Regulatory GTP-binding Proteins (ADP-Ribosylation Factor, G, and RAS) Are Not Activated Directly by Nucleoside Diphosphate Kinase*

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The expression of nucleoside diphosphate kinase (NDK) genes has been implicated as a negative regulator of murine and human tumor metastases and is critical to proper development in Drosophila melanogaster. Molecular mechanisms for the role(s) of NDK in these complex processes have not yet been elucidated, but several reports have suggested that these and many other signal transduction pathways may be activated by NDK acting directly on a regulatory GTP-binding protein(s). To test this hypothesis, we examined the ability of NDK to catalyze the phosphorylation of the GDP bound to the following three members of the superfamily of regulatory GTP-binding proteins: G, Ha-ras p21, and ARF.

We have found no evidence to support the hypothesis that NDK can directly activate any GTP-binding protein. Rather, evidence is presented which clearly shows that all of the GTP formed upon incubation of GTP-binding proteins with NDK is the result of NDK utilizing free GDP as substrate. The GDP bound to the regulatory proteins is not a substrate for NDK under conditions in which free nucleotides are rapidly and efficiently phosphorylated. The importance of appropriate controls for dissociation of GDP from the regulatory protein(s) during the NDK reaction and during the analysis of product is demonstrated.

We believe there is currently no experimental evidence to support the hypothesis that NDK can directly activate a regulatory GTP-binding protein.

Nucleoside diphosphate kinase (NDK) is a ubiquitous enzyme first described in 1953 (1, 2). NDK catalyzes the reaction

\[ \text{NTP} + \text{NDP} \rightarrow \text{NDP} + \text{NTP} \]

thus, shuttling the \( \gamma \)-phosphate between different nucleotides. The mechanism of catalysis by NDK has been studied extensively and involves formation of a phosphorylated enzyme intermediate in a classic ping-pong mechanism (for reviews see Refs. 3, 4). In mammalian tissues, the enzyme is a hexamer of 17-kDa subunits migrating on sizing columns with an apparent molecular mass of 100,000-120,000 Da. Multiple species with activity can be identified by ion-exchange chromatography. These isozymes differ both in equilibrium kinetics and activation energies. It is likely that these multiple isozymes result from as few as two gene products. In human erythrocytes, two distinct NDK protein chains have been identified and purified to homogeneity (5). One has an acidic pI (A) and the other a basic pI (B). Hexamers formed from different combinations of these monomers (e.g., A, A, B, ..., B) appear to account for the multiple isozymes and a mixture of these two gene products appears to recreate the complex mixture of isoforms found in crude cell extracts. The A monomer is the product of the nm23-H1 gene, the human gene homologue of the mouse gene, nm23-1 (6, 7). B is the product of nm23-H2, a second gene cloned from a human cDNA library using an nm23-1 probe (8).

Initially the function of NDK was thought to be the maintenance of nucleoside triphosphates at the expense of adenosine triphosphate. However, both the abundance and distribution of NDK activity appear somewhat inconsistent with this proposed function. For example, NDK activity is about 10-fold greater than that of the glycolytic enzymes in red blood cells (3, 9), and NDK constitutes between 0.1-0.2% of total cellular protein in bovine brain (10). Other reports have found discrepancies between the nucleotide pools found in certain cells and the predicted values based on in vitro derived kinetic constants for NDK and the different nucleotide substrates (3, 4). These data have prompted speculation (3, 4) on additional, regulatory roles for NDK in cellular physiology. Interest in the possible regulatory roles for this enzyme has recently increased dramatically as NDK genes have been implicated as negative regulators of tumor metastasis in mice (7, 11) and as an important regulatory gene in fly development (6, 12). It is difficult to imagine how either of these complex processes might be regulated by NDK activity affecting nucleoside triphosphate pools. With the large number of regulatory GTP-binding proteins, and proposed functions for them, it has been proposed (e.g., 13-18) that one mechanism by which NDK may effect either specific or pleiotropic regulation of cellular functions is through direct action on a GTP-binding protein or proteins.

NDK-mediated activation of GTP-binding proteins implies that NDK catalyzes the transfer of phosphate from an NTP to GDP bound to the GTP-binding protein, thereby activating the protein without the need for nucleotide exchange. As the release of GDP has been shown to be the rate-limiting step in the activation of a number of regulatory GTP-binding proteins, the formation of GTP \textit{in situ} is predicted to dramatically increase the activity of the GTP-binding protein. Two approaches have been used to investigate this potential regulatory role for NDK. First, the formation of GTP has...
been examined. Thus, NDK has been shown to catalyze the transfer of phosphate from ATP to GDP that had been bound to ras (19), EF-α (19), tubulin (20, 21), G, (15), G, (19), and, most recently (18), ADP-ribosylation factor (ARF), a 21-kDa GTP-binding protein that regulates protein traffic in eukaryotes. Second, NDK has been shown to activate GTP-binding proteins in vitro. For example, ATP-dependent activation of adenylate cyclase in platelet membranes (22), acetylcholine-activated K+ channels in cardiac myocytes (23), and NADPH-oxidase in HL-60 membranes (24) were all reported to be NDK-mediated. ADP-ribosylation factor activity (18) and the GTPase activity of G, (15) have been reported to increase with added NDK and ATP. However, in very few of these studies has an attempt been made to document that the GDP is actually bound to the GTP-binding protein when phosphorylated by NDK. This is a critical distinction as addition or production of GTP in solution, in the absence of receptor and agonist, is known to have little or no effect on any GTP-binding protein-mediated effector system. A clear demonstration of a GTP-binding protein being activated by NDK absolutely requires proof that the phosphorylation of GDP occurs while bound to the regulatory protein. We believe that this condition has not been met by any published report to date. Omission or improperly controlled tests of this condition may lead to erroneous conclusions regarding the actions of NDK.

In this report, we have examined GDP bound to ARF, G, and Ha-ras p21 as potential substrates for NDK, specifically testing whether phosphate is transferred to the bound GDP. We were unable to demonstrate such a transfer under conditions in which phosphate was efficiently transferred to free nucleoside diphosphate. Several artifacts are described and documented which we believe account for all of the published data that supported the conclusion that a GTP-binding protein is activated by NDK when, in fact, such an event may not occur.

MATERIALS AND METHODS

Reagents

Recombinant mARFlp (25), nm23-1p (18), and Gp53 were purified from bacteria or Sf9 cells as previously described. nm23-H1p and nm23-H2p were expressed in bacteria and purified by the same procedure previously described for nm23-1p (18). Recombinant Ha-ras p21 and (G12V) Ha-ras p21, purified from bacteria as described (27), were the generous gifts from Dr. Richard Michitsch (Oncogene Science Inc., Manhasset, NY) and Drs. Douglas Lowy and Alex Papageorge (NCI, Bethesda, MD). G, G,, Gα, and rod outer segment discs, and urea-washed discs containing rhodopsin were purified from bovine retina as described (28, 29). Bovine liver nucleoside diphosphokinase (catalog no. N-2635), ATP, GTP, t-a-dimyristoyl phosphatidylcholine, and Sephadj G-25 were purchased from Sigma. [α-32P]GTP, made by Du Pont-New England Nuclear, was used to prepare [α-32P]GDP as described in Hamel and Lin (30). PEI-cellulose thin layer plates were purchased from J. T. Baker Chemical Co. BA-85 nitrocellulose filters (25 mm) were obtained from Schleicher & Schuell.

Loading Proteins with [α-32P]GDP

ARF—ARF was loaded with [α-32P]GDP as described in Ref. 18. Ras—Between 0.5 and 3 μg of recombinant ras p21 was incubated in 25 mM HEPES, pH 7.4, containing 2.5 mM MgCl2, 1 mM dithiothreitol, and approximately 10 μCi of [α-32P]GDP (1000–6000 Ci/mmol) in a volume of 100 μl for 2 h at 37 °C. The solution was cooled to 4 °C, and free nucleotide was removed and buffer was exchanged on a Sephadj G-25 column equilibrated in 25 mM HEPES, pH 7.4, containing 1 mM EDTA, 2 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol, and 0.25% sodium cholate. Gβ-g—Gα (1 μM) was added to a solution containing purified recombinant Gβγ (10 nM) and rhodopsin (10 nM; present in ureawashed rod outer segment discs in a buffer containing 10 mM MOPS, pH 7.5, 1 mM EDTA, 3 mM MgSO4, 1 mM dithiothreitol, 3 mg/ml BSA, and 1 μM [α-32P]GTP at 30 °C for 120 min). The membranes and rhodopsin were removed by centrifugation at 75,000 rpm in a Beckman TL-100.1 rotors at 4 °C for 10 min. Free nucleotides were removed by gel filtration on Sephadj G-25 developed in the binding reaction buffer to which 100 mM NaCl was added. Analysis of bound nucleotides revealed all of the radionucleotide was in the form of GDP, due to intrinsic hydrolysis. The heterotrimer was loaded in the same manner except the purified G, was used and no βγ subunits were added.

Nucleoside Diphosphate Kinase Assay

Coupled Spectrophotometric Assay—NDK activity was determined by the method of Agarwal et al. (4) which uses dTTP as substrate in an enzyme-coupled assay resulting in oxidation of NAD by lactate dehydrogenase.

Radioisotopic Assay—Enzyme activity was measured as GTP formation from [α-32P]GDP. Except where noted otherwise in the text, reactions contained 25 mM HEPES, pH 7.4, 2.5 mM MgCl2, 1 mM dithiothreitol, 1 mM ATP, and 100 μM GTP with the indicated concentrations of nucleotide diphosphate substrate and nucleoside diphosphate kinase in a volume of 100 μl. Reactions were initiated in one of three ways as noted in the text: 1) direct application of a 5–10-μl sample to a PEI-cellulose plate; 2) adding the sample to ethanol to achieve a final ethanol concentration of 50 or 67%; or 3) adding the sample to formic acid to a final formic acid concentration of 2, 4, or 6 M. Guanine nucleotides were separated by ascending chromatography on PEI-cellulose plates developed in 2 M LiCl, 2 M formic acid (1:1, v/v). The nucleotides were visualized by autoradiography, identified by comigration with nucleotide standards, and quantified by scintillation spectroscopy. The amount of GTP formed is typically expressed as the percentage of total guanine nucleotide.

Nitrocellulose Filter Trapping Assay

This assay was performed essentially as described in Ref. 31. Samples of 10–20 μl were diluted into 2 ml of ice-cold 20 mM Tris, pH 7.4, 10 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol (TNMD) with or without 200 μg/ml bovine serum albumin as indicated in the text. The samples were filtered on nitrocellulose filters, and the filters were washed six times with 2 ml of cold TNMD.

G-25 Sephadex Gel Filtration Chromatography

Samples of 100 μl or less were fractionated on a 10 × 80-mm Sephadex G-25 column previously equilibrated with and developed in 20 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM MgCl2, 1 mM dithiothreitol, 100 mM NaCl, and 20 μg/ml BSA. Fractions (0.2-0.4 ml) were collected into tubes containing 20 μl of concentrated formic acid.

RESULTS

We have initiated a formal test of the hypothesis that nucleoside diphosphate kinase can directly activate one or more regulatory GTP-binding proteins. Because activation of a wild type regulatory GTP-binding protein requires the production of a GTP-ligated protein (32) we have addressed whether, in a purified system, NDK can use ATP to produce GTP from GDP while the guanine nucleotide remains bound to a regulatory protein. To test this, we have employed [α-32P]GDP-bound proteins as substrates in NDK reactions after removal of unbound labeled nucleotide. We considered any GTP formed could have been produced by one or more of three possible reactions: 1) the GDP bound to the protein was phosphorylated by NDK while bound; 2) the GDP was released from the protein during the incubation with NDK and was phosphorylated by NDK while free in solution; or 3) the GTP was produced during the analysis of products or "post-reaction." Clearly conditions 2 and 3 must be eliminated or...
accurately assessed before it is possible to conclude that possibility 1 has occurred. We chose to examine three regulatory GTP-binding proteins as representatives of this large superfamily. G1 and Gα were used as representatives of the heterotrimeric G protein family as conditions were known which would allow only slow exchange of bound GDP from this protein. Both the monomeric α subunit and the heterotrimer were used to test for any effects of the presence of βγ subunits on the reaction. Ha-ras p21 was used as a representative of the RAS superfamily of monomeric GTP-binding proteins due to its significant role in oncogenesis. Both the proto-oncogenic form, Ha-ras p21, and an oncogenic form, (G12V) Ha-ras p21 were used as they have very different effects on cells in vivo, and the latter has a lower rate of intrinsic GDP hydrolysis. Finally, human ARF1p was used as a representative of the ARF subfamily of the RAS superfamily as it is quite distinct from the other subfamilies in the RAS superfamily and has unique guanine nucleotide binding properties which make it a likely candidate for an NDK-activated regulatory protein (18).

**GDP Dissociation as a Potential Source of Artifact in NDK Assays**—Conditions were sought which would minimize the rate of GDP dissociation from the regulatory proteins to allow a clear distinction between the rate of phosphorylation of bound GDP from that of free GDP. To monitor dissociation of GDP, proteins were loaded with [α-32P]GDP in a standard exchange reaction at 30 °C, as described under "Materials and Methods." Unbound nucleotides were then removed by gel filtration at 4 °C. The preloaded proteins were then incubated at 30 °C in the presence of 100 μM GTP (this typically represents a 1,000-10,000-fold isotopic dilution) to exclude dissociation and reassociation as the pathway for formation of bound [α-32P]GTP. After the indicated periods of time, 32P bound to protein was assessed either by nitrocellulose filter trapping or by fractionation on Sephadex G-25, as described under "Materials and Methods." In addition to those agents previously identified (e.g. Mg”, ionic strength, phospholipids (25, 32, 33)), the rate of GDP dissociation from hARF1p (Fig. 1) or G, was found to be sensitive to the total protein concentration. Stabilization of nucleotide-bound hARF1p at low concentrations was achieved by addition of a carrier protein, such as BSA. Albumin has no effect on the rate or extent of nucleotide exchange on ARF in a standard exchange reaction. As seen in Fig. 1, incubation of 30 nM [α-32P]GDP-ARF at 30 °C resulted in the loss of bound GDP. In the experiment shown, more than 40% of the nucleotide had dissociated after 60 min. This is likely the result of denaturation of the hARF1p in dilute solutions rather than exchange as it is readily prevented by protein addition, and no binding to hARF1p has been observed under these conditions. As little as 1-2 μg/ml protein dramatically slows the dissociation rate, and at total protein concentrations of greater than 10 μg/ml less than 10% of the bound GDP had dissociated (Fig. 1A and B). Protein stabilization of GDP-bound ARF was non-specific, with ARF, BSA, and NDK all effectively preventing GDP-dissociation when present at 25 μg/ml or greater (Fig. 1B). Similar results were obtained when G or Go were tested. At concentrations of 100 nM or less with no other protein present, all the preloaded GDP had dissociated within 2 h at 30 °C. Addition of 3 mg/ml BSA completely prevented nucleotide dissociation from G or Go at 4 °C and substantially reduced dissociation at 30 °C.

The denaturation of ARF in dilute solution motivated the re-evaluation of the nitrocellulose filter trapping assay as a quantitative method for the determination of guanine nucleotide binding. This is a standard means of monitoring nucleotide binding to proteins (31) in which protein-bound nucleotide and free nucleotide are rapidly and efficiently separated.

**Fig. 1. Effect of added protein on the rate of dissociation of GDP from ARF.** Panel A, time course: 30 nM [α-32P]GDP-ARF was incubated in 25 mM HEPES, pH 7.4, containing 2.5 mM MgCl₂, 1 mM ATP, 100 μM GTP, 1 mM dithiothreitol, and either no further addition (circles) or 100 μg/ml BSA (squares) at 30 °C. 1-μl samples were taken and diluted into 2 ml of ice-cold TNMD containing 200 μg/ml BSA at the indicated times. Bound [α-32P]GDP was determined by nitrocellulose filter trapping, as described under "Materials and Methods." Panel B, [α-32P]GDP-ARF (10-30 nM) was incubated in 25 mM HEPES, pH 7.4, 1 mM ATP, 100 μM GTP, and 1 mM dithiothreitol with either BSA (circles), ARF (squares), or bovine liver NDK (triangles) at the indicated concentrations. After 1 h at 30 °C, the mixtures were chilled on ice and fractionated on Sephadex G-25 at 4 °C as described under "Materials and Methods." The protein-bound (void volume) and free (included volume) nucleotides were determined and presented as the percentage of GDP dissociating from the ARF during the incubation.

Typically, the binding reaction is stopped by a combination of dilution and lowering the temperature. Inclusion of high concentrations of Mg", which often slows nucleotide dissociation, is also useful to maintaining binding after dilution. For the assay to accurately reflect binding, exchange and denaturation must be minimal while the protein is in the stop solution and during filtration. If these conditions are met, recovery of binding sites is independent of time in stop solution. These properties have been documented previously for G proteins, ras p21, and ARF under specific conditions. Typically, ARF in a solution containing 3 mM DMPC and 0.1% sodium cholate (conditions required for nucleotide exchange on ARF (34)) is diluted 100-200-fold into exchange stop solution (TNMD) at 4 °C. As shown in Fig. 2, ARF-GDP recovery is independent of time in TNMD in this case. If, however, ARF is incubated in an NDK reaction mixture without other protein or DMPC and then diluted into TNMD, recovery of the ARF-GDP complex decreases exponentially with time. BSA (100 μg/ml) added to either the NDK reaction mixture or in the TNMD stabilizes ARF, and recovery is independent of time in TNMD at 4 °C. Thus, addition of protein or DMPC is sufficient to stabilize ARF when in dilute solutions. The earlier characterization of the stability and guanine nucleotide binding properties of ARF proteins, performed in a combination of DMPC and cholate, clearly cannot be extended to those conditions which lack phospholipids.
FIG. 2. GDP dissociation from ARF in TNMD at 4°C. Ten-

µl samples of \([\alpha-^32P]\)GDP-ARF (100 nM) in 25 mM HEPES, 2.5 mM

MgCl₂, 1 mM ATP, 100 µM GTP, 1 mM dithiothreitol, and either no

further addition (circles), 3 mM DMPC, and 0.1% sodium cholate

(squares) or 100 µg/ml BSA (triangles) was added to 2 ml of TNMD

or TNMD containing 200 µg/ml BSA (open circles) at 4°C. At the

indicated times, the amount of bound nucleotide was determined by

the nitrocellulose filter-trapping methods as described under “Mate-

rials and Methods.”

These data indicate that close attention must be paid to the

conditions used to test for dissociation of GDP, including total protein concentration, phospholipids, salts, and metals. Without such controls the amount of free GDP produced as a possible substrate for NDK can be grossly underestimated (18).

Post-reaction Product Formation as a Potential Source of

Artifacts in NDK Reactions—The method used to terminate

the NDK reaction prior to analysis of product formation was also

examined. Two methods of stopping NDK reactions, addition of the reaction mixture to ethanol (e.g. 20) or directly to the surface of a PEI-cellulose plate (e.g. 18, 35), were tested to determine if complete arrest of NDK activity had occurred. Formic acid was also examined for the ability to instantly arrest any NDK activity as it would not interfere with sub-

sequent nucleotide separations. Each of the GTP-binding proteins tested, G₀, ARF, or Ha-ras p21, were preloaded with \([\alpha-^32P]\)GDP, as described under “Materials and Methods” and used as substrates to test for any NDK activity occurring after the reactions were presumably stopped. Two solutions, one containing (G12V)Ha-ras p21- \([\alpha-^32P]\)GDP (0.3 pmol) in a buffer containing ATP and GTP, the other containing either 10 or 100 ng bovine liver NDK, were added simultaneously either to ethanol or to the surface of a PEI-cellulose plate, or to 20 µl of 75% ethanol or to 20 µl of 9 M formic acid. Nucleotides were then analyzed and quanti-

tized as described under “Materials and Methods.” The percentage of \([\alpha-^32P]\)GDP converted to \([\alpha-^32P]\)GTP is shown. Panel B, NDK reaction mixtures containing 10 mM MOPS pH 7.5, 1 mM EDTA, 3

mM MgSO₄, 1 mM dithiothreitol, 3 mg/ml BSA, 100 mM NaCl, 1 mM

ATP, 100 µM GTP, and 100 mM heat-denatured (90°C, 5 min) \([\alpha-

32P]\)GDP-Gα were prepared on ice. Four µl of the reaction was added to 10 µl of H₂O (lanes 1 and 2), ethanol (final 67%, lanes 3 and 4), 3

m formate (final 2 M, lanes 5 and 6), 6 m formate (final 4 M, lanes 7

and 8), or 9 m formate (final 6 M, lanes 9 and 10). One µl of 10 µg/

ml nm23-H2p was added to each sample, and 10 µl was analyzed by chromatography on PEI-cellulose plates, as described under “Mate-

rials and Methods.” Panel C, the conditions were identical to those in panel B, but the substrate was native \([\alpha-^32P]\)GDP-Gα.

immediately, with either spotting onto PEI-cellulose plates or addition to ethanol. Lanes 5–10 show that formate at each concentration tested completely arrested the conversion of free GDP to GTP. Although not demonstrated directly, it is likely that denaturation of the GTP-binding proteins is occurring (facilitated by ethanol) prior to the complete loss of NDK activity, and the consequent free GDP is then converted by residual NDK. At higher concentrations of NDK, there is more activity remaining in the “stopped” reaction to produce the GTP (Fig. 3A, solid bars). For all three GTP-binding proteins examined, the contribution of post-reaction GTP formation could be eliminated by stopping the reactions with formic acid.

Testing GDP Bound to Proteins as Possible Substrate for

NDK—With the appropriate controls for GDP dissociation and post-reaction product formation, it was possible to test the hypothesis that GDP could be converted to GTP while remaining bound to a regulatory protein. The regulatory protein was loaded with \([\alpha-^32P]\)GDP and incubated at 30°C with the indicated concentrations of NDK, ATP, and GTP. Formation of GTP was determined after separation of products on PEI-cellulose, and the amount of GDP that had dissociated during the reaction was determined by gel filtra-
tion. When ARF was examined as a substrate for NDK, no phosphorylation of the bound GDP was detected. Any GTP formed from GDP bound to ARF observed in previous studies was likely the result of intra-incubation and post-incubation dissociation of GDP. In Fig. 4A, the effect of eliminating the post-reaction artifact on GTP formation is shown. Filled symbols represent data obtained when the experiment was performed exactly as previously reported (18) and without controlling for dissociation or post-reaction artifacts. These reactions were run in the absence of BSA or DMPC and stopped by directly spotting on PEI-cellulose plates. When the reactions were stopped in formic acid (open symbols) less GTP was formed, and increasing the NDK concentration led to a decrease in the net GTP produced. The latter is likely the result of the higher protein concentration resulting in less phosphorylation of free GDP (not shown). After 60 min, samples were taken from each of the three reactions, shown in Fig. 4B, and analyzed by gel filtration to determine the proportions of bound and free nucleotides. In each case the amount of GTP formed during the reaction exactly correlated with the amount of dissociated nucleotide (not shown). These results indicate that any GTP formed in the reaction was formed in solution and not while bound to the ARF.

Using the same technique, the GDP bound to Ha-ras p21 and (G12V) Ha-ras p21, were tested as substrates for NDK. Again, when the appropriate controls for nucleotide dissociation were used, there was no GTP formed on the protein in any case. In Fig. 5A, the phosphorylation of free GDP (100 μM, squares) was compared to that of GDP bound to either 100 nM (G12V) Ha-ras p21 (triangles) or 100 nM Ha-ras p21 (circles), in the presence of 1 mM ATP, 100 μM GTP, NDK, and 100 μM BSA. Within 10 min, 25 ng/ml NDK converted more than 80% of the free GDP to GTP. However, less than 25% of GDP from either ras protein was phosphorylated by 10 μg/ml NDK after 60 min at 30 °C. This was similar to the amount of GDP dissociating from (G12V) Ha-ras p21 in this experiment. As seen in Fig. 5A, the rate of GTP formation from 100 nM GDP that had been bound to (G12V) Ha-ras p21 was the same whether 1 μg/ml (triangles) or 10 μg/ml (inverted triangles) NDK were present with 22% of the GDP converted to GTP after 60 min at 30 °C. Hence, the rate of GDP formation was not limited by the availability of catalytic sites on the enzyme but, rather, was limited by the GDP dissociation rate. When samples from this experiment were analyzed by gel filtration (Fig. 5B), at the start of the reaction (0 min, triangles) all [α-32P]GDP eluted in the void volume (bound to protein). After 60 min (circles), 22% of the radio-

![Fig. 4. Contribution of GDP dissociation and post-reaction artifacts on the production of GTP from ARF/GDP by NDK. Panel A, [α-32P]GDP-ARF (150 nM) was incubated with bovine liver NDK at either 0.25 μg/ml (circles) or 2.5 μg/ml (squares) in 25 mM HEPES, pH 7.4, 2.5 mM MgCl2, 1 mM ATP, 100 μM GTP, and 1 mM dithiothreitol at 30 °C for the indicated times. The reactions were terminated either by direct spotting of samples (10 μl) on PEI-cellulose plates (filled symbols) or by addition of 10-20 μl of 9 M formic acid (open symbols), and GTP formation was quantified as described under "Materials and Methods." Panel B, reactions were identical to those in panel A, but each sample was stopped by addition to formate at the indicated times. Each reaction contained NDK at either 0.25 μg/ml (circles) or 2.5 μg/ml (squares) and either no (open symbols) or 100 μg/ml BSA (filled symbols).](image)

![Fig. 5. Comparison of GDP and GDPORas as substrates for NDK. Panel A, Ha-ras p21 (100 nM, circles) or (G12V) Ha-ras p21 (100 nM, triangles, inverted triangles) was preloaded with [α-32P]GDP and incubated, as described in the legend to Fig. 4 with the addition of 100 μg/ml BSA and 1 μg/ml (triangles) or 10 μg/ml (circles, inverted triangles) bovine liver NDK. Alternatively, free [α-32P]GDP (squares) was incubated in the same buffer with 25 ng/ml liver NDK. Samples were taken at the indicated times and the NDK reaction was terminated in formate prior to analysis of products by thin layer chromatography, as described under "Materials and Methods." The percentage of the [α-32P]GDP converted to [α-32P]GTP is shown. Panel B, samples (80 μl) from the above incubation containing (G12V) Ha-ras p21 and 10 μg/ml NDK were taken at 0 min (triangles) and 60 min (circles) but prior to formate addition, and bound and free nucleotides were separated by gel filtration at 4 °C, as described under "Materials and Methods."](image)
activity was recovered in the salt volume (fractions 10–15), having dissociated from (G12V)Ha-ras p21. In fact, whenever examined, any [α-32P]GTP formed from protein-bound [α-32P]GDP exactly correlated with the amount of GDP dissociated. Similar protocols were used to test for the ability of NDK to activate Gi or Goα, as seen in Fig. 6. Three different recombinant NDK proteins, nm23-1p (lanes 1–4), nm23-H1p (lanes 5–8), and nm23-H2p (lanes 9–12), were incubated with [α-32P]GDP·Goα (panel A) or with free [α-32P]GDP (panel B), produced by heat denaturation of the Goα, in the standard NDK reaction buffer for 0 min (lanes 1, 2, 5, 6, 8, and 10) or 5 min (lanes 3, 4, 7, 8, 11, and 12) at 30 °C before stopping in formate. Reaction times were kept short because dissociation of nucleotide from Gi occurs more readily than from ARF or ras proteins. As seen in panel A, the free nucleotide was completely converted to product within 5 min at 30 °C. Only recombinant NDK proteins, nm23-lp (lanes 1–4), nm23-Hlp (lanes 5–8), and nm23-H2p (lanes 9–12), were incubated with [α-32P]GDP·Goα, an ARF. NDK complex was predicted to appear at an apparent mass of >120,000 Da. However, NDK had no effect on the migration of [α-32P]GDP·ARF. Thus, in addition to the failure to find any evidence for conversion of bound GDP to GTP, we were also unable to find any evidence for physical interaction between ARF and NDK either by competition studies or by looking for a change in the migration of ARF in the presence of NDK and resolution by either gel filtration or sucrose density gradients. Effects of Different Preparations of NDK—Most of the experiments reported above were performed with a commercially available preparation of NDK purified from bovine liver. We have also tested the protein products of the murine nm23–1 and human nm23-H1 and nm23-H2 genes, purified from bacterial strains which over-produced each protein, for NDK activity and in protocols described above (e.g., see Fig. 6). In the standard NDK assay (4), using dTDP as substrate the specific activities of these preparations of NDK were: bovine liver (Sigma N-2635), 1.60 units/μg; nm23-1p, 4.48 units/μg; nm23-H1p, 3.01 units/μg, and nm23-H2, 4.63 units/μg. We have found no significant differences between any of these preparations with regard to their activities relating to any GTP-binding protein. Thus, we believe that the results reported above may be extended for all NDK.

**DISCUSSION**

We have described a rigorous test of the hypothesis that regulatory GTP-binding proteins can be directly activated by NDK and have found no evidence to support it. GDP bound to representatives of the trimeric G protein (both monomeric
and heterotrimeric G or RAS superfamilies (including ras p21 and ARF) was found to be unaffected by NDK, using conditions in which the NDK was fully functional and utilized free guanine nucleotide as substrate. During the course of these studies, we have documented a number of artifacts which we believe have contributed to the erroneous conclusions that NDK acts on GTP-binding proteins. We cannot exclude the possibility that another GTP-binding protein can be directly activated by an NDK, although we currently consider such a possibility unlikely. Re-examination of our own previous work (18) as well as that of others (e.g. 13, 15, 19, 36) has left us unable to cite a single faultless piece of evidence which supports the conclusion that a GTP-binding protein can be directly activated by any NDK through a mechanism which may have physiological significance.

In addition, results from functional and structural studies of GTP-binding proteins provide strong arguments against a role for NDK as a regulator of these proteins. A paradigm describing the regulation of heterotrimeric G protein activation via membrane receptors has emerged from a number of well-controlled kinetic studies of reconstituted G protein-coupled systems (32, 37–39). Results from these and other studies provide strong evidence for the release of bound GDP as the rate-limiting step in activation of G proteins and the step which is most dramatically effected by receptors. Much less is known about the mechanism of activation of members of the RAS superfamily, but it is likely that the rate of GDP dissociation also is limiting. These conclusions make the hormone receptors (in the case of G proteins) or "exchange factors" (in the case of the members of the RAS superfamily) the catalysts for the signal generation inherent in both classes of regulatory proteins. In contrast, the role proposed for NDK would short circuit this regulation by generating the active catalysts for the signal generation inherent in both classes of regulatory proteins. We note that in our previous report (18) apparent K_m of 0.16 μM for NDK activation of ARF was found in reality to be the concentration of ARF which prevented loss of prebound GDP by 80% (0.16 μM ARF = 4 μg/ml, see Fig. 1B). Dissociation was not detected after gel filtration in the previous report as it was only performed on samples containing high concentrations of NDK which stabilized the ARF-GDP.

When nucleotide dissociation was carefully monitored, the nucleotides bound to ARF, Ha-ras, G_s, or G_{α} were not found to be substrates for NDK. It is difficult to assess the extent to which the other artifact reported above, post-reaction product formation, have affected results in other studies. Certainly in those instances where ethanol or spotting in thin layer plates was the means of terminating the reaction, the results must be viewed with skepticism.

In many previous studies examining formation of GTP from GDP that had been bound to G_s, ras, and EF_α (19), nucleotide exchange was not measured and therefore cannot be excluded as the likely cause of apparent phosphorylation of bound GDP. Hence, these studies are incomplete as they cannot exclude the likelihood of NDK acting solely on GDP after its dissociation from each binding protein. In other studies, dissociation of GDP from GTP-binding proteins has been improperly measured. For instance, exchange was defined (15) as loss of [3H]GDP from G, determined as loss of radiolabel trapped to nitrocellulose filters. However, no free unlabeled nucleotide was included to prevent the rebinding of [3H]GTP after NDK acted on the free nucleotide. Another apparent error was introduced in at least one study (20) which failed to take into account the contribution made by the bound GDP in the determination of the specific activity of guanine nucleotides. It has been reported that many GTP-binding proteins are purified as a 1:1 molar complex with GDP (34, 44, 45). Therefore, to obtain an accurate determination of guanine nucleotide-binding sites, the nucleotide which starts bound to the protein must be included in the calculation of specific activity, unless the bound nucleotide can be shown to remain bound throughout the incubation (e.g. the non-exchangeable site of tubulin). Others have attempted to avoid these difficulties by indirectly assessing the NDK-catalyzed phosphorylation of GDP on heterotrimeric G proteins (15). In these experiments, the transfer of 32P from [γ-32P]ATP to GDP and subsequent release of 32P, upon hydrolysis of GTP has been used to measure the ability of NDK to use G proteins as substrates. However, such studies cannot unambiguously be interpreted to demonstrate NDK phosphorylation in situ unless the GTPase rate measured exceeds the GDP dissociation rate. This criterion was not met for the affect of NDK on G_s (46). As we have shown with ARF, the rate of formation of GTP was not affected by increasing NDK; rather, the reaction was limited by the GDP dissociation rate.

In a number of cases the ability of NDK to affect the activity of a GTP-binding protein was assessed. However, these studies also suffered from at least one conceptual flaw in experimental design which precludes the conclusion that NDK was acting as a direct activator of a GTP-binding protein. We note that in our previous report (18, Fig. 2) of the functional consequences of NDK addition to the ARF assay, the reaction required the addition of GTP, DMPC, and cholate, a condition which facilitates GDP-GTP exchange. ARF activity was unaffected by added NDK unless conditions were used which promoted nucleotide exchange. We cannot exclude, but rather favor, the possibility that in these cases the NDK is simply acting as a nucleotide triphosphate regenerating system, removing the inactivating GDP to a point where the GTP can compete more effectively and become a more potent activator. This explanation is particularly attractive in the case of ARF as it has a much greater affinity for GDP than GTP (25), and even under the best conditions only a small fraction of the protein will bind GTP without removal of the released GDP. Such a mechanism is unlikely to be of physiological significance as cells have other nucleoside triphosphate regenerating systems. Thus, we conclude that there are currently no data to support the hypothesis that NDK plays any role in the activation of ARF proteins.

Results from other attempts to document the effects of NDK on a G protein-mediated process in membranes are ambiguous. A source of confusion in these studies is in the
use of GTPγS, or ATPγS which can produce GTPγS via NDK (47) in crude in vitro systems. The ability of ATPγS to activate a GTP-binding-protein-mediated activity, e.g., activation of adenylate cyclase (22), NADPH oxidase (24), or G-mediated potassium conductance (23), was interpreted as evidence of NDK catalyzed thio-phosphate transfer to bound GDP or to GDP in a pool that can be "channeled" to the binding site (26, 48). The finding that removal of free GDP by an NTP regenerating system eliminated the effect of ATPγS was thought to further support the latter conclusion (16). However, these arguments fail to consider that while added GDP or GTP reaches a steady-state with any binding sites present in the reaction, GTPγS does not. In the case of the trimeric G proteins, GTPγS binding under most conditions is essentially irreversible. Furthermore, GTPγS, unlike GTP, is not hydrolyzed by GTP-binding proteins. Consequently, even in the presence of excess GTP there is still a slow but irreversible increase in the activation of a G protein when low levels of GTPγS are formed. Hence, these effects on the trimeric G proteins, GTPγS binding under most conditions is not well retained when low levels of GTPγS are formed. Hence, these effects mediated potassium conductance of NDK may have distinct functions in the cytosol and in the nucleus. It is hoped that researchers may now focus more of their attention on more productive lines of research that may lead to a better understanding of human tumor metastasis, development, and the physiological functions of NDK.

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The importance of Mg2+ in stabilizing the binding of GTPγS to G proteins has been well documented previously (32). It is worth noting, however, that GTPγS binding to ARF, in particular, and apparently other smaller GTP-binding proteins is not well retained even in solutions containing 20 mm magnesium (R. A. Kahn, unpublished observation).