 μl of reaction buffer (50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM DTT, and 5 mM MgCl2) with [γ-32P]GTP at 30 °C for 30 min, and then CheY protein was added (lanes 3, 5, 6, and 8). After incubation for another 30 s, 5 μl of gel sample buffer was immediately added to each reaction mixture. Samples were then heated to 37 °C for 1 min and applied directly to 16% SDS-PAGE. Protein amounts used were: CheA, 1.25 μg; NDP kinase (Ndk), 0.7 μg; CheY, 1.1 μg. Radiotopes used were [γ-32P]GTP (6 μCi, 5000 Ci/mmol) (lanes 1–6) and [γ-32P]ATP (6 μCi, 6000 Ci/mmol) (lanes 7 and 8). Autoradiogram of a dried SDS-PAGE gel is shown.

**FIG. 8.** Binding of phospho-OmpR formed with NDP kinase and EnvZ in the presence of GTP to the ompF promoter region. Purified proteins indicated at the top of the gel were mixed and incubated for 20 min; 1.8 μg of EnvZ(C), 0.48 μg of NDP kinase, and 0.25 μg of OmpR were used. ATP or GTP (a final concentration of 0.3 mM) was added. The reaction mixture was then incubated for 50 min at 30 °C, followed by the addition of the Klenow-[α-32P]dGTP-labeled ompF promoter region from –100 to –64; Ref. 29). The final mixtures were incubated for 20 min at 25 °C and then subjected to gel electrophoresis as described under "Materials and Methods." Lane 1 represents free probe, lanes 2–7 contain GTP, and lane 8 contains ATP. Autoradiogram of the dried gel is shown. The position of protein-DNA complexes is indicated by an arrow.

**DISCUSSION**

Prokaryotic signal transducing systems contain a large family of protein histidine kinases and their response regulators. These histidyl-aspartyl phosphorelay systems and signaling circuits enable bacteria to adapt to rapidly changing environments (42). While extensive studies have been directed toward the phosphotransferase activity of histidine protein kinases, understanding of the regulation of histidine kinases is still incomplete. In this report, we demonstrate for the first time that NDP kinase can act as a protein kinase to activate bacterial histidine kinases in the His-Asp phosphorelay signal transduction system not only in vivo but also in vitro. We have also shown that, besides ATP, GTP can be used as a phosphate donor for the His-Asp phosphorelay signal transduction in the presence of NDP kinase. It is important to note that as listed below, one can exclude the possibility that NDP kinase converted ADP contaminated in the reaction mixtures used in the present experiments into [γ-32P]ATP, which in turn phosphorylated EnvZ(C). (a) Although an EnvZ(C)-G1 mutant protein was unable to be phosphorylated with either ATP or GTP, it can be phosphorylated with GTP in the presence of NDP kinase. This result can only be explained by the protein kinase-like function of NDP kinase. (b) When phosphorylated NDP kinase free of nucleotides was mixed with EnvZ(C), EnvZ(C) was effectively phosphorylated, indicating that the phosphate group was directly transferred from phosphorylated NDP kinase to EnvZ(C). (c) When NDP kinase was incubated with [γ-32P]GTP or NDP kinase was incubated with EnvZ(C) and [γ-32P]GTP, these reaction mixtures were unable to generate any detectable [γ-32P]ATP. In addition, after NDP kinase was incubated with [γ-32P]GTP, the flow-through fraction obtained by centrifuging the reaction mixture with use of Microcon-3 (3-kDa molecular mass cutoff) was unable to phosphorylate EnvZ(C) (data not shown).

While EnvZ and CheA represent typical bacterial histidine protein kinases in the His-Asp phosphorelay system, there are two other classes of bacterial histidine kinases: phosphoenolpyruvate phosphotransferase systems and metabolite-activated histidine kinases such as NDP kinase. Phosphorelay between two histidine residues has been shown in the bacterial phosphoenolpyruvate/sugar phosphotransferase system (43) and within a single molecule in the case of the E. coli ArcB protein (44). The present results indicate that the histidine-phosphorelay between NDP kinase and the C-terminal signaling domain of EnvZ is reversible and quite efficient.
This interaction appears to be specific, since a mutation in EnvZ(C) (Asn-347 → Asp) blocks the phosphorylation reaction even if the histidine residue for the phosphorylation site remains intact. It should be noted that direct phosphorylation does not occur between NDP kinase and cognate response regulators for the His-Asp phosphorelay system such as OmpR and CheY.

While we have demonstrated that EnvZ and CheA histidine protein kinases can be phosphorylated by NDP kinase in the presence of GTP, it is also possible that NDP kinase could phosphorylate other histidine kinase protein kinases as well. Cross-talk among members of the His-Asp phosphorelay has been documented in other E. coli systems such as CheA which can serve as a phosphodonor for NtrC (45), OmpR (46), and SpoOA (47), and EnvZ can serve as a phosphodonor for NtrC (48). This report now demonstrates that cross-talk can also exist between families of histidine kinases as well. Recently, NDP kinase from rat liver was found to phosphorylate ATP-citrate lyase from PC12 cell cytosol on a histidine residue (15). Nm23/NDP kinase from rat liver was found to phosphorylate ATP-citrate lyase from PC12 cell cytosol on a histidine residue (15). Nm23/NDP kinase has a 100-fold higher affinity for ATP than histidine kinase, as demonstrated by proteoliposome activity (16). However, the link between tumor metastasis suppression and NDP kinase activity still remains to be elucidated under which physiological conditions NDP kinase plays a role in the stress response and adaptation by activating a His-Asp phosphorelay signal transduction pathway.

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