GTPase-Dependent Signaling in Bacteria: Characterization of a Membrane-Binding Site for Era in *Escherichia coli*

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Era is an *Escherichia coli* GTPase that is essential for cell viability and is peripherally associated with the cytoplasmic membrane. Both immunoelectron microscopy and subcellular-fractionation experiments have shown that Era is present in cytoplasmic as well as membrane-associated pools. These data led to speculation that the mechanism of action of Era may require cycling between membrane and cytoplasmic sites. In order to investigate this possibility, an in vitro binding assay was developed to characterize the binding of Era to membrane fractions. Competition and saturation binding experiments suggest that a site that is specific for Era and capable of binding up to 5 ng of Era per microgram of membrane protein is present in membrane preparations. The binding curve is complex, indicating that multiple equilibria describe the interaction. The binding of Era to this putative receptor is dependent on guanine nucleotides; binding cannot be measured in the absence of nucleotide, and neither ATP nor UTP can substitute. Subfractionation of cell walls showed that the guanine nucleotide-dependent binding site was present in fractions enriched in cytoplasmic membrane. These data provide evidence that Era may be involved in a GTPase-receptor-coupled membrane-signaling pathway that is essential for growth in *E. coli*.

Membrane-associated GTPases are important regulators of membrane signaling in eukaryotic cells. Many different low-molecular-weight ligands elicit cellular responses by binding to G protein-coupled receptors (24). It is now known that these GTPases and their targets are present in all vertebrates and invertebrates (5, 24). Furthermore, investigations in *Saccharomyces cerevisiae* and mammalian cells have demonstrated that intracellular vesicle transport is controlled by small GTPases such as Ypt1, Sec4, Arf, and members of the rab family (9, 12, 17, 30, 33, 34). ras and ras-like proteins represent another distinct class of membrane-associated GTPases that are ubiquitous in eukaryotic cells and are essential for normal cell growth (3) and development (4, 18, 35). A large body of experimental evidence has shown that GTPases modulate cellular responses by cycling between GTP- and GDP-bound states (6, 7). The GTP-bound form of a GTPase is required for pathway activation. Hydrolysis of the γ -phosphate of GTP converts the GTP-bound GTPase (GTPase·GTP) to GTPase·GDP, rendering these proteins inactive. Recently there have been many reports describing factors that are required to regulate these conversions (2, 11, 13, 19, 21, 22, 27–29, 37, 39, 40). In contrast, except for the heterotrimeric G proteins, much less is known about the factors that GTPases interact with on the membrane.

Surprisingly, GTPase-dependent membrane-signaling pathways in bacteria have not been elucidated in detail. In *Escherichia coli*, an essential GTPase of unknown function (Era) which binds GTP and GDP and hydrolyzes the γ -phosphate bond of GTP has been described (1, 10, 16, 20, 26, 38). Subcellular fractionation (26) and immunoelectron microscopy (15) have provided evidence that Era is a peripheral membrane protein that is localized at the cytoplasmic surface of the inner membrane. There is no information concerning the

mechanism by which Era interacts with the membrane or whether it does so in a manner consistent with a role as a GTPase involved in membrane signaling.

In this report, an experimental approach that is aimed at characterizing the putative membrane Era receptor is described. By using the purified Era protein as a probe, and an exquisitely sensitive immunodetection system, an Era-specific binding site was found to cofractionate with the inner membrane. All these data, taken together, support a model in which Era functions as a molecular switch responsible for modulating an essential membrane-dependent pathway in *E. coli*.

MATERIALS AND METHODS

Strains and growth conditions. CTER411 (16) was grown in Luria broth medium (5 g of yeast extract, 10 g of tryptone [Difco], and 5 g of NaCl per liter) supplemented with ampicillin (50 μ g/ml) and kanamycin (50 μ g/ml). Cells were grown to early log phase at 42°C and then shifted to 27°C for 3 h to deplete Era (16). Growth was monitored turbidimetrically by using a Klett-Summerson calorimeter containing a red number 66 filter. The *E. coli* K-12 strain SB221 has been described previously (25).

Preparation of pure Era protein. Era was purified from overproducing cells as previously described (26), with the exception that a more efficient expression vector (pNG128) was employed in these experiments. In pNG128, the Era-encoding sequence was cloned downstream of the phage lambda promoter P_R . pNG128 also contains the temperature-sensitive repressor of P_R , c1857. At 30°C no elevated expression of Era can be observed, but within 1 h after shifting to 42°C, Era levels reach at least 10% of the total cellular protein. Longer incubations at 42°C result in an even higher level of expression. pNG128 contains the *ori* region and ampicillin resistance gene derived from pBR322.

Preparation of an Era-free membrane fraction. Era-depleted membranes were prepared from CTER411 grown as described above. All membrane fractionation steps were per-

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formed at 0 to 4°C. CTER411 cells were harvested by centrifugation, and the cell pellet was resuspended in lysis buffer (10 mM Na₂HPO₄ [pH 7.1], 1 mM EDTA, and 0.01 mg of lysozyme per ml) and incubated on ice for 30 min. Cell extract was prepared by disrupting cells by sonication followed by centrifugation at 1,000 × *g* for 10 min to remove cell debris. The supernatant was subjected to centrifugation at 100,000 × *g* for 80 min. The resulting membrane pellet was homogenized in a minimal volume of 10 mM Na₂HPO₄ (pH 7.1) and stored at -70°C.

Binding assay. Various concentrations of pure Era protein were added to CTER411 membranes in 10 mM Na₂HPO₄ (pH 7.1) in a total volume of 50 µl. The binding mixture was incubated at 0°C for 1 h followed by centrifugation at 288,000 × *g* for 14 min in a TL100 ultracentrifuge (Beckman). The membrane pellet was resuspended in 5 µl of Na₂HPO₄ buffer, and the suspension was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17.5% polyacrylamide gel), followed by immunoblotting. Era binding was quantitated by densitometry. Nonspecific aggregation was measured by performing the same assay in the absence of membranes. A known amount of pure Era was included on every immunoblot as an internal standard for transfer efficiency.

Preparation of ³⁵S-Era. ³⁵S-Era was prepared from the overproducing strain SB221/pNG128. The cells were grown in 100 ml of M9 minimal medium (supplemented with leucine, tryptophan, vitamin B₁, and ampicillin) at 30°C until mid log phase. The culture was shifted to 42°C for 1 h, and then 400 µCi of ³⁵S-methionine (Amersham) was added, and the incubation was continued for 10 min. The cells were harvested and resuspended in buffer A (20 mM Tris-HCl [pH 7.5], 20 mM KCl, 5 mM MgCl₂, 0.01 mM GDP) containing 0.3 mM phenylmethylsulfonyl fluoride. This suspension was subjected to sonication in order to lyse the cells. The cytosol fraction was separated from the membrane fraction by centrifugation at 100,000 × *g* for 80 min. ³⁵S-Era was purified from the supernatant by successive extraction with ammonium sulfate solutions of various concentrations from 85 to 15% (14). The extract and precipitate of each step were saved and analyzed by SDS-17.5% PAGE. Era was identified as the major protein in the 25% ammonium sulfate extract. It was estimated that more than 90% of the protein and the radioactivity of this fraction could be accounted for by Era protein.

Competitive binding experiment. CTER411 membranes (117.6 µg) were incubated with 9.7 × 10⁻⁸ M ³⁵S-Era in the presence or absence of various concentrations of unlabeled Era or CheR (provided in pure form by the laboratory of A. Stock, Center for Advanced Biotechnology and Medicine, Piscataway, N.J.) in a total volume of 0.625 ml at 0°C for 1 h. At the end of the incubation, the binding mixture was centrifuged at 288,000 × *g* for 14 min. The pellets were resuspended in Na₂HPO₄ buffer and subjected to scintillation counting. The same set of experiments were performed in the absence of membranes to determine the level of nonspecific aggregation of ³⁵S-Era.

Nucleotide dependence of Era-membrane binding. Pure Era was depleted of endogenous nucleotide by Sephadex G-25 column chromatography and employed in binding assays. Nucleotide-free Era was preincubated with GDP or guanosine 5'-O-(3-thiotriphosphate) (GTPγS) at a molar ratio of 1:1,000 for 45 min at 0°C followed by the normal binding assay described above. The nucleotide dependence of Era binding to membranes was also investigated by employing activated silica beads that were precoupled to membranes. Glutaraldehyde-activated silica beads (Boehringer Mannheim Co.) were pre-

coated with Era-free membranes. Approximately 20 mg of the silica beads was incubated with 312 µg of Era-free membrane protein in 1 ml of 10 mM Na₂HPO₄ (pH 7.1) and 0.1% NaCl. Constant mixing was maintained by gentle rotation at room temperature for 3 h. The beads were washed with 1 ml of 1.5% NaCl four times, and then unreacted sites were blocked with 0.3 M ethanolamine (pH 7.5) at room temperature for 40 min. The beads were then washed with 0.9% NaCl followed by 0.5 M propionic acid and finally washed again with 0.9% NaCl. The beads coated with Era-free membranes were then resuspended in a minimum volume of 10 mM Na₂HPO₄ and used in the nucleotide-dependent Era-binding assay as described above. A 5-mg amount of beads was employed in each binding assay. As a control for nonspecific binding, beads were prepared as described above except that no membrane protein was added to the coupling reaction mixture.

Subfractionation of Era-membrane binding sites by a discontinuous sucrose gradient. Era-free membranes were brought to a sucrose concentration of 20% (wt/wt) in 300 µl of Na₂HPO₄ buffer and then layered onto a 4.7-ml discontinuous sucrose gradient prepared in 10 mM Na₂HPO₄ (pH 7.1) containing 25, 40, and 55% (wt/wt) sucrose. The gradient was centrifuged at 117,000 × *g* for 16 h at 4°C in a Beckman SW65 rotor. Fractions were collected, diluted, and repelleted by ultracentrifugation. These membrane pellets were washed with Na₂HPO₄ buffer and employed in Era-binding assays.

Electrophoresis and immunoblotting. SDS-PAGE was performed with a 17.5% polyacrylamide slab gel (25). Proteins were visualized by Coomassie blue staining. For immunoblotting, proteins were transferred to nitrocellulose by semidry electroblotting with a Hoefer Scientific TE70 apparatus. Era protein was detected following immunoblotting by employing the Amersham ECL detection system as described previously (16). The purification and use of anti-Era immunoglobulin G has also been described previously (15).

Protein determination. Protein was determined by the method of Bradford (8), with bovine serum albumin as the standard.

RESULTS

In preliminary experiments aimed at developing an *in vitro* Era-membrane binding assay, we attempted to remove endogenous Era by high-salt-concentration washes. This method was not satisfactory for preparation of Era-free membranes in a reproducible manner. Therefore the strain CTER411 was employed to obtain membrane fractions depleted of Era *in vivo*. In CTER411, the chromosomal copy of Era was placed under control of a thermally inducible promoter (16). Previous experiments have shown that CTER411 grown at 42°C produces wild-type levels of Era. Within 2 h after a temperature shift to 27°C, Era levels are reduced by more than 90% (16). Figure 1 shows the membrane proteins obtained from a culture harvested after such a downshift (lane 1) and from a culture maintained at 42°C (lane 2). While the total membrane protein patterns are nearly indistinguishable between these conditions, it can be seen that membranes harvested from cells growing at 27°C contain no detectable Era (Fig. 1, lanes 4 and 5). As expected, cultures grown at 42°C contain wild-type levels of Era in the membrane fraction (Fig. 1, lanes 6 and 7). Era is present in membranes at levels that are below the limit of detection by Coomassie blue staining, and therefore the difference in Era content can be detected only by immunoblotting (15) (Fig. 1, lanes 1 and 2). In all subsequent experiments, membranes which were prepared from cells grown at 27°C and which contained no detectable Era were employed. Each

FIG. 1. SDS-PAGE analysis of membrane fractions prepared from CTER411. Cell membranes were prepared as described in Materials and Methods from cells growing at 27°C (lanes 1, 4, and 5) or 42°C (lanes 2, 6, and 7). The total membrane content was examined by Coomassie blue staining (lanes 1 and 2). Era content was examined by immunoblotting (lanes 3 to 7). Lane 3 contains 5.7 ng of pure Era protein; the arrowhead indicates the position of migration of Era. Lanes 1 and 4 contain 6.2 μ g of protein, lanes 2 and 6 contain 5.6 μ g of protein, lane 5 contains 9.3 μ g of protein, and lane 7 contains 8.4 μ g of protein. Electrophoresis and immunoblotting are described in Materials and Methods.

preparation of Era-free membranes was checked by immunoblotting to be certain that Era was reduced to levels below the limit of detection.

Era-membrane binding is saturable and specific. Increasing amounts of electrophoretically pure Era were mixed with a fixed concentration of Era-free membranes and incubated as described in Materials and Methods. Membranes were subsequently collected by ultracentrifugation and assayed for Era content by immunoblotting. These data were quantitated by

Saturation of CTER411 Membrane

FIG. 2. Binding of exogenous Era to Era-free membranes. Increasing amounts of pure Era protein were added to 1.1 μ g of CTER411 membranes as indicated. After a 60-min binding assay at 0°C, membranes were collected by ultracentrifugation and subjected to SDS-PAGE. Bound Era was quantitated by immunoblotting; each point represents an average of three independent determinations. Error bars indicate standard deviations.

Competitive Binding

FIG. 3. Inhibition of 35 S-Era-membrane binding by nonradioactive Era. In vivo-labeled 35 S-Era was prepared as described in Materials and Methods. A 2,400-cpm sample (2.2 μ g) of 35 S-Era was added to 117.6 μ g of Era-free membranes, and the mixture was incubated for 60 min at 0°C. Binding reaction mixtures also contained concentrations of nonradioactive Era (■) or CheR (▲) ranging from 0 to 50 $\times 10^{-7}$ M as indicated. Following the binding incubation, membranes were collected by ultracentrifugation and 35 S-Era binding was measured by scintillation counting.

densitometric analysis. Figure 2 shows a graphic representation of the results, which demonstrate that exogenous Era can saturate membrane-binding sites in vitro. At saturation, approximately 5 ng of Era per μ g of total membrane protein was bound. If Era interacted with a single receptor site at a 1:1 stoichiometry, the saturation curve should be hyperbolic and a single equilibrium constant could be calculated. Scatchard analysis of the data shown in Fig. 2 gives rise to two lines with different slopes. In the absence of additional knowledge concerning the mechanism by which Era interacts with membrane, further conclusions cannot be drawn from these data; however, the dissociation constants were calculated at 10 and 50 $\times 10^{-8}$ M. In this in vitro binding assay, at least two distinct equilibria were required to describe the binding that was being measured.

Cultures containing cells overexpressing Era were used as a source to prepare Era that was radiolabeled with [35 S]methionine in vivo (see Materials and Methods). 35 S-Era was added to Era-free membranes, both alone and in the presence of increasing amounts of nonradioactive Era or nonradioactive CheR. CheR is a soluble *E. coli* protein that interacts with membrane receptors responsible for transducing signals required for chemotaxis (36). Binding reactions were carried out as described above, and 35 S-Era present in the membrane pellet was quantitated by scintillation counting. Figure 3 shows that nonradioactive Era could compete efficiently for 35 S-Era binding, whereas addition of nonradioactive CheR resulted in reduction of 35 S-Era binding to a level of 70% of the signal observed in the absence of competing ligand. Increasing the amount of CheR did not result in further reduction of 35 S-Era binding (Fig. 3). These samples were also subjected to SDS-PAGE followed by autoradiography in order to be certain that 100% of the radioactivity found in the membrane pellet was due to 35 S-Era. Coomassie blue staining showed that CheR was a major component of the membrane pellet, and its concentration increased in proportion to the amount added in the reaction mixture. Thus, although CheR was not an effective competitor for the Era receptor, it was apparently binding to

FIG. 4. Nucleotide dependence of Era-membrane binding. (A) Pure Era protein was depleted of endogenous nucleotide by column chromatography and employed in membrane-binding assays. Lanes 2 through 7 show the results from binding assays containing 132 ng of nucleotide-free Era. Era was preincubated with 3.9 μ M GDP, or 3.9 μ M GTP γ S, for 45 min at 0°C as indicated. After preincubation, 6.5 μ g of Era-free membrane (MEMB) was added to some samples as indicated. Following ultracentrifugation, pellets were subjected to SDS-PAGE. Era content was evaluated by immunoblotting. (B) Era-membrane binding was assessed by measuring binding to silica beads precoated with Era-free membranes. Reaction mixtures (lanes 2 through 7) were prepared as described in the legend to panel A, with respect to Era and nucleotide content. Samples from lanes 2, 4, and 6 also contained membrane-coated beads (MEMB) (prepared as described in Materials and Methods). Samples from lanes 3, 5, and 7 contained uncoated beads as a control. Following a 60-min binding incubation, beads were collected by gravity, washed extensively, and then eluted with 1% SDS buffer. The SDS eluate was subjected to electrophoresis and immunoblotting. For both panels A and B, lane 1 contains 3.42 ng of pure Era as a marker (indicated by the arrowheads).

its receptor in a dose-dependent manner. The simplest interpretation of this observation is that the CheR and Era membrane-binding sites were not identical. Control experiments performed in the absence of added membrane demonstrated that most of the radioactivity pelleting in samples which contained more than 10-fold molar excess of nonradioactive Era could be accounted for by nonspecific protein-protein aggregation. At 10×10^{-7} M nonradioactive Era (10-fold molar excess), these controls showed that one-fifth of the residual signal was due to aggregation, and at 20×10^{-7} M, two-thirds of the residual signal was due to aggregation. This was not the case for samples containing nonradioactive CheR. Thus, the results shown in Fig. 3 represent an underestimate of the effectiveness of nonradioactive Era, compared with CheR, as a specific competitor for binding. From these data it can be concluded that nonradioactive Era specifically competed for 35 S-Era membrane association.

The binding of Era to its putative receptor is guanine nucleotide dependent. The Era protein used in the reactions described above was purified in the presence of 10 μ M GDP. GDP was removed from Era by Sephadex G-25 column chromatography, and then Era was employed in the binding assay. The binding of nucleotide-free Era was compared with the binding of Era $\cdot$ GDP and Era $\cdot$ GTP γ S. Membrane pellets were analyzed for Era content by immunoblotting (Fig. 4A). It can be seen that nucleotide-free Era has little or no affinity for membranes (Fig. 4A, lane 6), but addition of GDP fully restores binding (lane 4). However, the affinity of Era $\cdot$ GTP γ S for membranes could not be assessed because addition of

GTP γ S caused the pure protein to pellet even in the absence of added membrane (Fig. 4A, lane 3). This property is not shared by either Era $\cdot$ GDP or nucleotide-free Era (compare lanes 3, 5, and 7 in Fig. 4A). Apparently the GTP-bound form of Era has physical properties which are very different from those of either of the other two forms of the protein.

In order to assess whether Era $\cdot$ GTP γ S could associate with membranes, Era-free membranes were linked to activated glass beads. These membrane-coated beads were employed in the binding assay and then collected by gravity, washed extensively, and boiled in SDS electrophoresis sample buffer. The SDS eluate was subjected to SDS-PAGE followed by immunoblotting. Figure 4B shows that Era only has affinity for membrane-coated beads when either GTP γ S or GDP is added to the reaction mixture (lanes 2 and 4). As expected, nucleotide-free Era has no affinity for membrane-coated beads (Fig. 4B, lane 6), and guanine nucleotide-bound Era did not bind to uncoated beads (lanes 3 and 5). These assays were also carried out in the presence of UTP and ATP, and neither nucleotide could substitute for guanine nucleotide (not shown). The amount of bound Era recovered when reaction mixtures were supplemented with GTP γ S was slightly, but very reproducibly, greater than the amount of bound Era recovered when reaction mixtures were supplemented with GDP. Addition of total, dialyzed (nucleotide-depleted), cytoplasmic protein to the reaction mixture enhanced the effect of GTP γ S (not shown). Therefore, there are probably additional soluble factors that participate in a functional complex with Era at the putative membrane-binding site. From these data it can be concluded that the binding reaction was guanine nucleotide dependent.

Era binding to fractionated membranes. Previous experiments provided evidence that Era is a soluble protein that interacts peripherally with the cytoplasmic membrane (15, 26). These data led to the prediction that the Era-membrane binding sites should reside in the inner membrane subfraction of the total cell wall preparation. The inner membrane is less dense (and contains less protein) than the outer membrane (32). The outer membrane will readily pellet through 40% (wt/wt) sucrose and can be collected on a 55% (wt/wt) sucrose cushion. Era-free membranes were applied to a sucrose step gradient containing 25, 40, and 55% (wt/wt) sucrose. Following centrifugation, fractions were recovered from the gradient and diluted, and membranes were repelleted. The amount of membrane protein recovered was determined, and Era-binding assays were performed with resuspended material. Light membranes recovered at the 25 to 40% (wt/wt) sucrose interface and in the upper half of the 40% (wt/wt) sucrose step contained about 30% of the total protein pelleted and had a density corresponding to that of the inner membrane. These fractions contain about 80% of the total amount of Era bound for all fractions (Fig. 5A). The remaining pelletable protein was recovered in the more dense outer-membrane fractions (about 70% of the protein and 15% of the total Era bound) (Fig. 5A). The Era receptors found in the light membrane fractions were shown to contain guanine nucleotide-dependent binding sites (Fig. 5B). These data demonstrate that the less dense subfractions of *E. coli* cell wall preparations were enriched in the Era receptor. These results are consistent with immunoelectron microscopic data showing Era associated with the inner surface of the cytoplasmic membrane (15).

DISCUSSION

The experiments described here have shown that a specific and saturable binding site for Era exists in fractions containing cytoplasmic membranes. The fact that this binding can be

FIG. 5. Subfractionation of Era-membrane binding sites by discontinuous sucrose gradient centrifugation. (A) A 2.35-mg amount of Era-free membrane protein was brought to a sucrose concentration of 20% (wt/wt) and layered onto a 4.7-ml discontinuous sucrose gradient containing 2.1 ml of 25% sucrose, 2.1 ml of 40% sucrose, and 0.5 ml of 55% sucrose (wt/wt). The materials at the top (20% fraction), the 25% layer, the interface between 25 and 40% (Int 25-40%), the upper half of the 40% layer (U 40%), the lower half of the 40% layer (L 40%), and the 40- to 55% interface (Int 40-55%) were collected separately, diluted, and pelleted by ultracentrifugation. The protein content of the pellets was determined, and 5 μ g of membrane protein was employed in Era-membrane binding assays. The amount of added Era that bound was determined by quantitating immunoblots of polyacrylamide gels as described for our previous experiments. The total amount of Era binding for all fractions was determined, and the percentage of the total amount bound was calculated for each fraction (shaded bars). Also, the total amount of membrane protein recovered in all pellets was determined, and the percentage found in each fraction was calculated (hatched bars). (B) The membranes recovered in the Int 25-40% and U 40% fractions were pooled and examined for GDP-dependent Era-membrane binding as described in the legend to Fig. 4A. Lane 1 contains pure Era marker (arrowhead), and lanes 2 through 5 contain 132 ng of nucleotide-free Era. Reaction mixtures also contain 3.9 μ M GDP and/or 5 μ g of fractionated membrane (MEMB) as indicated. Era content was assessed by immunoblotting.

measured only in the presence of guanine nucleotides strongly supports the idea that the *in vitro* assay was a true measure of an important *in vivo* function of Era.

The studies aimed at investigating the nucleotide specificity of the binding reaction provided several important observations concerning the function of Era. It was demonstrated that the purified protein possessed hydrodynamic properties in the presence of GTP γ S very different from those in the presence of GDP (Fig. 4). Pure Era $\cdot$ GTP γ S in the absence of exogenous membrane was efficiently collected in the pellet upon ultracentrifugation. This was not true for any other form of the pure protein. The simplest explanation for this observation is that Era $\cdot$ GTP γ S forms larger intermolecular aggregates. This phenomenon may be a specific polymerization or a nonspecific artifact arising from the exposure of a hydrophobic domain upon protein binding to GTP γ S. Regardless of the correct explanation, the observation directly implies that the GTP-bound form of Era is distinct from the GDP-bound form of the protein. This is considered to be a defining feature of GTPases that act as molecular switches (6, 7). It must be emphasized that the aggregation property of Era was specific for Era $\cdot$ GTP and was not observed for Era $\cdot$ GDP (except at extremely high protein concentrations). All experiments in which ultracentrifugation was employed to collect bound Era were performed with Era $\cdot$ GDP in order to be certain that protein-protein aggregation was not interfering with our conclusions concerning membrane binding. The second interesting observation that arose from the investigations of nucleotide specificity of the binding reaction was that Era $\cdot$ GTP γ S and Era $\cdot$ GDP did not have equivalent membrane-binding properties. In all assays, the amount of bound Era was greater in the presence of GTP γ S than it was in binding reactions containing GDP. This observation was consistent even when ultracentrifugation was not employed to collect bound Era (Fig. 4B) and, therefore, cannot be explained by excess intermolecular aggregation of Era. Furthermore, this effect was enhanced when total, nucleotide-depleted, cytoplasmic protein was added back to binding reaction mixtures such as those used in the reactions described in the legend to Fig. 4B. This, coupled with the complex nature of the saturation binding curve (Fig. 2), suggests that the Era-membrane interaction involves multimeric or higher-order structures.

Recently, experiments with eukaryotic GTPases, such as p21^{ras}, have suggested that these enzymes form functional complexes with multiple proteins that include both soluble factors and membrane receptors (for a review, see reference 31). While membrane-associated signaling pathways in prokaryotes and eukaryotes vary widely in their purpose, it seems reasonable that certain aspects of the signaling mechanism itself might be similar in the two types of cells. It is intriguing to speculate that Era, upon activation and GTP binding, might then associate with one or more proteins at the membrane and facilitate propagation of an extracellular signal. Furthermore, Era might function as a common relay point in multiple pathways in accordance with the numerous pleiotropic effects observed when Era is depleted *in vivo* (23). In any case, the nucleotide-dependent association of Era with the inner membrane may provide an important clue in elucidating the elusive function of this protein.

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REFERENCES

- Ahn, J., P. E. March, H. Takiff, and M. Inouye. 1986. A GTP-binding protein of *E. coli* has homology to yeast RAS proteins. *Proc. Natl. Acad. Sci. USA* **83**:8849–8853.
- Albright, C. F., B. W. Giddings, J. Liu, M. Vito, and R. A. Weinberg. 1993. Characterization of a guanine nucleotide dissociation stimulator for a ras-related GTPase. *EMBO J.* **12**:339–347.
- Barbacid, M. 1987. *ras* genes. *Annu. Rev. Biochem.* **56**:779–827.
- Beitel, G., S. Clark, and H. R. Horvitz. 1990. The *Caenorhabditis elegans* *let-60* acts as a switch in the pathway of vulval induction. *Nature (London)* **348**:503–509.
- Birnbaumer, L. 1992. Receptor-to-effector signaling through G proteins: roles for $\beta\gamma$ dimers as well as α subunits. *Cell* **71**:1069–1072.
- Bourne, H. R., D. A. Sanders, and F. McCormick. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature (London)* **348**:125–132.
- Bourne, H. R., D. A. Sanders, and F. McCormick. 1991. The GTPase superfamily: a conserved structure and molecular mechanism. *Nature (London)* **349**:117–127.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell* **62**:317–329.
- Chen, S.-M., H. E. Takiff, A. M. Barber, G. C. Dubois, J. C. A. Bardwell, and D. L. Court. 1990. Expression and characterization of RNase III and Era proteins: products of the *rnc* operon of *Escherichia coli*. *J. Biol. Chem.* **265**:2888–2895.
- Diekmann, D., S. Brill, M. D. Garrett, N. Totty, J. Hsuan, C. Monfries, C. Hall, L. Lim, and A. Hall. 1991. *Bcr* encodes a GTPase-activating protein for p21^{rac}. *Nature (London)* **351**:400–402.
- Donaldson, J. G., R. A. Kahn, J. Lippincott-Schwartz, and R. D. Klausner. 1991. Binding of ARF and β -COP to Golgi membranes: possible regulation by a trimeric G protein. *Science* **254**:1197–1199.
- Emkey, R., S. Freedman, and L. A. Feig. 1991. Characterization of a GTPase-activating protein for the Ras-related Ral protein. *J. Biol. Chem.* **266**:9703–9706.
- Englard, S., and S. Seifter. 1990. Precipitation techniques. *Methods Enzymol.* **182**:285–300.
- Gollop, N., and P. E. March. 1991. Localization of the membrane binding sites of Era in *Escherichia coli*. *Res. Microbiol.* **142**:301–307.
- Gollop, N., and P. E. March. 1991. A GTP-binding protein (ERA) has an essential role in growth rate and cell cycle control in *Escherichia coli*. *J. Bacteriol.* **173**:2265–2270.
- Hall, A. 1990. The cellular functions of small GTP-binding proteins. *Science* **249**:635–640.
- Han, M., and P. W. Sternberg. 1990. *let-60*, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a ras protein. *Cell* **63**:921–931.
- Hart, M. J., K. Shinjo, A. Hall, T. Evans, and R. A. Cerione. 1991. Identification of the human platelet GTPase activating protein for the CDC42Hs protein. *J. Biol. Chem.* **266**:20840–20848.
- Inada, T., K. Kawakami, S.-M. Chen, H. E. Takiff, D. L. Court, and Y. Nakamura. 1989. Temperature-sensitive lethal mutant of Era, a G protein in *Escherichia coli*. *J. Bacteriol.* **171**:5017–5024.
- Jones, S., M. L. Vignais, and J. R. Broach. 1991. The CDC25 protein of *Saccharomyces cerevisiae* promotes exchange of guanine nucleotide bound to Ras. *Mol. Cell. Biol.* **11**:2641–2646.
- Leonard, D., M. J. Hart, J. V. Platko, A. Eva, W. Henzel, T. Evans, and R. A. Cerione. 1992. The identification and characterization of a GDP-dissociation inhibitor (GDI) for the CDC42Hs protein. *J. Biol. Chem.* **267**:22860–22868.
- Lerner, C. G., and M. Inouye. 1991. Pleiotropic changes resulting from depletion of Era, an essential GTP-binding protein in *Escherichia coli*. *Mol. Microbiol.* **5**:951–957.
- Lochrie, M. A., and M. I. Simon. 1988. G protein multiplicity in eukaryotic signal transduction systems. *Biochemistry* **27**:4957–4965.
- March, P. E., and M. Inouye. 1985. Characterization of the *lep* operon of *E. coli*: identification of the promoter and the gene upstream of the signal peptidase I. *J. Biol. Chem.* **260**:7206–7213.
- March, P. E., C. G. Lerner, J. Ahnn, X. Cui, and M. Inouye. 1988. The *E. coli* Ras-like protein (Era) has GTPase activity and is essential for cell growth. *Oncogene* **2**:539–544.
- Mizuno, T., K. Kaibuchi, T. Yamamoto, M. Kawamura, T. Sakoda, H. Fujioka, Y. Matsuura, and Y. Takai. 1991. A stimulatory GDP/GTP exchange protein for smg p21 is active on the post-translationally processed form of c-Ki-ras p21 and rhoA p21. *Proc. Natl. Acad. Sci. USA* **88**:6442–6446.
- Morii, N., K. Kawano, A. Sekine, T. Yamada, and S. Narumiya. 1991. Purification of GTPase-activating protein specific for the *rho* gene products. *J. Biol. Chem.* **266**:7646–7650.
- Rubinfeld, B., S. Munemitsu, R. Clark, L. Conroy, K. Watt, W. J. Crosier, F. McCormick, and P. Polakis. 1991. Molecular cloning of a GTPase activating protein specific for the Krev-1 protein p21^{ras}. *Cell* **65**:1033–1042.
- Salminen, A., and P. J. Novick. 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell* **49**:527–538.
- Schlessinger, J. 1993. How receptor tyrosine kinases activate Ras. *Trends Biochem. Sci.* **18**:273–275.
- Schnaitman, C. A. 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. *J. Bacteriol.* **104**:890–901.
- Segev, N., J. Mulholland, and D. Botstein. 1988. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell* **52**:915–924.
- Serafini, T., L. Orci, M. Amherdt, M. Brunner, R. A. Kahn, and J. E. Rothman. 1991. ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell* **67**:239–253.
- Simon, M. A., D. D. L. Bowtell, G. S. Dodson, T. R. Lavery, and G. M. Rubin. 1991. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell* **67**:701–716.
- Stock, J. B., M. G. Surette, W. R. McCleary, and A. M. Stock. 1992. Signal transduction in bacterial chemotaxis. *J. Biol. Chem.* **267**:19753–19756.
- Strom, M., P. Vollmer, T. J. Tan, and D. Gallwitz. 1993. A yeast GTPase-activating protein that interacts specifically with a member of the Ypt/Rab family. *Nature (London)* **361**:736–739.
- Takiff, H. E., S. Chen, and D. L. Court. 1989. Genetic analysis of the *rnc* operon of *Escherichia coli*. *J. Bacteriol.* **171**:2581–2590.
- Trahey, M., and F. McCormick. 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* **238**:542–545.
- Ullrich, O., H. Stenmark, K. Alexandrov, L. A. Huber, K. Kaibuchi, T. Sasaki, Y. Takai, and M. Zerial. 1993. Rab GDP dissociation inhibitor as a general regulator for the membrane association of Rab proteins. *J. Biol. Chem.* **268**:18143–18150.