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Characterization of Membrane-Associated *Pseudomonas aeruginosa* Ras-Like Protein Pra, a GTP-Binding Protein That Forms Complexes with Truncated Nucleoside Diphosphate Kinase and Pyruvate Kinase To Modulate GTP Synthesis

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We report the purification and characterization of a protein from the membrane fraction of *Pseudomonas aeruginosa* showing intrinsic guanosine triphosphatase (GTPase) activity. The protein was purified as a 48-kDa polypeptide capable of binding and hydrolyzing GTP. The N-terminal sequence of the purified protein revealed its similarity to the *Escherichia coli* Ras-like protein (Era), and the protein cross-reacted with anti-Era antibodies. This protein was named *Pseudomonas* Ras-like protein (Pra). Anti-Pra antibodies also cross-reacted with *E. coli* Era protein. Pra is autophosphorylated in vitro, with phosphotransfer of the terminal phosphate from [γ-^{32}P]GTP but not [γ-^{32}P]ATP. Pra is capable of complex formation with the truncated 12-kDa form of nucleoside diphosphate kinase (Ndk) but not with the 16-kDa form. Purified Pra was also shown to physically interact with pyruvate kinase (Pk); Pk and Pra can form a complex, but when the 12-kDa Ndk, Pk, and Pra are all present, Pk has a higher affinity than Pra for forming a complex with the 12-kDa Ndk. The 12-kDa Ndk-Pra complex catalyzed increased synthesis of GTP and dGTP and diminished synthesis of CTP and UTP or dCTP and dTTP relative to their synthesis by uncomplexed Ndk. Moreover, the complex of Pra with Pk resulted in the specific synthesis of GTP as well when Pra was present in concentrations in excess of that of Pk. Membrane fractions from cells harvested in the mid-log phase demonstrated very little nucleoside triphosphate (NTP)-synthesizing activity and no detectable Ndk. Membranes from cells harvested at late exponential phase showed NTP-synthesizing activity and the physical presence of Ndk but not of Pk or Pra. In contrast, membrane fractions of cells harvested at early to late stationary phase showed predominant GTP synthesis and the presence of increasing amounts of Pk and Pra. It is likely that the association of Pra with Ndk and/or Pk restricts its intrinsic GTPase activity, which may modulate stationary-phase gene expression and the survival of *P. aeruginosa* by modulating the level of GTP.

The *ras* genes have attracted a great deal of attention in recent years because of their involvement in the malignancy of human cells (16, 22). The Ras proto-oncogene protein (p21) in mammalian cells is a GTP-binding protein with a molecular size of 21 kDa and is frequently activated in human oncogenesis. Ras and Ras-like proteins are associated with membranes and are known as membrane-associated guanosine triphosphatases (GTPases) (12, 23). Although it is generally accepted that the members of the GTPase superfamily are extremely important in regulating membrane-signalling pathways in cells (21, 23), very little is known about the cellular functions of membrane-associated GTPases in bacterial systems. In order to understand the possible functions of *ras* genes, a number of prokaryotic proteins have been studied as models. Ras-like proteins play important roles in the physiologies of lower eukaryotic microorganisms and bacteria; for example, *Escherichia coli* Ras-like protein (Era) is a GTP-binding protein that is essential for cell growth and viability (1, 24). In *Bacillus subtilis*, the *spo0B* operon encodes a GTP-binding protein (Obg) which is required for sporulation and essential for cell viability (32, 35). In *Saccharomyces cerevisiae*, Ras is also necessary for viability (19), whereas in *Schizosaccharomyces pombe* and *Dictyostelium discoideum*, Ras is essential for appropriate responses in mating (13, 14) and chemoattraction (33). In *Drosophila melanogaster*, *ras* genes are involved in development (6, 27).

GTP binding and GTP hydrolysis are intrinsic biochemical properties of Ras proteins. GTP is a key signalling molecule and a precursor of the regulatory compound ppGpp in bacteria (8). Proteins which bind guanosine nucleotides (G proteins) are involved in hormone action (9), visual transduction (30), protein synthesis (26), and microtubule assembly (7) in eukaryotes and serve as important key regulatory elements (18). Although guanosine nucleotide-binding proteins in eukaryotes are well studied, there is less information on the roles of these proteins in the life cycles of prokaryotes. We have previously reported that in *Pseudomonas aeruginosa* 8830, the 16-kDa nucleoside diphosphate (NDP) kinase (Ndk) is cleaved by an intracellular protease and converted to a 12-kDa form (29). This 12-kDa form is predominantly associated with the cell membrane and forms a complex with pyruvate kinase (Pk), which predominantly synthesizes GTP (29, 31). Cell membrane-associated GTPases and G proteins may be directly involved with GTP-generating complexes, such as the membrane-associated Ndk-Pk complex, in stringently regulating the intracellular levels of GTP. Moreover, GTP is involved in the synthesis of alginate as a precursor of GDP mannose (25, 31).

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Since the membrane-associated Ndk-Pk complex predominantly generates GTP, it was of interest to examine whether a GTPase activity might also be associated with this membrane-bound complex. In this report, we demonstrate the membrane association of a Ras-like protein which has intrinsic GTPase activity and which is capable of forming complexes with the 12-kDa Ndk as well as Pk, thereby modulating Ndk's and Pk's GTP-synthesizing activities and presumably the extents of their gene expression in the stationary phase.

MATERIALS AND METHODS

Bacterial strains, media, chemicals, and reagents. *P. aeruginosa* 8830 is a stable alginate-producing strain (11). The organism was maintained on *Pseudomonas* isolation agar plates (Difco Laboratories, Detroit, Mich.) and routinely grown in Luria-Bertani broth at 37°C with shaking at 250 rpm. [α-^{32}P]- or [γ-^{32}P]GTP, [α-^{32}P]- or [γ-^{32}P]ATP (3,000 Ci/mmol each), and inorganic phosphate ($^{32}P_i$, 1 mCi) were obtained from Amersham Inc., Arlington Heights, Ill. The NDPs ADP, CDP, GDP, and UDP and the nucleoside triphosphates (NTPs) ATP, CTP, GTP, and UTP and their deoxy derivatives (dNDPs and dNTPs) were obtained from Sigma Chemical Co., St. Louis, Mo. All other reagents were of the highest quality commercially available.

Proteins and antibodies. The 12-kDa Ndk and Pk were purified from membrane and cytoplasmic fractions of *P. aeruginosa*, respectively, as previously described (29, 31). Anti-Ndk, anti-Pk, and anti-Pra polyclonal antibodies were obtained commercially (Cocalico Biologicals Inc., Reamstown, Pa.). Purified Era (1) was a generous gift from M. Inouye of Robert Wood Johnson Medical School, Piscataway, N.J., and anti-Era antibodies were kindly supplied by D. Court and B. Powell from the Frederick Cancer Research and Development Center.

Purification of Pra from *Pseudomonas aeruginosa* 8830. Twenty-five liters of cells was used for the purification of Pra. Cells were grown in 4.0-liter batches of Luria-Bertani broth to an A_{600} of 1.2. The cells were harvested by centrifugation at 15,000 $\times$ *g* for 30 min at 4°C. The pellet was washed once with ice-cold buffer A (50 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.5% glycerol) and subsequently suspended in the same buffer to obtain a homogeneous cell suspension.

Preparation of membrane fraction. Cells were lysed with a French press (Power Laboratory Press; American Instruments Co., Silver Spring, Md.) at 18,000 lb/in^2 for 3 to 5 min, and this process was repeated three times for each sample. The cell lysate was centrifuged at 10,000 $\times$ *g* for 30 min to remove cell debris and unbroken cells, and the resulting supernatant was collected in fresh plastic bottles. The supernatant thus obtained was centrifuged at 50,000 $\times$ *g* at 4°C for 1 h to sediment membranes. The resulting pellet obtained was suspended in ice-cold buffer A and used as the membrane fraction for all subsequent experiments. The membrane fraction was stored at −70°C.

Ammonium sulfate fractionation. The membrane fraction was subjected to 30% $(NH_4)_2SO_4$ precipitation overnight with light stirring at 4°C, and the precipitated proteins were collected by centrifugation at 10,000 $\times$ *g* at 4°C for 30 min. The supernatant of the 30% $(NH_4)_2SO_4$ fraction was subjected to 50% $(NH_4)_2SO_4$ precipitation and kept gently swirling at 4°C for 2 to 3 h, followed by centrifugation at 10,000 $\times$ *g* at 4°C for 30 min. The supernatant from the 30 to 50% fractionation was further subjected to 70% $(NH_4)_2SO_4$ precipitation; the precipitated protein was recovered by centrifugation, and the resulting pellet was kept at −70°C.

Column chromatography. Before column chromatography, the pellets were completely resuspended in appropriate volumes of ice-cold buffer A and dialyzed against 4 liters of ice-cold buffer A. Four hundred milligrams of protein from the 50 to 70% $(NH_4)_2SO_4$ fraction was applied to a Q-Sepharose column (2 by 7 cm) which had been equilibrated overnight with TMD (50 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 1 mM DTT). The flow rate was 1 ml/min. The column was eluted with a linear gradient of NaCl in TMD (0 to 100 mM) in a total volume of 80 ml, with the flow rate adjusted to 0.2 ml/min. All fractions were checked for GTPase activity, and those with GTPase activity were pooled, although a significant level of refinement of the protein over that of the crude extract was not obtained with this matrix. Ammonium sulfate (final concentration, 70%) was added to 22 ml of pooled active fractions and immediately mixed. EDTA was added to a final concentration of 0.1 mM for chelating trace amounts of metals from the $(NH_4)_2SO_4$. The pooled fractions were next applied to a hydrophobic-interaction column (TSK-phenyl; Bio-Rad, Hercules, Calif.) previously equilibrated with buffer A plus 1.0 M $(NH_4)_2SO_4$. The column was eluted with linearly decreasing concentrations of $(NH_4)_2SO_4$ in buffer A (1.0 M–0.0 M). The flow rate was adjusted to 0.5 ml/min. Fractions eluted with GTPase activity were pooled together. Sixteen milliliters of active pooled fractions from the TSK-phenyl column was subjected to overnight dialysis with 2 liters of 50% glycerol in TMD (pH 8.0) at 4°C with gentle but constant stirring. By this method, the active pooled fractions were concentrated to 7.5 ml. We constructed a GDP-agarose column (1.5 by 3.5 cm) consisting of GDP insolubilized in 4% beaded agarose, activated by cyanogen bromide (Sigma Chemical Co.), and equilibrated with TMD buffer. The 7.5 ml of concentrated protein sample was applied to the column; the column was eluted with 10 ml (each) 100, 200, and 400 mM KCl in TMD (pH 8), and each fraction was assayed for GTPase activity.

Immunoaffinity purification. Active fractions from the GDP-agarose column were pooled, and the protein content was determined to be 500 μg by the method of Bradford (5). Purified Ndk (1 mg/ml) was added in excess to this solution, followed by mixing, incubation on ice for 5 min, and the addition of anti-Ndk antibodies (1:5,000). The precipitated immunocomplex was centrifuged at 20,000 $\times$ *g* at 4°C for 20 min and collected. To this, 1 ml of 1 mM GTP dissolved in TMD buffer was added in an attempt to dissociate an Ndk-Pra complex. The solution was incubated at 37°C for 2 to 3 min and kept on ice for 5 min, followed by centrifugation at 20,000 $\times$ *g* at 4°C for 20 min. The resultant supernatant was collected, and its protein content and GTPase activity were determined. The fraction of Pra precipitated was approximately 80%, and almost all of the protein was released by GTP addition. The final preparation of Pra was not contaminated with Ndk, as was determined by Western blot analysis. Purified Pra was stored at −70°C in buffer A containing 50% glycerol.

GTPase assay. GTPase activity was measured in a 20-μl reaction volume in TMD buffer in which 1.0 μCi of [γ-^{32}P]GTP (3,000 Ci/mmol) was incubated with 10 μg of the protein for 30 to 60 min at 25°C. At the end of the incubation, the reaction was terminated by the addition of 2 μl of 40% sodium dodecyl sulfate (SDS) solution, and the reactants were separated by polyethylenimine thin-layer chromatography (TLC) with a mobile phase of 1 M LiCl–1 M HCOOH. Radioactivity counting was done with a Radioanalytic Imaging System (Ambis Systems Inc.) as described previously (31). The amount of $^{32}P_i$ released (in picomoles per milligram of protein) due to hydrolysis of GTP was calculated. Nonenzymatic degradation of GTP was measured by simultaneous incubation of [γ-^{32}P]GTP in the buffer in absence of membrane proteins. The N-terminal sequence analysis of the purified GTPase protein was carried out by K.-L. Ngai at the Genetic Engineering facility of the University of Illinois.

GTP binding assay. GTP binding was performed by the nitrocellulose filter binding method. The binding reaction was carried out in TMD buffer. One microgram of purified protein was spotted on the center of each piece (2 by 2 cm) of a Hybond-C+ membrane filter (Amersham, Inc., Arlington Heights, Ill.) and allowed to air dry for 10 min. Each filter was put onto a petri plate, and 10 ml of binding buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM DTT) was added. The plate was incubated at 25°C for 10 min, followed by addition of 1 ml of 10 mM GTP. One microcurie of [γ-^{32}P]GTP (3,000 Ci/mmol) was added to each plate and gently but thoroughly mixed. The binding reaction was monitored at 0, 15, 30, 45, 60, 90, and 120 min at 25°C. After completion of the binding reaction, each filter was washed for 30 min with an excess of TMD buffer (100 ml) several times and unbound excess [γ-^{32}P]GTP was removed. The filter was air dried, covered with plastic wrap, and subjected to autoradiography.

Autophosphorylation. Autophosphorylating activity of the purified protein was measured by incubating 1 μg of the purified protein with 1 μCi of [γ-^{32}P]GTP (3,000 Ci/mmol) at 25°C in a total reaction volume of 20 μl made with TMD buffer. One microgram of the purified protein was also incubated with 1 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) in the same buffer. The reaction was allowed to continue for 10 min and was terminated by the addition of 2 μl of 10% SDS. The terminated reaction mixture was loaded onto an SDS–15% polyacrylamide gel and run at 70 V for 3 to 4 h, followed by autoradiography.

Glycerol density gradient centrifugation. Glycerol density gradient centrifugation was carried out by a modified method of Bowman et al. (4) as described previously (31, 36). A batch gradient of glycerol was prepared by successive layering of 60, 50, 40, 30, 20, 10, and 5% glycerol solutions, each with a 1-ml volume in a Beckman 10-ml ultracentrifuge tube. The purified protein sample to be analyzed was layered carefully on top of the 5% band. The gradient tubes were centrifuged at 60,000 $\times$ *g* for 2 h at 4°C. Fractions were carefully collected by withdrawing 1-ml aliquots from the top of each gradient to microcentrifuge tubes. All fractions were analyzed by Western blot analysis with anti-Ndk antibodies, anti-Pk antibodies, and anti-Pra antibodies.

Effect of purified Pra on NTP and dNTP-synthesizing activity of Ndk or Pk. Both NTP- and dNTP-synthesizing activities were assayed in 20-μl reaction volumes containing 250 μM (each) CDP, GDP, and UDP or 250 μM (each) dCDP, dGDP, and dTDP. The reaction was initiated by adding different amounts of purified protein and 1 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) along with 125 μM nonradioactive ATP and incubated for 1 min, followed by the addition of 2 μl of 10$\times$ SDS stop buffer. One microliter of the reaction mixture was spotted onto a polyethylenimine TLC plate, and the products of the reaction were separated in 0.75 M KH_2PO_4 buffer (pH 3.75) as described previously (29, 31). The TLC plates were air dried, covered with plastic wrap, and exposed to X-ray film.

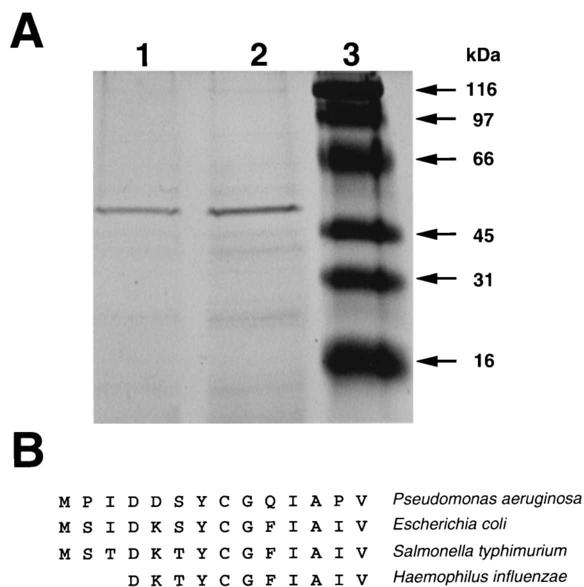
Immunological detection of Pra levels in various growth phases of *P. aeruginosa*. *P. aeruginosa* 8830 was grown in L broth at 37°C for 24 h, and 100-ml samples were withdrawn at various time intervals. One milliliter was used for measurement of the A_{650}, the remaining 99 ml was harvested by centrifugation, and the resulting pellet was washed once with ice-cold phosphate buffer. The cell suspension was subsequently lysed by sonication, and the total membrane fraction was isolated as described above. Various membrane fractions and supernatant samples from different growth phases were analyzed by Western blotting with anti-Ndk, anti-Pk, and anti-Pra antibodies.

TABLE 1. Purification of a membrane-associated GTP-hydrolyzing protein from *P. aeruginosa* 8830

Step no.	Purification step	Sp act (pmol of P_i/mg/h)	Fold purification
1	Membrane preparation	5,911	1.0
2	50 to 70% $(NH_4)_2SO_4$ pelleting	29,559	5.0
3	Phenyl-Sepharose column chromatography	88,665	15.0
4	GDP-agarose column chromatography	242,351	41.0
5	Immunoaffinity	1,695,113	286.7

RESULTS

Purification and identification of a GTP-hydrolyzing protein from cell membranes of *P. aeruginosa*. We have previously shown that a complex of 12-kDa Ndk with Pk in the *P. aeruginosa* membrane is responsible for altering the NTP-synthesizing specificity of Ndk to GTP (29, 31). We initially detected a GTPase activity in the membrane and attempted to isolate a protein with GTP-hydrolyzing activity from such fractions using standard methods. Following $(NH_4)_2SO_4$ fractionation, the GTP-hydrolyzing activity was enhanced and was found to be maximum in a 50 to 70% $(NH_4)_2SO_4$ pellet (Table 1); this fraction also showed Ndk and Pk activities. Further attempts to isolate this GTP-hydrolyzing activity, including the use of Q-Sepharose, phenyl-Sepharose, and GDP-affinity columns met with limited success (Table 1). Finally, fractions showing GTP-hydrolyzing activity following use of the GDP-affinity column were pooled and subjected to immunoaffinity purification with anti-Ndk antibodies. This process resulted in a purification of the GTP-hydrolyzing protein by approximately 286-fold (Table 1). Analysis of the purified protein employing SDS-polyacrylamide gel electrophoresis revealed a predominant polypeptide band with a molecular mass of approximately 47 to 48 kDa (Fig. 1A).



M P I D D S Y C G Q I A P V	*Pseudomonas aeruginosa*
M S I D K S Y C G F I A I V	*Escherichia coli*
M S T D K T Y C G F I A I V	*Salmonella typhimurium*
D K T Y C G F I A I V	*Haemophilus influenzae*

FIG. 1. (A) Purification of a GTP-hydrolyzing protein from *P. aeruginosa* 8830. The protein was purified from *P. aeruginosa* cell membranes according to the protocol described in Materials and Methods and summarized in Table 1. The purified protein was analyzed for size and electrophoretic homogeneity. Lane 1, 1 μg of purified protein; lane 2, 2 μg of purified protein; lane 3, molecular mass standards. (B) Sequence comparison of amino acids located at the N terminus of the *P. aeruginosa* GTP-hydrolyzing protein with various bacterial Ras-like proteins.

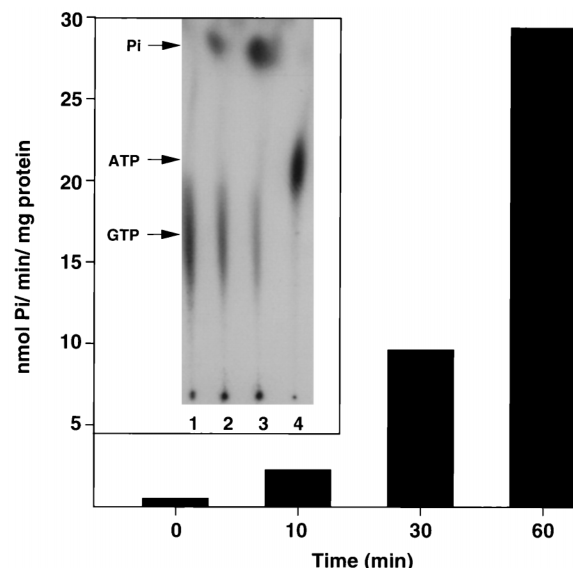


FIG. 2. GTPase activity of purified Pra. Twenty picomoles of Pra was incubated with 1 μCi of [γ-^{32}P]GTP (3,000 Ci/mmol) and 125 μM nonradioactive GTP at 25°C for various time intervals (0 to 60 min), and the release of $^{32}P_i$ was measured on an Ambis densitometric scanner (inset, lanes 1 to 3 correspond to 0, 30, and 60 min of incubation). Inset lane 4 shows no P_i release when the same amount of Pra is incubated with 1 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 125 μM nonradioactive ATP for 60 min. The level of release of P_i in the absence of Pra was negligible, and this value has been deducted in calculating Pra-induced P_i release.

The N-terminal sequence of 14 amino acids of the purified GTP-hydrolyzing protein was obtained and utilized in a BLAST search of the PIR protein database. This sequence was found to have high homology with Ras-like proteins from *E. coli*, *Salmonella typhimurium*, and *Haemophilus influenzae* (Fig. 1B). Because of its amino acid sequence, molecular mass, and biochemical properties, which were similar to those of proteins such as Era from *E. coli* (24), the GTP-hydrolyzing protein purified from *P. aeruginosa* was termed Pra for *Pseudomonas* Ras-like protein. Additionally, purified Era cross-reacted with anti-Pra antibodies, while purified Pra cross-reacted with anti-Era antibodies (data not shown).

Interactions of Pra with GTP. We examined the ability of Pra to hydrolyze GTP by incubating the protein with [γ-^{32}P] GTP for various time intervals (0, 10, 30, and 60 min) at 25°C and observing the release of ^{32}Pi. As shown in Fig. 2, the decreasing amount of [γ-^{32}P]GTP in the assay over time (Fig. 2 inset, lanes 1 to 3) is accompanied by an increase in the amount of ^{32}Pi (Fig. 2). Quantitation of the GTPase activity showed that Pra hydrolyzed GTP in a time-dependent manner, with maximal activity observed at around 60 min (Fig. 2). Additionally, the purified Pra protein showed no ATP-hydrolyzing activity (Fig. 2 inset, lane 4).

In order to see if the binding of Pra with GTP is important for its GTPase activity, a time course analysis of binding of [γ-^{32}P]GTP with Pra was conducted, as described in Materials and Methods. Maximal binding at 25°C was found only after 30 to 60 min. The binding was limited to GTP; no binding was observed with ATP, CTP, or UTP (data not shown).

We next examined the ability of the purified Pra protein to be autophosphorylated with [γ-^{32}P]GTP as a substrate. Incubation of Pra with [γ-^{32}P]GTP resulted in the rapid autophosphorylation of the protein (Fig. 3, lanes 1 and 2); however,

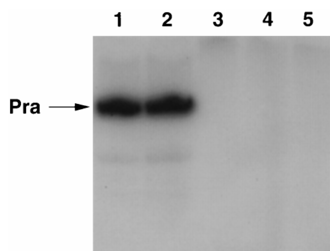


FIG. 3. Autophosphorylation of Pra in the presence of [γ-^{32}P]GTP. Lane 1, 20 pmol of Pra plus 1 μCi of [γ-^{32}P]GTP; lane 2, 40 pmol of Pra plus 1 μCi of [γ-^{32}P]GTP; lane 3, 40 pmol of Pra plus 1 μCi of [γ-^{32}P]ATP; lane 4, 40 pmol of bovine serum albumin plus 1 μCi of [γ-^{32}P]GTP; lane 5, 40 pmol of Pra plus anti-Pra antibodies plus 1 μCi of [γ-^{32}P]GTP.

autophosphorylation was not observed when Pra was incubated with [γ-^{32}P]ATP (Fig. 3, lane 3). The addition of anti-Pra antibodies abolished the autophosphorylation of the Pra protein (Fig. 3, lane 5).

Complex formation of Pra with Ndk and Pk. Since Pra was purified via immunoaffinity with anti-Ndk antibodies, we next examined whether Pra formed a complex with Ndk or Pk. We have previously shown that the cytoplasmic *P. aeruginosa* 16-kDa Ndk is truncated at the stationary phase to a 12-kDa form which is found in the cellular membrane fraction (29). This 12-kDa Ndk, and not the 16-kDa cytoplasmic Ndk, forms a complex with Pk; the Ndk-Pk complex alters the specificity of nucleotide synthesis by Ndk from all NTPs to GTP (31). We utilized glycerol gradient centrifugation (31) to assess the formation of complexes of Pra with 16-kDa Ndk, 12-kDa Ndk, and Pk; the formation of complexes is accompanied by a shift in the sedimentation of the proteins to a higher-concentration glycerol fraction (4). Following glycerol gradient centrifugation, fractions were collected and Western dot blots of each fraction were set up in triplicate; the blots were incubated with either anti-Ndk antibodies (Fig. 4A), anti-Pra antibodies (Fig. 4B), or anti-Pk antibodies (Fig. 4C). In previous experiments, we have shown that both the 16-kDa Ndk and the 12-kDa Ndk sediment in the 5% glycerol fraction (fraction 1) while Pk sediments in the 30% glycerol fraction (fraction 4) (31). In this experiment, we found that purified Pra was detected in the 10% glycerol fraction (Fig. 4B, fraction row Pra). Incubation of the 16-kDa Ndk with Pra did not result in a shift in the mobility of either protein; the 16-kDa Ndk was observed in the 5% fraction (fraction 1), while Pra was observed in the 10% glycerol fraction (fraction 2) (Fig. 4A and B, row Ndk 16 + Pra). Incubation of the 12-kDa Ndk with Pra resulted in a shift in mobility, as both of these proteins were observed in the 40% glycerol fraction (fraction 5) (Fig. 4A and B, row Ndk 12 + Pra). As we have shown previously (31), the 12-kDa Ndk, and not the 16-kDa Ndk, forms a complex with Pk; these results were confirmed in this experiment (Fig. 4A and C; rows Ndk 16 + Pk and Ndk 12 + Pk). Coincubation of the 16-kDa Ndk with Pra and Pk resulted in a shift in the mobilities of Pra and Pk, but not that of Ndk (Fig. 4A to C, row Ndk 16 + Pk + Pra). This suggested that Pra can also form a complex with Pk but not with 16-kDa Ndk as shown before; we confirmed this observation by incubating these two proteins together (Fig. 4B and C; row Pk + Pra). Coincubation of the 12-kDa Ndk with Pra and Pk resulted in complex formation of Ndk and Pk, suggesting that the 12-kDa Ndk has a higher affinity for Pk than Pra (Fig. 4A to C; row Ndk 12 + Pk + Pra), so that Pra cannot engage in complex formation and remain in fraction 2 when both the 12-kDa Ndk and Pk are present. The 12-kDa Ndk and Pk, however, form a complex and are found in the 50% glycerol fraction as reported previously (31).

Complex formation of *E. coli* Ras-like protein Era, which is similar to Pra, with the 12-kDa Ndk or Pk. The *E. coli* Ras-like protein Era has been shown to shuttle between the cytoplasm and the membrane and is essential for cell viability (21). Its biochemical function(s) is, however, not known (20, 21, 28). Since Pra and Era show sequence similarity at the N terminus and since these two proteins can cross-react with each other's antibodies, it was of great interest to see if Era could show cellular functions similar to those of Pra. The conversion of *E. coli* Ndk to any truncated form that may associate with the membrane has not been reported. Nevertheless, Era could form a complex, albeit with lower efficiency, with the *P. aeruginosa* 12-kDa Ndk but not with the *P. aeruginosa* 16-kDa Ndk, as measured by glycerol gradient centrifugation (Fig. 5). Similar to the results with Pra shown in Fig. 4, addition of Era to

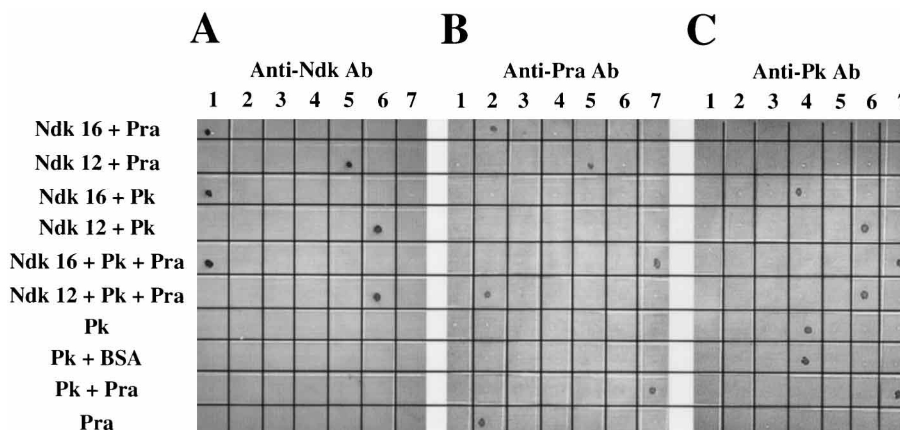


FIG. 4. Glycerol density gradient centrifugation analysis of complex formation among Pra, Ndk, and Pk. The glycerol batch gradient was prepared as described in Materials and Methods. The lanes have been marked according to the samples they represent. Column numbers 1 to 7 denote the fraction numbers and also represent increasing glycerol percentages. (A) Dot blot analysis of fractions 1 to 7 (glycerol, 5, 10, 20, 30, 40, 50, and 60%, respectively) probed with anti-Ndk antibodies; (B) same fractions as in panel A probed with anti-Pra antibodies; (C) same fractions as in panel A probed with anti-Pk antibodies. Five-microliter samples of the collected fractions were spotted onto a Hybond-C+ nitrocellulose filter (Amersham, Inc.) and probed with the antibodies. In all columns, 40 pmol of the 16- or 12-kDa Ndk, 40 pmol of Pra, and 40 pmol of Pk were used for complex formation. The antibodies were used at a dilution of 1:250 to enable detection without the need to concentrate samples diluted by centrifugation and separation in glycerol. Bovine serum albumin was used at twice the concentration of Pk in the complex formation assays.

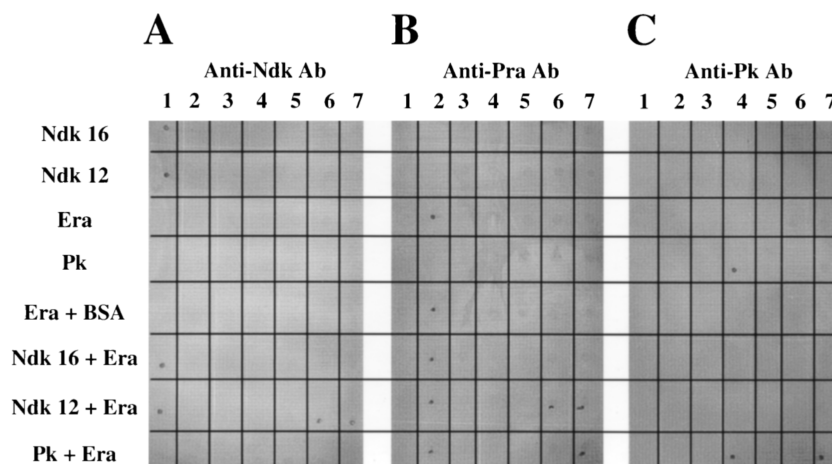


FIG. 5. Glycerol gradient centrifugation analysis of complex formation among Ndk, Pk, and *E. coli* Era. The methods of gradient formation, fraction collection, and dot blot analysis with anti-Ndk, anti-Pra, and anti-Pk antibodies are similar to those described in the legend to Fig. 4.

the 12-kDa Ndk shifts part of the Ndk band to higher concentrations of glycerol (50 and 60% glycerol, corresponding to fractions 6 and 7) and this complex cross-reacts with both anti-Ndk and anti-Pra antibodies (Fig. 5A and B, row Ndk 12 + Era). Like Pra (Fig. 4, row Pk + Pra), Era also forms a complex with *P. aeruginosa* Pk (Fig. 5, row Pk + Era) and the complex, although incomplete, sediments in fraction 7.

Effect of purified Pra or Era on NTP and dNTP synthesis by the 12-kDa Ndk and Pk. Ndk is responsible for the synthesis of all NTPs and dNTPs except for ATP in the cell. Since we found that the 12-kDa Ndk could form a complex with Pra (in the absence of Pk), we decided to determine if this interaction could affect the synthesis of NTPs and dNTPs by Ndk. Incubation of the 12-kDa Ndk with [γ-^{32}P]ATP and nonradioactive CDP, GDP, and UDP resulted in phosphotransfer and formation of [γ-^{32}P]CTP, [γ-^{32}P]GTP, and [γ-^{32}P]UTP (Table 2). Addition of increasing amounts of Pra to this reaction mixture resulted in the stimulation of GTP synthesis at low concentrations of Pra but inhibition of GTP synthesis at high concentrations of Pra. In contrast, Pra inhibited terminal phosphate transfer to CDP and UDP at any concentration (Table 2). Similar results were noted when phosphotransfer was measured from [γ-^{32}P]ATP to dNDPs such as dCDP, dGDP, and dTDP.

To examine if Era, like Pra, can modulate NTP, and particularly GTP, synthesis by Pk or the 12-kDa Ndk through complex formation, we tested the effects of adding different concentrations of Pra or Era to a fixed concentration of Pk (Fig. 6). We previously demonstrated that Pk can catalyze phosphotransfer from [γ-^{32}P]ATP to CDP-GDP-UDP in the presence of phosphoenolpyruvate (PEP) and Mn^{2+} ions (31). This is shown in lane A of Fig. 6. Addition of increasing amounts of Pra led to the predominant formation of GTP (Fig. 6, lanes D to G) when up to 300 pmol of Pra was incubated with 50 pmol of Pk. However, higher concentrations of Pra abolished phos-

TABLE 2. Effect of Pra on the synthesis of NTPs and dNTPs by the 12-kDa Ndk[a]

Protein(s)	Relative quantity of:				Relative quantity of:			
	GTP	ATP	CTP	UTP	dGTP	dATP	dCTP	dTTP
Ndk	72.0	10.3	40.7	33.0	84.0	4.0	44.7	40.4
Ndk with 1.0 pmol of Pra	112.3	66.4	8.8	8.5	146.9	40.0	13.4	12.3
Ndk with 10.0 pmol of Pra	108.8	114.4	3.1	5.4	80.3	86.8	4.9	4.3
Ndk with 120.0 pmol of Pra	59.7	117.0	1.0	2.2	89.8	90.9	3.7	1.8

[a] Ten microcuries (3,000 Ci/mmol) of [γ-^{32}P]ATP was incubated with a mixture containing 5 μmol each of nonradioactive CDP, GDP, and UDP (or dCDP, dGDP, and dTDP) and with 2.5 μmol of ATP (or dATP) in the presence of 50 pmol of the 12-kDa Ndk alone or in the presence of 1.0, 10.0, or 120.0 pmol of Pra. The mixture was incubated for 3.0 min, after which the reaction was stopped and [γ-^{32}P]NTPs were separated by TLC as described in Materials and Methods. The amounts of individual NTPs (GTP, ATP, CTP, and UTP) (or dNTPs) at the termination of the reaction were determined by Ambis densitometric scanning and quantitated as shown here.

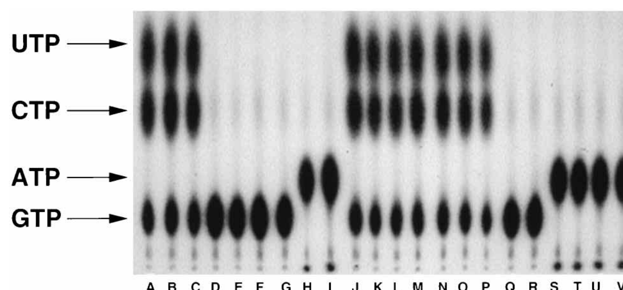


FIG. 6. NTP synthesis by Pk and its modulation by Pra and Era. Various amounts of Pra and Era were added to an NTP-synthesizing assay system with 50 pmol of Pk in the presence of 1 mM PEP and 50 μM Mn^{2+}. The NTP-synthesizing system consisted of adding 1 μCi of [γ-^{32}P]ATP, 125 μM nonradioactive ATP, and a mixture of 250 μM (each) CDP, GDP, and UDP and incubating the mixture for 3 min. The reaction was terminated, and the reactants were separated by TLC as described previously (29, 31). Lanes: A, 50 pmol of Pk plus PEP plus CDP-GDP-UDP; B, same reactants as in lane A plus 20 pmol of Pra; C, same reactants as in lane A plus 60 pmol of Pra; D, same reactants as in lane A plus 120 pmol of Pra; E, same reactants as in lane A plus 200 pmol of Pra; F, same reactants as in lane A plus 250 pmol of Pra; G, same reactants as in lane A plus 300 pmol of Pra; H, same reactants as in lane A plus 400 pmol of Pra; I, same reactants as in lane A plus 500 pmol of Pra; J, 50 pmol of Pk plus PEP plus CDP-GDP-UDP; K, same reactants as in lane J plus 20 pmol of Era; L, same reactants as in lane J plus 60 pmol of Era; M, same reactants as in lane J plus 120 pmol of Era; N, same reactants as in lane J plus 200 pmol of Era; O, same reactants as in lane J plus 250 pmol of Era; P, same reactants as in lane J plus 300 pmol of Era; Q, same reactants as in lane J plus 400 pmol of Era; R, same reactants as in lane J plus 500 pmol of Era; S, same reactants as in lane J plus 600 pmol of Era; T, Pk plus CDP-GDP-UDP but no PEP; U, Pra plus PEP plus CDP-GDP-UDP; V, Era plus PEP plus CDP-GDP-UDP.

FIG. 7. Effects of Pra and Era on NTP synthesis by the 12-kDa Ndk. Fifty picomoles of the 12-kDa Ndk was incubated with increasing concentrations of Pra or Era in an NTP-synthesizing system which consisted of 10 μCi of [γ-^{32}P]ATP, 125 μM nonradioactive ATP, and a mixture of 250 μM (each) CDP, GDP, and UDP incubated for 3 min. (A) Lanes 1 to 9 contain 50 pmol of the 12-kDa Ndk plus CDP-GDP-UDP without (lane 1) or with (lanes 2 to 9) Pra in the following amounts: 1.0 pmol (lane 2), 2.5 pmol (lane 3), 7.5 pmol (lane 4), 10 pmol (lane 5), 20 pmol (lane 6), 60 pmol (lane 7), 120 pmol (lane 8), and 150 pmol (lane 9). (B) Lanes 1 to 9 contain 50 pmol of the 12-kDa Ndk plus CDP-GDP-UDP without (lane 1) or with (lanes 2 to 9) the following amounts of Era: 1.0 pmol (lane 2), 2.5 pmol (lane 3), 7.5 pmol (lane 4), 10 pmol (lane 5), 20 pmol (lane 6), 60 pmol (lane 7), 120 pmol (lane 8), and 150 pmol (lane 9).

photransfer from [γ-^{32}P]ATP to GDP, preventing NTP synthesis (Fig. 6, lanes H and I). In contrast, Era had little effect up to a concentration of 300 pmol per 50 pmol of Pk (Fig. 6, lanes J to P). Above this concentration, Era changed the specificity of NTP synthesis by Pk to predominantly GTP (Fig. 6, lanes Q and R). Above a 500-pmol concentration, Era abolished phosphotransfer from [γ-^{32}P]ATP to CDP-GDP-UDP (Fig. 6, lane S). Pk had no NTP-synthesizing ability in the absence of PEP (Fig. 6, lane T), while neither Pra nor Era by itself could catalyze phosphotransfer to the CDP-GDP-UDP mixture from [γ-^{32}P]ATP (Fig. 6, lanes U and V).

We also tested the effects of increasing concentrations of Pra and Era on the specificities of NTP synthesis by both the 16-kDa and the 12-kDa forms of Ndk. Both Pra and Era had no apparent effect on NTP synthesis by the 16-kDa form of Ndk (data not shown). However, both of these proteins had a pronounced effect on NTP synthesis by the 12-kDa Ndk. In the assay system, 50 pmol of the 12-kDa Ndk was incubated with increasing amounts of Pra or Era in an NTP synthesis experiment as described in Materials and Methods. The effects of increasing concentrations of Pra from 7.5 to 60 pmol shifted the specificity of NTP synthesis by the 12-kDa Ndk to GTP (Fig. 7A, lanes 4 to 7). When the concentration of Pra was increased to above 60 pmol in the assay system, the NTP-synthesizing activity of the 12-kDa Ndk was completely inhibited (Fig. 7A, lanes 8 and 9). The effect of Era on the NTP-synthesizing activity of the 12-kDa Ndk was similar to that of Pra (Fig. 7B). However, a higher concentration of Era (20 pmol) was required to alter the specificity of synthesis to GTP (Fig. 7B, lane 6); as with Pra, concentrations of Era of 120 pmol or higher inhibited the NTP-synthesizing activity of the 12-kDa Ndk (Fig. 7B, lanes 8 to 9).

Role of membrane-associated Ndk, Pk, and Pra in NTP synthesis. We also examined NTP synthesis in vivo in the membrane fraction of *P. aeruginosa* at various stages of growth. We had previously shown that the membrane fractions of overnight-grown *P. aeruginosa* synthesize predominantly GTP and very little CTP and UTP from [γ-^{32}P]ATP and a mixture of GDP, CDP, and UDP and that this synthesis is due to the formation of a complex containing the 12-kDa Ndk and Pk (31). Shankar et al. (29) have shown that Ndk is predominantly cytoplasmic and has a size of 16 kDa during the exponential phase of growth but that it is converted to a 12-kDa form at the onset of stationary phase and is docked to the membrane. It was therefore of interest to see if the membrane fractions from early-log-phase cells could make either GTP or all three NTPs, depending upon the presence of either form of Ndk. The results in Fig. 8 show an analysis of NTP synthesis in membranes with antibodies to study the roles of Ndk, Pk, and Pra. After 6 h of growth (mid-log phase), no NTP-synthesizing activity is present in the membrane fraction (Fig. 8, lane A). When cultures are harvested after 8 h (late log phase), NTP-synthesizing activity is present in the membrane fraction (Fig. 8, lane E); this activity is lost following the addition of anti-Ndk antibodies but not anti-Pra or anti-Pk antibodies (Fig. 8, lanes F to H), suggesting that the activity is attributable only to Ndk. At 10 h, when cultures have entered stationary phase, predominantly GTP synthesis is observed (Fig. 8, lane I). The GTP-synthesizing activity is lost following the addition of anti-Ndk antibodies (Fig. 8, lane K), and the specificity for GTP synthesis is lost when anti-Pk antibodies are added (Fig. 8, lane L), suggesting that an Ndk-Pk complex is responsible for GTP synthesis. Anti-Pra antibodies had no effect. We have previously shown that the GTP-synthesizing activity (also shown in Fig. 8, control lanes Y to β) is attributable to a complex of Ndk and Pk (31). Our observations of membrane fractions at 12, 16, and 20 h of growth indicate that Pra plays a role in NTP-synthesizing activity well into the stationary phase. Addition of anti-Pra antibodies to the NTP synthesis reaction mixtures with membranes isolated at 12 h onwards resulted in a loss of the [γ-^{32}P]GTP generated (Fig. 8, lanes N, R, and V), with the bulk of the radioactivity being released as γ-^{32}P$_i$ (data not shown). However, the presence of small amounts of [γ-^{32}P]

FIG. 8. Time course of formation of GTP-synthesizing complexes involving the 12-kDa Ndk, Pra, and Pk in the membrane of *P. aeruginosa* 8830. Ten micrograms of membrane extract was used with or without the corresponding antibodies, each at a dilution of 1:1,000. Antibodies were added prior to the addition of the NDPs and [γ-^{32}P]ATP. CDP, GDP, and UDP were used at a concentration of 250 μM, and ATP was used at 125 μM; radioactive ATP was used at 10 μCi (3,000 Ci/mmol) of [γ-^{32}P]ATP per assay. Lanes A to X contain membrane extracts isolated from cells after the following periods of growth: 6 h (lanes A to D), 8 h (lanes E to H), 10 h (lanes I to L), 12 h (lanes M to P), 16 h (lanes Q to T), and 20 h (lanes U to X). Lanes Y to β are from a control experiment consisting of the purified 12-kDa Ndk and Pk, which form a complex and generate predominantly GTP (lane Y). This GTP formation by the Ndk-Pk complex is unaffected by anti-Pra antibodies (lane Z) but is abolished by anti-Ndk antibodies (lane α), while anti-Pk antibodies change the specificities to the syntheses of all three NTPs (lane β).

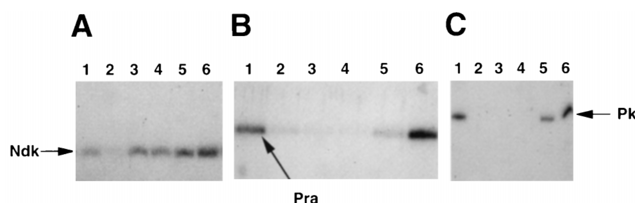


FIG. 9. Detection of the 12-kDa Ndk, Pra, and Pk in membrane fractions of *P. aeruginosa* 8830 at different growth time points. Membrane fractions were isolated as described in Materials and Methods and proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted, and subjected to Western blot analysis. Anti-Ndk, anti-Pra, and anti-Pk antibodies were each utilized at a dilution of 1:1,000. Panels A to C consist of identical Western blots probed with different antibodies. Lanes 1, purified Ndk, Pra, or Pk; lanes 2, membrane isolated from cells grown for 6 h; lanes 3, membrane from cells grown for 8 h; lanes 4, membrane from cells grown for 10 h; lanes 5, membrane from cells grown for 12 h; lanes 6, membrane from cells grown for 16 h.

CTP and [γ-^{32}P]UTP was noted (Fig. 8, lanes N, R, and V); such small amounts of [γ-^{32}P]CTP and [γ-^{32}P]UTP are not present in the NTP synthesis reaction mixtures to which no antibodies were added (Fig. 8, lanes M, Q, and U), suggesting that Pra may be involved in the predominant synthesis of GTP in the stationary phase as a complex with the 12-kDa Ndk. Results at 12, 16, and 20 h of growth with mixtures in which anti-Ndk or anti-Pk antibody was added clearly showed that Ndk is essential for NTP synthesis, while Pk at a late stage of growth promotes GTP synthesis in the membrane (Fig. 8, lanes G and H, K and L, O and P, S and T, and W and X). In order to determine if Pk and Pra are associated at different times in the membrane, thereby modulating GTP synthesis to different extents, and keeping in mind that the Ndk-Pk complex has a higher stability so that once formed, Pra cannot displace Pk from such a complex (Fig. 4), we used Western blots to analyze the *P. aeruginosa* membrane extracts for the presence of Ndk, Pk, and Pra at various growth time points (Fig. 9). Ndk was not detectable at the 6-h time point (Fig. 9A, lane 2), commensurate with the lack of NTP synthesis (Fig. 8, lanes A to D), but amounts of the protein increased at each succeeding time interval (Fig. 9A, lanes 3 to 6). In contrast, the Pra levels were low until the cells reached stationary phase (Fig. 9B, lanes 2 to 6), while the levels of Pk were similarly low until the onset of the stationary phase (Fig. 9C, lanes 2 to 6).

DISCUSSION

Guanine nucleotides are critical elements in gene expression, and many regulatory proteins or enzymes, termed G proteins, bind such nucleotides, particularly GTP. Hydrolysis of the protein-bound GTP to GDP often provides the energy, particularly in eukaryotes, for various cellular functions, such as oncogenesis, ion channel opening, tubulin polymerization, etc. One group of such proteins comprises the oncogene Ras proteins that transduce signals across the cellular membrane (3, 17). In prokaryotes, GTP-binding proteins, such as IF-2, EF-Tu, and EF-G, mediate various steps in peptide chain initiation and elongation (26) while Ras-like proteins, such as Era or Sgp, mediate some vital, but undefined functions of the cell (10, 20). Homologs of Era in *Streptococcus mutans* and *Coxiella burnetti* that can substitute for the essential functions of Era are also known (28, 37). The isolation of a *Pseudomonas* Ras-like protein, Pra, capable of cross-reacting with the *E. coli* Era antibody is therefore not entirely surprising. G proteins capable of association with cellular membranes and having GTPase activity are well known in prokaryotes (23), and both *E. coli* Era and the *B. subtilis* protein Obg are believed to be essential for cell viability (20, 32, 35). Thus, while knockout mutations in the *era* gene have not been isolated in the absence of a partly functional *era* gene, depletion of Era in temperature-sensitive mutants at high temperatures shows depressed synthesis of heat shock proteins, increased capability of the metabolism of some tricarboxylic acid cycle intermediates, and a lack of thermal induction of ppGpp pools (20). Exactly how Era depletion leads to all of these pleiotropic changes in the cell has not been fully evaluated.

While *era* and the *era* homologs were isolated based on their sequence homology with the *ras* genes (24), Pra was isolated based on its membrane-associated GTPase activity and its immunoprecipitation with anti-Ndk antibodies. We previously demonstrated that during the log phase of growth, the 16-kDa Ndk is cytoplasmic and produces all of the NTPs. At the onset of the stationary phase, a protease which has been purified (29) cleaves the 16-kDa Ndk to a 12-kDa form which is membrane associated. Site-directed mutational analyses have suggested that there are specific chaperones which might interact with the 12-kDa Ndk for its membrane association (31a). The 12-kDa Ndk forms a complex with Pk which modulates NTP synthesis to predominantly GTP synthesis (31). An important question was if membrane-associated Pk was the only protein that modulates NTP synthesis in the membrane. Since preliminary experiments demonstrated clear GTPase activity in the membrane, it was of obvious interest to purify the GTPase protein(s) and examine its nature of association with the GTP-generating Ndk-Pk complex or with Ndk and Pk individually. Indeed, the ability of Pra to form complexes separately with the 12-kDa Ndk or Pk (Fig. 4), altering the kinases' NTP-synthesizing specificities (Table 2 and Fig. 6 and 7), is an interesting example of the modulation of NTP synthesis by various enzyme complexes in the membrane. Since Pk has a higher affinity for complex formation with the 12-kDa Ndk than Pra (Fig. 4), and since the relative amounts of Pk are somewhat higher than those of Pra at the onset of stationary phase (Fig. 9), the membrane generates large amounts of GTP at this phase of growth (Fig. 8, lanes I, M, Q, and U). This GTP might then be converted to ppGpp by the RelA protein (8), which can then activate the stationary phase sigma factor RpoS (15) to allow cells to survive in the stationary phase. The time course of interaction among Ndk, Pk, and Pra and the extents of their complex formation during growth remain to be investigated.

Since Pra forms complexes with the 12-kDa Ndk and Pk, and since it cross-reacts with *E. coli* anti-Era antibodies and the *E. coli* Era cross-reacts with anti-Pra antibodies, an interesting question was if *E. coli* Era would show properties similar to those of Pra with respect to its complex formation with Ndk and Pk and modulation of their NTP-synthesizing abilities. It is to be noted that Pra at high concentrations inhibits GTP synthesis by Pk while promoting GTP synthesis at lower concentrations (Fig. 6). Thus, low concentrations of Pra may have effects on overall GTP synthesis different from those at higher concentrations, which occur in the late stationary phase (Fig. 9). High concentrations of Pra may allow subunit aggregation, leading to appreciable GTPase activity, as is seen with FtsZ, another GTP-binding protein with GTPase activity (34). The fact that anti-Pra antibodies had no effect on NTP synthesis in membrane fractions harvested from cells at 8 h of growth and 10 h of growth (onset of stationary phase) (Fig. 8, lanes F and J) appears to suggest that Pra is not a major determinant of NTP synthesis during the log phase or at the onset of the stationary phase of growth. However, when the anti-Pra antibodies were included in the NTP-synthesizing reaction mixture with membrane fractions isolated from stationary-phase cells (Fig. 8, lanes N, R, and V), GTP synthesis was completely

abolished, presumably through enhanced GTPase action, since most of the radioactivity appeared as P_i (data not shown). Thus, anti-Pra antibodies may cause structural alterations in the complex, leading to activation of the intrinsic GTPase activity. The role of Pra in the complex would then seem to keep such GTPase activities in check, thus promoting GTP synthesis. Exactly how such complexes may modulate NTP synthesis at the onset or early stationary phase and how such changes in the NTP levels may lead to alterations in gene expression or macromolecular metabolism at the stationary phase is at present unclear. Since Pra can interact with either the 12-kDa Ndk or Pk, modulating the kinases' GTP-synthesizing activities, but fails to form complexes when the 12-kDa Ndk and Pk are already in complexed form to generate GTP (Fig. 4), the modulation of GTP synthesis appears to depend on the relative appearance and the relative concentrations of these proteins in the cell. Further studies of the compositions or the natures of the membrane-associated Ndk complexes with various proteins are needed to obtain insights on their roles in cellular signaling processes.

The abilities of *E. coli* Era, like Pra, to form complexes with the *P. aeruginosa* 12-kDa Ndk and Pk and to modulate their NTP-synthesizing activities appear to indicate that Era may regulate GTP levels in *E. coli* in the stationary phase, as Pra seems to do in *P. aeruginosa*. While Ndk in *E. coli* has been studied extensively (2), very little information is available with regard to its membrane location or modulation of NTP synthesis due to complex formation with other proteins. It would be interesting to determine if, like *P. aeruginosa* Ndk (29), *E. coli* Ndk also undergoes proteolytic cleavage at the onset of the stationary phase to be transported to the membrane for complex formation with Pk or Era for effective modulation of GTP synthesis. Since GTP is a signalling molecule, modulation of its synthesis by Era would likely explain the pleiotropic effects of the depletion of Era in *E. coli* cellular metabolism (20).

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