lated from isotopically heterogeneous materials or that Re was leached at some stage following their accretion.

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The Effect of GTPase Activating Protein upon Ras Is Inhibited by Mitogenically Responsive Lipids

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Bacterially synthesized c-Ha-*ras* protein (Ras) was incubated with guanosine triphosphatase (GTPase) activating (GA) protein in the presence of various phospholipids. The stimulation of Ras GTPase activity by GA protein was inhibited in some cases. Among the lipids most active in blocking GA protein activity were lipids that show altered metabolism during mitogenic stimulation. These included phosphatidic acid (containing arachidonic acid), phosphatidylinositol phosphates, and arachidonic acid. Other lipids, including phosphatidic acid with long, saturated side chains, diacylglycerols, and many other common phospholipids, were unable to alter GA protein activity. The interaction of lipids with GA protein might be important in the regulation of Ras activity during mitogenic stimulation.

CELLULAR *RAS* PROTEIN (RAS) appears to be critical during mitosis (*1*). Although it is not known how Ras functions, the regulation of Ras activity could be essential in the control of cellular proliferation (*2*). A cytoplasmic GTPase activating (GA) protein stimulates the rate at which Ras converts bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP) (*3*). Since this conversion is believed to inactivate Ras, GA protein might be involved in negatively regulating Ras activity. On the other hand, studies with *ras* mutants suggest that Ras might control the activity of GA protein (*4, 5*). In either case, the interaction between these two proteins could be involved in the control of cellular proliferation, although it has not been shown that the interaction between Ras and GA protein can be modified during mitogenic stimulation. Microinjection of antibody to Ras, however, indicated that the mitogenic action of several lipid-related materials was dependent upon cellular Ras activity (*6*). It was postulated that phospholipid metabolism might be related to the biological activation of cellular Ras and therefore to the interaction between GA protein and Ras.

GTPase activating protein (often called GAP) activity has been identified in crude cytoplasmic extracts from a variety of cell types, it has been purified, and the gene has been cloned (*7, 8*). GA protein increases the GTPase activity of purified, bacterially synthesized Ras in solution or of cellular Ras associated with crude membrane preparations. We initially used crude mouse brain cytosol as our source of GA protein (*9*) and then also used bacterially synthesized, cellular Harvey Ras. In the first analysis, liposomes of phosphatidic acid with saturated fatty acid side chains (stearic acid) were

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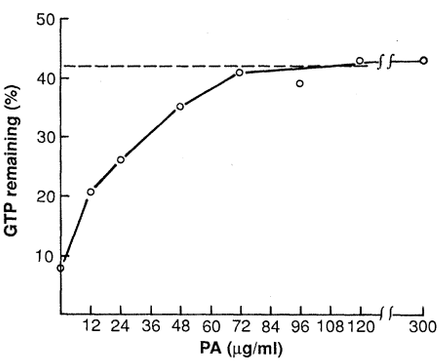

Fig. 1. Inhibition of GA protein activity by increasing concentrations of phosphatidic acid. (PA) Bacterially synthesized Ras was bound to [α-^{32}P]GTP and then incubated with crude GA protein in the presence of various concentrations of phosphatidic acid. After 1 hour, the Ras was precipitated with a specific antibody and the ratio of bound nucleotides was determined with TLC. The broken line indicates the percentage of GTP remaining bound to Ras in the absence of GA protein or phospholipid. The ability of GA protein to increase GTPase and decrease the amount of bound GTP was inhibited approximately 50% by 24 μg/ml phosphatidic acid. Nucleotide-free Ha-Ras (2 μ*M*) was incubated for 20 min at 30°C with 1 μ*M* [α-^{32}P]GTP (3000 Ci/mmol; Amersham) in 50 μl of tris-HCl, *p*H 7.5, buffer containing 2 m*M* dithiothreitol without added $MgCl_2$. GTPase reaction was initiated by addition of $MgCl_2$ and crude GA protein preparation (*3–5, 9*) and the indicated concentrations of phosphatidic acid (γ-stearoyl β-arachidonoyl) in 150 μl of reaction buffer [final concentrations: 20 m*M* tris-HCl, *p*H 7.5, 3 m*M* $MgCl_2$, 0.15*M* NaCl, 1 m*M* dithiothreitol (*23*)]. After incubation at 30°C for 1 hour, Ras was immunoprecipitated by monoclonal antibody Y13-259 and protein A–Sepharose beads coated with rabbit antibody to rat immunoglobulin G (*3, 4*). Bound nucleotides were released from the immunoprecipitate by boiling for 3 min. Bound nucleotides were resolved on a polyethyleneimine cellulose TLC plates (EM Science) in 1*M* potassium phosphate, *p*H 3.4, visualized by autoradiography and quantitated. Liposomes were formed as in (*12*). Ha-*ras* protein was prepared in a bacterial expression system as described (*24*).

incubated with Ras that had been loaded with [α-^{32}P]GTP. This lipid was selected because it is highly mitogenic when added to cultures of NIH 3T3 cells (*6, 10*). The extent of conversion of bound GTP to GDP was determined after 30 min by precipitating Ras with a specific antibody, dissociating the bound nucleotides, and resolving them by thin-layer chromatography. As expected, the GA protein preparation increased Ras GTPase and thus the proportion of GDP bound to Ras after incubation. This increase in the rate of Ras GTPase induced by GA protein was not affected by added liposomes.

Phosphatidylinositol normally contains arachidonic acid (a 20-carbon fatty acid with four *cis* double bonds) and a saturated fatty acid. Since the metabolism of this lipid increases upon mitogenic stimulation (*11*), liposomes (or micelles) of phosphatidic acid with this fatty acid structure were next tested. As with the saturated phosphatidic acid tested above, this lipid was deposited on the bottom of a tube by drying from a chloroform solution and then suspended in tris-HCl buffer, *p*H 7.5, by gentle sonication (*12*). The increased conversion of GTP to GDP induced by GA protein was greatly reduced in the presence of this lipid. The concentration of lipid was directly related to the extent of GA protein inhibition (Fig. 1). A 30% inhibition of GA protein activity was observed in the presence of 12 μg/ml of phosphatidic acid, while 60 μg/ml completely inhibited GA protein activity. With 60 μg/ml, the amount of GTP associated with Ras after incubation in the presence of GA protein was equal to the GTP associated with Ras incubated without GA protein. Even 300 μg/ml of added phosphatidic acid was not able to further increase the proportion of GTP bound to Ras after incubation (*13*). Apparently, the lipid was able to completely block the effect of GA protein upon Ras but was unable to affect the GTPase activity of Ras itself.

It is clear from our data that only phosphatidic acid with the proper structure can affect GA protein activity. We therefore tested a number of phosphatidic acids from two commercial sources. Phosphatidic acids with saturated fatty acid moieties of various length were tested along with two phosphatidic acids containing unsaturated side chains. Little or no activity was observed with the compounds containing saturated side chains. Dioleoyl and dilauroyl phosphatidic acid exhibited only limited activity compared to the molecule containing arachidonic-stearic acid (Fig. 2A and Table 1A).

A variety of other lipids were next tested for inhibition of GA protein activity. Phospholipids that are present in membranes at high concentrations were tested, as were some of their mitogenically active metabolites (Table 1A). The highest activity identified was associated with lipids related to phosphatidylinositol, while phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and several diacylglycerols were totally inactive. Even the diacylglycerol that contained arachidonic and stearic acids (which differs from the most active phosphatidic acid by a single phosphate group) was essentially inactive. Phosphatidylinositol was able to inhibit GA protein activity but not as well as phosphatidylinositol 4-monophosphate or phosphatidylinositol 4,5-diphosphate. Dose response analysis indicated the following relations in GA protein inhibition: phosphatidic acid (arachidonoyl, stearoyl) > phosphatidylinositol monophosphate > phosphatidylinositol diphosphate > phosphatidylinositol (Fig. 2B). It is interesting that the metabolism of phosphatidic acid and the phosphatidylinositol phosphates increases in mitogenically stimulated cells (*11*).

The selectivity of GA protein inhibition by the various lipids might indicate the biological importance and mechanism of the lipid-GA protein interaction. It is possible, however, that this selective inhibition of GA protein by the lipids is more a consequence of the physical conformation of the active lipids within the reaction mixture than of their molecular structures. Consequently, liposomes and micelles containing active phosphatidic acid were generated and tested for inhibitory activity. Liposomes were generated by mixing (6:1 weight ratio) phosphatidylcholine and phosphatidic acid (arachidonoyl, stearoyl) and drying and sonicating them. Molecular exclusion chromatography confirmed that liposomes had been formed. Only those liposomes containing phosphatidic acid, however, inhibited GA

Table 1. Inhibition of GA protein activity by various lipids. The percentage of inhibition of GA protein activity indicates how efficiently the added lipid was able to block the ability of GA protein to increase GTP hydrolysis (after incubation for 1 hour as in Fig. 1). This was determined as follows: (the proportion of GTP remaining bound to Ras after incubation with lipid and GA protein, minus this proportion with GA protein and Ras but without lipid) divided by (the proportion of GTP remaining bound to Ras incubated with no addition minus this proportion with added GA protein only). Although numerous determinations were performed with analogous results, these values are averages of three experiments, whereas the fatty acids and dilauroyl and dioleoyl phosphatidic acids are averages of two determinations.

Lipid	Fatty acid composition	Concentration (μg/ml)	Inhibition (%)
(**A**) *Phospholipids*			
Phosphatidylserine	Mixture	100	0
Phosphatidylethanolamine	Mixture	100	0
Phosphatidylcholine	Mixture	150	0
	Stearic, arachidonic	150	0
Phosphatidic acid	Dipalmitic	100	2
	Distearic	100	2
	Dilauric	100	20
	Dioleoic	100	43
	Stearic, arachidonic	100	100
Phosphatidylinositol	Stearic, arachidonic	100	100
	Linoleic, palmitic	100	100
Phosphatidylinositol 4-monophosphate	Mixture	100	100
Phosphatidylinositol 4,5-diphosphate	Mixture	100	100
Diacylglycerol	Dilineoleic(1,3)	100	3
	Oleoic, acetic	100	2
	Stearic, arachidonic	160	4
(**B**) *Fatty acids*			
Lauric (12:0)*		50	0
Palmitic (16:0)		50	0
Stearic (18:0)		50	0
Nonadecanoic (19:0)		50	0
Arachidic (20:0)		50	0
Arachidonic (20:4)		50	88
Linolenic (18:3)		50	40
Linoleic (18:2)		50	33
Eicosadienoic (20:2)		50	33
γ-Linolenic (18:3)		50	29
Eicosapentaenoic (20:5)		50	21
Eicosatrienoic 8,11,14 (20:3)		50	20
Eicosenoic (20:1)		50	18
Eicosatrienoic 11,14,17 (20:3)		50	9

*Fatty acids are designated first by the number of carbon atoms followed by the number of double bonds.

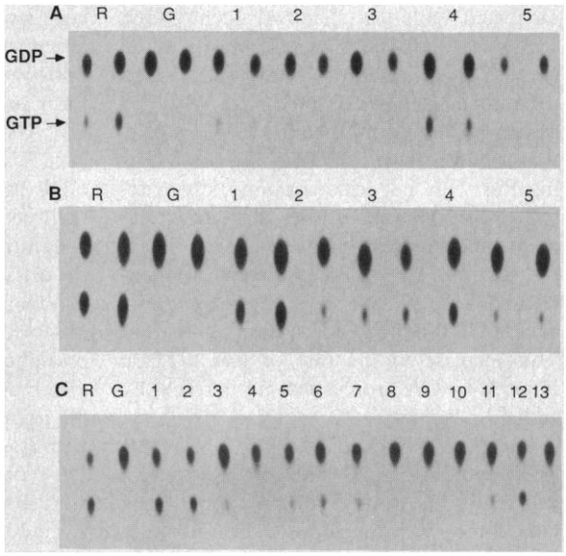

Fig. 2. Inhibition of GA protein activity by various phosphatidic acids (A), phosphoinositides (B), and free fatty acids (C). (**A**) Inhibition of GA protein activity by various phosphatidic acids (in duplicate). Ras bound to [α-^{32}P]GTP was incubated with crude GA protein (all lanes except R). In addition, various phosphatidic acids were added to lanes: 1, dioleoyl; 2, dilauroyl; 3, distearoyl; 4, γ-stearoyl β-arachidonoyl; 5, dipalmitoyl phosphatidic acids. GTPase activity assay was performed as in Fig. 1. GDP migrates in this system most rapidly and appears near the top. The extent of GTP hydrolysis in the absence of GA protein (or added lipid) is in lane R. Disappearance of the lower GTP spot in lane G indicates that GA protein had stimulated GTPase activity (in the absence of added lipid). To ensure the stability of phosphatidic acid, the lipids were obtained commercially (Sigma and Avanti Polar Lipids) as sodium salts and were converted to the free acid form immediately prior to assay by dissolving the lipids in chloroform:methanol (2:1), mixed with 0.1*M* phosphate buffer, *p*H 3.0. The organic phase was then washed repeatedly with aqueous buffer, and liposomes (or micelles) were formed from the organic phase (*12*). Lane R contains only Ras, while lane G contains Ras and GA protein but no added lipid. Inhibition of GA protein activity results in the preservation of GTP (lower spot) in the reaction. Full inhibition is apparent when GTP in the reaction is equivalent to that seen in the absence of added GA protein (lane R). (**B**) Inhibition of GA protein activity by phosphatidic acid and phosphoinositides. Duplicates of each reaction are shown. Ras bound to [α-^{32}P]GTP was incubated with GA protein (except lane R). Lipids (60 μg/ml) were added to lanes: 1, γ-stearoyl β-arachidonoyl phosphatidic acid; 2, phosphatidylinositol; 3, phosphatidylinositol 4-monophosphate; and 4, phosphatidylinositol 4,5-diphosphate. At this concentration only the phosphatidic acid is fully inhibitory. At higher concentrations (100 μg/ml) full inhibition can be observed with each lipid (see Table 1A). (**C**) Inhibition of GA protein activity by free fatty acids. Ras bound to [α-^{32}P]GTP was incubated with GA protein (except lane R). Fatty acids (50 μg/ml) were added to lanes: 1 and 2, arachidonic acid; 3, 11-eicosenoic acid; 4, *cis* 11,14,17-eicosatrienoic acid; 5, γ-linolenic acid; 6, *cis* 5,8,11,14,17-eicosapentaenoic acid; 7,11,14 eicosadienoic acid; 8, nonadecanoic acid; 9, arachidonic acid; 10, stearic acid; 11, *cis* 8,11,14-eicosatrienoic acid; 12, linoleic acid; and 13, choline.

protein. Liposomes of pure phosphatidylcholine were not inhibitory. In addition, mixed micelles were formed from 1-*O*-octyl-β-D-glucopyranoside and several phosphatidic acids (14:1, molecular ratio). Those phosphatidic acids found to be inhibitory in pure form were the only ones able to inhibit GA protein in mixed micelles. The inhibition observed, therefore, appears to be a consequence of the molecular structure, not the physical form, of these lipids. These observations are consistent with a relation between the inhibition of GA protein activity, mitogenic stimulation, and the concentration of these lipids. There is no direct evidence, however, that the production of these lipids is necessary for mitogenesis or that they are directly involved in the biological activation of Ras. Furthermore, we have been unable to obtain and test phosphatidylinositol 3-monophosphate, which is known to be generated by an active tyrosine kinase oncogene and is therefore likely to be of mitogenic importance (*14*).

The phosphatidic acid most active in inhibiting GA protein contained arachidonic acid, as do most phosphatidylinositols. The importance of arachidonic acid in this inhibition was tested directly by determining the ability of the free fatty acid to inhibit GA protein. At a concentration of 50 μg/ml, free arachidonic acid inhibited GA protein by 88% (Table 1B). Other fatty acids had much less inhibitory activity. Long chain, saturated fatty acids were completely inactive, whereas some long chain, unsaturated fatty acids were partially inhibitory (Fig. 2C and Table 1B). The significance of this observation is unknown. Although protein kinase C is activated by diacylglycerols, it also responds to the free fatty acids that we have found inhibit GA protein activity (*15*). In the case of protein kinase C it is postulated that there might be multiple levels of control (*15*). Alternately, the free fatty acids might mimic the action of the phospholipids normally involved in biological activity. In support of this possibility, those fatty acids able to directly alter GA protein activity are those most active when part of a phosphatidic acid molecule (Table 1).

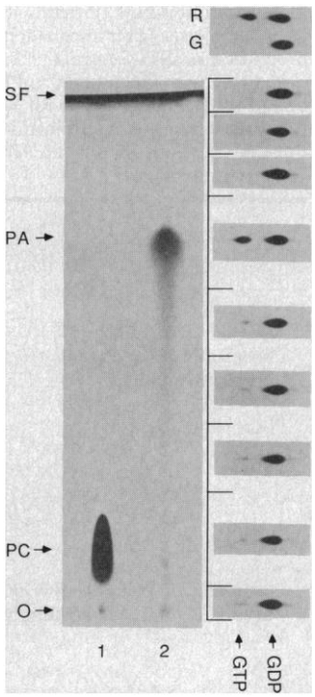

Fig. 3. Enzymatic generation of phosphatidic acid from inactive phosphatidylcholine. Phospholipase D was incubated with γ-stearoyl β-arachidonoyl phosphatidylcholine to generate phosphatidic acid. The original material (lane 1) and the reaction products (lane 2) were resolved on silica gel TLC plates. The conversion of phosphatidylcholine to phosphatidic acid was efficient. Analysis in the presence of Ras and GA protein confirmed that this treatment generated a material able to inhibit GA protein from the originally inactive phosphatidylcholine. The chromatogram of the reaction products (lane 2) was then separated into the nine regions designated by lines to the right of the chromatogram. The lipids were extracted from each of these chromatographic regions and tested for their ability to inhibit GA protein. The results of the GA protein assay are to the right of the corresponding chromatographic regions. The ability to inhibit GA protein is indicated by preservation of GTP in the assay as seen in the left-most autoradiographic spot. For comparison the conversion of GTP to GDP can be seen in the presence of Ras only (lane R) or in the presence also of GA protein with no added lipid (lane G). Hydrolysis of γ-stearoyl β-arachidonoyl phosphatidylcholine by phospholipase D has been described (*22*). A suspension of 10 mg of phosphatidylcholine in 0.5 ml of 0.2*M* acetate buffer (*p*H 5.6) was incubated with 0.4 ml of phospholipase D solution (from cabbage, 2500 U/ml; Sigma) and 0.4 ml of ethyl ether. The reaction mixture was shaken vigorously and left at room temperature overnight. After removal of most of the ether in a stream of nitrogen, lipids were extracted with methanol:chloroform (1:1) and chromatographed on a silica TLC plate, which was developed in chloroform:methanol:acetic acid (13:3:1). The lipids were visualized by soaking the TLC plate in 30% methanol containing 0.03% Coomassie blue in 0.1*M* NaCl for 5 min. An unstained, parallel chromatogram was separated into nine regions. The silica gel was scraped from each region, and lipids were extracted in chloroform:methanol (1:1) and tested for their ability to inhibit GA protein activity as in Fig. 2. Abbreviations: SF, solvent front; PA, phosphatidic acid; PC, phosphatidylcholine; and O, origin.

Several analyses were performed to further characterize the inhibition of GA protein activity by phosphatidic acid (β-arachidonoyl-γ-stearoyl). We found that this lipid inhibited the stimulation of Ras GTPase by GA protein within 5 min of incubation at as

high or higher a level than that observed after the usual 30- to 60-min incubations. In addition, there was no apparent alteration in the rate of Ras nucleotide exchange by the added lipids. For this demonstration unlabeled GTP (100 μM) was added to the reaction at the time of GA protein addition. Only a slight alteration in the total amount or ratio of bound nucleotide was observed. In addition, as expected, the amount of GDP bound to Ras at the end of the incubation period was totally accounted for by the decrease in bound GTP, indicating that GDP bound to Ras was produced by the hydrolysis of GTP. Finally, purified GA protein (*7*) was sensitive to inhibition by phosphatidic acid. This result suggests that lipids directly affect GA protein without the involvement of another molecule.

Although it is clear that commercial lipid preparations inhibited GA protein activity, it is not certain that the primary lipid rather than a contaminant was responsible. To ensure that the indicated lipids were themselves the inhibitory elements in the preparations, two analyses were performed. First, the lipid preparation was resolved by thin-layer chromatography (TLC) [on silica gel G (Whatman) in chloroform:methanol:acetic acid 13:3:1]. Lipids present in various regions of the resulting chromatogram were then extracted from the plate and tested for their ability to inhibit GA protein activity. When phosphatidic acid (arachidonoyl, stearoyl) was tested, the inhibitory activity comigrated with the phosphatidic acid standard (Fig. 3). A similar analysis with phosphatidic acid (distearoyl) yielded no inhibitory activity.

The second analysis to show that phosphatidic acid is the inhibitory lipid in the preparations tested above involved its enzymatic generation. Beta-arachidonoyl γ-stearoyl phosphatidylcholine was tested for its ability to inhibit GA protein activity before and after treatment with phospholipase D, which cleaves a variety of phospholipids to yield phosphatidic acid. Thin-layer chromatography confirmed that the enzymatic conversion to phosphatidic acid was complete (Fig. 3). Analysis in the presence of Ras and GA protein then confirmed that the enzyme treatment had generated a material able to inhibit GA protein activity from the initially inactive phosphatidylcholine. To ensure that the active lipid generated by the phospholipase from phosphatidylcholine was phosphatidic acid, the reaction mixture was resolved on a silica TLC plate. The resulting chromatogram was separated into nine fractions. The lipids were extracted from each fraction and tested for the ability to inhibit GA protein. The only fraction from the TLC chromatogram able to inhibit GA protein was that fraction that contained the phosphatidic acid (Fig. 3).

Thus, a limited group of lipids could affect the in vitro relation between GA protein and Ras. The biological significance of this observation is not known. There are, however, several relevant observations. The possibility of a biochemical relation between Ras and phospholipids was predicted by microinjection studies with neutralizing antibody to Ras. These studies indicated that tyrosine kinase oncogenes and related growth factor receptors require cellular Ras activity within the cell to induce cellular proliferation (*1, 16*). Certain phospholipid-related materials were also mitogenic but were even more dependent upon Ras activity than tyrosine kinase oncogenes. Although thymidine labeling was inhibited 80 to 90% by the injected antibody when cell proliferation was induced by serum or oncogene transformation, the mitogenesis induced by phosphatidic acid (with saturated fatty acids) was inhibited by 99% (*6*).

On the basis of the microinjection data, we postulated that tyrosine kinases initiate a proliferative signal triggered by binding of a growth factor to its tyrosine kinase–containing receptor. The signal generated is then postulated to be received by Ras, which transfers the signal to soluble serine kinases in the cell. The proliferative signal generated by tyrosine kinases apparently involves phospholipid metabolism (*6, 16*). The effect of lipids upon the interaction of Ras and GA protein was, therefore, predicted by and supports this hypothesis.

In addition, phospholipid metabolism is often altered by mitogenic stimuli. Phosphatidylinositol phosphates are particularly sensitive to these stimuli, as are the concentrations of phosphatidic acids (*11*) and free arachidonic acid (*17*). These are the phospholipids that have the greatest ability to inhibit GA protein activity. It is not known if tyrosine kinases are involved in these phospholipid alterations, although these kinases might alter phospholipid metabolism. Lipocortins and calpactins, potential inhibitors of phospholipase A_2 and, thereby, arachidonic acid production, are major substrates of these kinases (*18*). In addition, tyrosine kinases associate with a phosphatidylinositol kinase (*19*), apparently phosphorylate a phosphatidylinositol diphosphate–specific phospholipase C (*20*), and contain a sequence pattern similar to putative tyrosine kinase control regions (*21*). This sequence pattern is also present in the bovine GA protein gene (*8*). Thus, GA protein and Ras might function together in the control of mitosis, in such a way that their interaction is dependent upon the lipid microenvironment surrounding Ras.

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9. Cytoplasmic extract containing GA protein activity was prepared from mouse brain. The cerebra from ten animals were homogenized in ice-cold hypotonic buffer (10 m*M* tris-HCl, *p*H 7.5, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 3000*g* for 10 min to remove the unbroken cells. The resulting supernatant was then centrifuged at 100,000*g* for 30 min to obtain a clarified supernatant fraction. The cytoplasmic extract containing GA protein activity (5 mg/ml) was stored at −80°C until used.
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12. Phospholipids (0.5 to 1.0 mg) and free fatty acids (200 μg) in chloroform were dried under a stream of nitrogen in a glass tube (12 by 75 cm). The thin layer of lipid coating the glass was suspended in 1 ml of 0.1*M* tris-HCl, *p*H 7.5, and sonicated in an ice bath for 30 s by inserting a titanium microtip (Fisher, Model 300 Sonic Dismembrator) into the bottom of the glass tube. Half-maximum power output was used for liposome-micelle preparation.
13. The ability of phosphatidic acid to inhibit GA protein activity varied among commercial preparations from Sigma by up to 20%. The variation could be at least partially overcome by dissolving dried phosphatidic acid in 3 mg of octylglucopyranoside per milliliter or extracting the chloroform solution of phosphatidic acid with water and then preparing the phosphatidic acid liposomes as in (*12*).
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23. The reaction conditions were as physiological as possible. NaCl or KCl (0.15*M*) was included in the

reaction buffer; pH was monitored and maintained at 7.5 throughout the reaction. Calcium was not critical for the inhibition of GA protein by lipids since addition of I m*M* EGTA or 50 μ*M* $CaCl_2$ did not alter the GTPase activity of GA protein in the presence or absence of phosphatidic acid.

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Switch Protein Alters Specificity of RNA Polymerase Containing a Compartment-Specific Sigma Factor

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During sporulation in *Bacillus subtilis*, expression of developmental genes *spoIVCB* and *cotD* is induced in the mother cell compartment of the sporangium at morphological stages IV and V, respectively. A 27-kilodalton RNA polymerase sigma factor called σ^K (or σ^{27}) has been found that causes weak transcription of *spoIVCB* and strong transcription of *cotD*. A 14-kD protein was also discovered that changes the specificity of σ^K-containing RNA polymerase, greatly stimulating *spoIVCB* transcription and markedly repressing *cotD* transcription. Both σ^K and the 14-kD protein are products of genes known to be required for expression of specific genes in the mother cell. Thus, σ^K directs gene expression in the mother cell and it is proposed that inactivation or sequestering of the 14-kD protein switches the temporal pattern of gene expression during the transition from stages IV to V of development.

A HALLMARK OF THE PROCESS OF ENdospore formation in the Gram-positive bacterium *Bacillus subtilis* is the formation of a sporangium composed of two compartments, the mother cell and the forespore (*1*). The compartments each receive a chromosome generated during the last vegetative round of DNA replication, but then undergo divergent developmental fates. The mechanisms governing temporal and spatial control of gene expression in the mother cell chamber of the sporangium have not been well studied. The sporulation genes *spoIIIC* and *spoIVCB*, the spore coat protein genes *cotA*, *cotC*, and *cotD*, and the germination gene *gerE* are examples of genes that are known or are inferred to be transcribed selectively in the mother cell (*2–6*). These genes fall into three temporal classes that are switched on at successive developmental stages. We report the use of cloned copies of *spoIVCB* (*7*)—which is switched on at stage IV (*3*)—and *cotD* (*5*)—which is switched on at stage V (*6*)—as templates for reconstructing compartment-specific gene transcription in vitro.

Run-off transcripts from linear templates bearing promoters for the mother cell–expressed genes *cotD* and *spoIVCB* were generated by RNA polymerase that had been partially purified (*8*) from sporulating cells of *B. subtilis* (Fig. 1). The transcripts were of the expected sizes (within 5 bases) for initiation at the known in vivo start sites of the *cotD* (*6*) and *spoIVCB* (*3*) genes, and the length of the run-off RNA's varied predictably with templates that extended for different distances downstream from the start sites (Fig. 1).

To identify the factor or factors responsible for *cotD* and *spoIVCB* transcription, the RNA polymerase was fractionated by gradient elution from a DNA-cellulose column (Fig. 2). The *cotD*-transcribing activity (A) eluted at slightly higher salt concentration (reaching a peak in fractions 24 to 26) than did *spoIVCB*-transcribing activity (Fig. 2B), which peaked in fraction 22. Moreover, longer exposure (*9*) of the same gel showed that the enzyme activity eluting at low salt (fractions 14 to 16) had almost exclusively *spoIVCB*-transcribing activity, whereas RNA polymerase in the higher salt-eluting fractions (fractions 30 to 34) had almost exclusively *cotD*-transcribing activity.

Proteins from the gradient elution were displayed by electrophoresis on an SDS-polyacrylamide slab gel (Fig. 2C). Several proteins showed an elution pattern that was approximately coincident with the distribution of *cotD*-transcribing activity, but reconstruction experiments showed that only one, a 27-kD polypeptide, directed *cotD* transcription. When, for example, proteins in gel slices in the 25- to 30-kD size range were eluted, renatured, and added to *B. subtilis* core RNA polymerase (Fig. 3A), only renatured protein corresponding to the 27-kD polypeptide activated *cotD* run-off transcription by core enzyme (Fig. 3B). Evidently, the 27-kD protein is a sigma factor for the *cotD* gene.

Because *spoIVCB* is expressed earlier than *cotD* during sporulation and because *spoIVCB*-transcribing activity eluted at lower salt concentration compared to *cotD*-transcribing activity (Fig. 2, A and B), another sigma factor may have been responsible for transcription of *spoIVCB*. However, proteins of many different sizes were tested (*9*), but only protein from the same 27-kD gel slice that stimulated *cotD* transcription (Fig. 3B) caused core RNA polymerase to transcribe from the *spoIVCB* promoter (Fig. 3C) (although the run-off transcription was weaker than that observed with *cotD*). One possible explanation was that the 27-kD gel slice contained two different proteins, one a sigma factor for *cotD* transcription that was

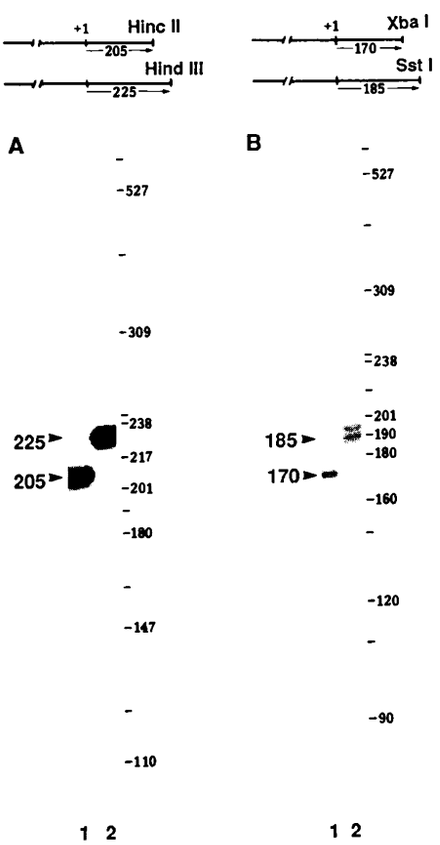

Fig. 1. Run-off transcription from templates containing the *cotD* and *spoIVCB* promoters. Linearized plasmid templates (2 μg) (top) were transcribed in 40-μl reaction mixtures (*17*) with RNA polymerase partially purified from sporulating cells of *B. subtilis* (*8, 18*). The products of run-off transcription were displayed by electrophoresis in a 5 percent polyacrylamide slab gel containing 8*M* urea and detected by autoradiography. (**A**) Run-off transcripts from the *cotD* promoter-containing template linearized with Hinc II (lane 1) or Hind III (lane 2). (**B**) Transcripts from the *spoIVCB* promoter-containing template linearized with Xba I (lane 1) or *Sst*I (lane 2).

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