21. The $(His)_6$-ykt6$^{(Phe42Glu)}$p expression construct was generated by replacing the wild-type *YKT6* fragment from $(His)_6$-Ykt6p with the corresponding fragment of *ykt6* (encoding ykt6$^{(Phe42Glu)}$p). SNARE binding assays were performed essentially as described previously (*14*, *24*). Purified recombinant SNARE proteins were added to the washed GST-Vti1p bead slurry in a final volume of 750 μl. $(His)_6$-Sed5p and $(His)_6$-Tlg1p were detected by immunostaining with an antibody against His_6 (*14*, *24*) and Phe42Glu-Ykt6p was detected by immunostaining with affinity-purified Ykt6p antiserum.
22. Band intensity was converted to arbitrary units by densitometry. The initial relative velocities at which Sed5p and Ykt6p were incorporated into the SNARE complex were determined from the slopes obtained by plotting arbitrary units against time. This was done for the data points up to and including 60 min (for Phe42Glu-Ykt6p) and 120 min (for Ykt6p), during which time the velocity was found to be constant. The relative differences in the observed velocities were expressed as a ratio of the Phe42Glu/Wild-type slopes.
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40. We thank F. Hung and Q. Zhang for assistance in protein purification and gel-filtration experiments, respectively, and C. Rock for comments on the manuscript. This work was supported by grants from the Research Grant Council of Hong Kong to D.K.B and M.Z. and by a grant from the Human Frontiers Science Program Organization to M.Z. The NMR spectrometer used in this work was purchased with funds donated to the Biotechnology Research Institute by the Hong Kong Jockey Club. The coordinates of the structure of Ykt6pN have been deposited in the Protein Data Bank (accession code 1h8m).

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Lysophosphatidylcholine as a Ligand for the Immunoregulatory Receptor G2A

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Although the biological actions of the cell membrane and serum lipid lysophosphatidylcholine (LPC) in atherosclerosis and systemic autoimmune disease are well recognized, LPC has not been linked to a specific cell-surface receptor. We show that LPC is a high-affinity ligand for G2A, a lymphocyte-expressed G protein–coupled receptor whose genetic ablation results in the development of autoimmunity. Activation of G2A by LPC increased intracellular calcium concentration, induced receptor internalization, activated ERK mitogen-activated protein kinase, and modified migratory responses of Jurkat T lymphocytes. This finding implicates a role for LPC-G2A interaction in the etiology of inflammatory autoimmune disease and atherosclerosis.

Lysophospholipids regulate a variety of biological processes including cell proliferation, tumor cell invasiveness, and inflammation (*1*, *2*). LPC, produced by the action of Phospholipase A_2 (PLA_2) on phosphatidylcholine, promotes inflammatory effects, including increased expression of endothelial cell adhesion molecules and growth factors (*3*, *4*), monocyte chemotaxis (*5*), and macrophage activation (*6*). As a component of oxidized low density lipoprotein (oxLDL), LPC plays an etiological role in atherosclerosis (*7*) and is implicated in the pathogenesis of the autoimmune disease systemic lupus erythematosus (SLE) (*8*). Despite physiologically high concentrations in body fluids (up to 100 μM) (*9*), extracellular actions of LPC through G protein–coupled receptors (GPCRs) are indicated (*10*, *11*). Although LPC action through a platelet activating factor (PAF) receptor(s) has been suggested (*10*, *11*), a specific LPC receptor has yet to be identified. OGR1 is a high-affinity receptor for sphingosylphosphorylcholine (SPC), a lysophospholipid structurally similar to LPC (*12*). OGR1 is closely related to G2A (*13*), TDAG8 (*14*), and GPR4 (*15*). G2A is a transcriptionally regulated GPCR expressed predominantly in lymphocytes, and its expression in response to stress stimuli and prolonged mitogenic signals suggests that it may negatively regulate lymphocyte growth (*13*). Genetic ablation of G2A function in mice further indicates a role for G2A in the homeostatic regulation of lymphocyte pools and autoimmunity (*16*).

To determine if G2A is a lysophospholipid receptor, we assessed signaling responses in cells ectopically expressing G2A (*17*). Human breast epithelial MCF10A cells were used because they do not express G2A or OGR1, and do not respond to SPC (*12*). Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined in MCF10A cells that were transfected with plasmids encoding green fluorescent protein–tagged G2A (G2A.GFP) (*18*) or GFP (*19*). LPC and SPC (0.1 μM) treatment induced transient $[Ca^{2+}]_i$ increases in G2A.GFP expressing cells only. Responses to lysophosphatidic acid (LPA) (1 μM), PAF (0.1 μM), and adenosine triphosphate (ATP) (20 μM) were not affected by G2A expression (Fig. 1A). Dose-dependent increases in $[Ca^{2+}]_i$ were observed in G2A.GFP-expressing cells [LPC, median effective concentration (EC_{50}) ~0.1 μM; SPC, EC_{50} ~0.4 μM] (Fig. 1B). Pretreatment of G2A.GFP-expressing cells with the PAF receptor antagonist BN 52021 (200 μM) blocked $[Ca^{2+}]_i$ elevation induced by PAF, but not that induced by LPC (1 μM), SPC (1 μM), LPA (1 μM), or ATP (20 μM) (Fig. 1C), indicating that LPC and SPC did not act through a PAF receptor. The pretreatment of G2A.GFP-expressing cells with LPC (1 μM) or SPC (10 μM) induced desensitization to subsequent stimulation with either agonist (1 μM) (Fig. 1D).

When G2A.GFP-expressing cells were pretreated with pertussis toxin (PTX, 100 ng/ml), an inhibitor of $G\alpha_i$, transient $[Ca^{2+}]_i$ increases induced by LPA (1 μM), LPC (0.1 to 5 μM), and SPC (1 to 5 μM) were inhibited (Fig. 1E). Calcium transients elicited by PAF (0.1 μM) or ATP (20 μM) were not affected. Pretreatment of

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G2A.GFP-expressing cells with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), abolished transient $[Ca^{2+}]_i$ increases induced by LPA, LPC, and SPC (up to 10 μM), but did not affect those induced by PAF (0.1 μM) and ATP (20 μM) (Fig. 1F). This suggests that PKC affects LPC and SPC signaling pathways by inducing G2A desensitization and/or inhibition of $G\alpha_i$. Several putative consensus PKC phosphorylation sites are present in G2A (*13*).

To determine binding affinities of LPC and SPC toward G2A, we performed radioligand binding assays (*12*, *20*). [^{3}H]LPC and [^{3}H]SPC bound to homogenates of human embryonic kidney (HEK) 293 cells expressing G2A.GFP (HEK 293 G2A.GFP) in a time-dependent manner and reached equilibrium after 60 min of incubation at 4°C (Fig. 2, A and B). Binding of [^{3}H]LPC and [^{3}H]SPC to HEK 293 G2A.GFP homogenates were saturable, and Scatchard analysis indicated a dissociation constant (K_d) of 65 nM for LPC and 230 nM for SPC (Fig. 2, C and D). The maximum binding capacities for LPC and SPC were about 1500 fmol/10^5 cells and 1840 fmol/10^5 cells, respectively. Competition analyses revealed that only SPC and various LPC species, but not 14:0 LPC, LPA, sphingosine-1-phosphate (S1P), lysophosphatidylinositol (LPI), sphingomyelin (SM), PAF, or lyso-PAF, competed for binding (Fig. 2, E and F).

GPCRs are internalized in response to ligand stimulation. In serum-starved HEK 293 G2A.GFP cells, G2A.GFP is expressed predominantly at the plasma membrane. LPC (0.1 μM), as well as SPC (1 μM), induced internalization of G2A.GFP in more than 90% of cells (*21*, *22*). Neither PAF, LPA, nor S1P induced receptor internalization.

ERK mitogen-activated protein (MAP) kinase activity is stimulated by SPC after transfection of otherwise unresponsive cell lines with OGR1 (*12*). Similarly, LPC does not stimulate ERK MAP kinase activation in a number of cell lines (*23*) (Fig. 3). G2A expression conferred responsiveness to these lysophospholipids in terms of ERK MAP kinase activation in Chinese hamster ovary (CHO) cells (*24*). A dose-dependent increase in ERK MAP kinase activity was observed in response to LPC and SPC (Fig. 3A), and activation was inhibited by PTX pretreatment, indicating the involvement of a $G\alpha_i$ family G protein (Fig. 3B).

LPC is thought to have chemoattractant properties toward T lymphocytes (*25*). Cellular transmigration of Jurkat T cells expressing GFP or G2A.GFP (both populations were 20% GFP-positive) through a polycarbonate membrane tissue-culture chamber toward the ligand was assessed over a 1-hour period (*26*). Although LPC suppressed transmigration of the GFP-positive fraction of Jurkat GFP populations, LPC (10 μM) stimulated transmigration of Jurkat G2A.GFP cells by four times that of Jurkat cells expressing GFP only (Fig. 4). SPC did not stimulate transmigration of Jurkat G2A.GFP cells (*27*), and the possible physiological functions of an SPC-G2A interaction have yet to be determined.

Different LPC species may have different affinities for G2A (Fig. 2, E and F); 14:0 LPC is not able to compete [^{3}H]–16:0 LPC binding, whereas 16:0 LPC, 18:0 LPC, and 18:1 LPC are potent competitors. Consistently, 14:0 LPC is unable to stimulate $[Ca^{2+}]_i$ increases in G2A expressing MCF10A cells (*27*). G2A also binds SPC with low affinity. The physiological significance of this promiscuity remains to be

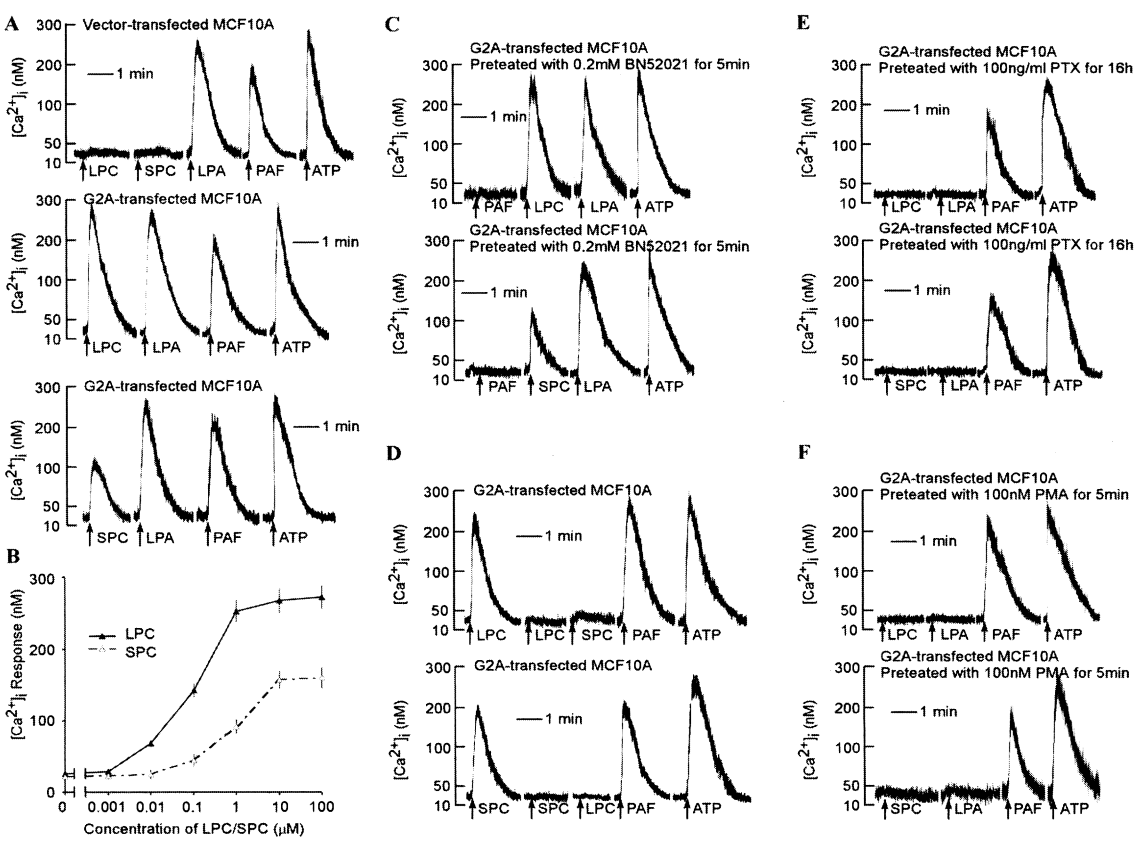


Fig. 1. LPC and SPC induce transient $[Ca^{2+}]_i$ increases in G2A-transfected MCF10A cells. (**A**) (Upper panel) Calcium responses of pEXV3 GFP (vector)-transfected MCF10A cells to 16:0 LPC (1 μM), SPC (1 μM), LPA (1 μM), PAF (0.1 μM), and ATP (20 μM). (Middle panel) Calcium responses of G2A-transfected MCF10A cells to 16:0 LPC (1 μM), LPA (1 μM), PAF (0.1 μM) and ATP (20 μM). (Lower panel) Calcium responses of G2A-transfected MCF10A cells to SPC (1 μM), LPA (1 μM), PAF (0.1 M), and ATP (20 μM). (**B**) 16:0 LPC and SPC dose responses in G2A-transfected MCF10A cells. (**C**) (Upper panel) The effect of PAF receptor inhibitor [BN52021 (Biomol, Plymouth Meeting, Pennsylvania)] on $[Ca_{2+}]_i$ increases induced by PAF (0.1 μM), 16:0 LPC (1 μM), LPA (1 μM), and ATP (20 μM). (Lower panel) The effect of BN52021 on $[Ca^{2+}]_i$ increases induced by PAF (0.1 μM), SPC (1 μM), LPA (1 μM), and ATP (20 μM). (**D**) Pretreatment of G2A-transfected MCF10A cells with 16:0 LPC (1 μM) (upper panel) or SPC (10 μM) (lower panel) induces desensitization to subsequent stimulation with 16:0 LPC or SPC (1 μM). (**E**) PTX (100 ng/ml, 16 hours) inhibits G2A-dependent $[Ca^{2+}]_i$ increases induced by 16:0 LPC (1 μM) (upper panel), SPC (1 μM) (lower panel), and LPA (1 μM) (both panels). (**F**) PMA pretreatment (100 nM, 5 min) inhibits G2A-dependent $[Ca^{2+}]_i$ increases induced by 16:0 LPC (1 μM) (upper panel), SPC (1 μM) (lower panel), and LPA (1 μM) (both panels). Data are representative of three independent experiments.

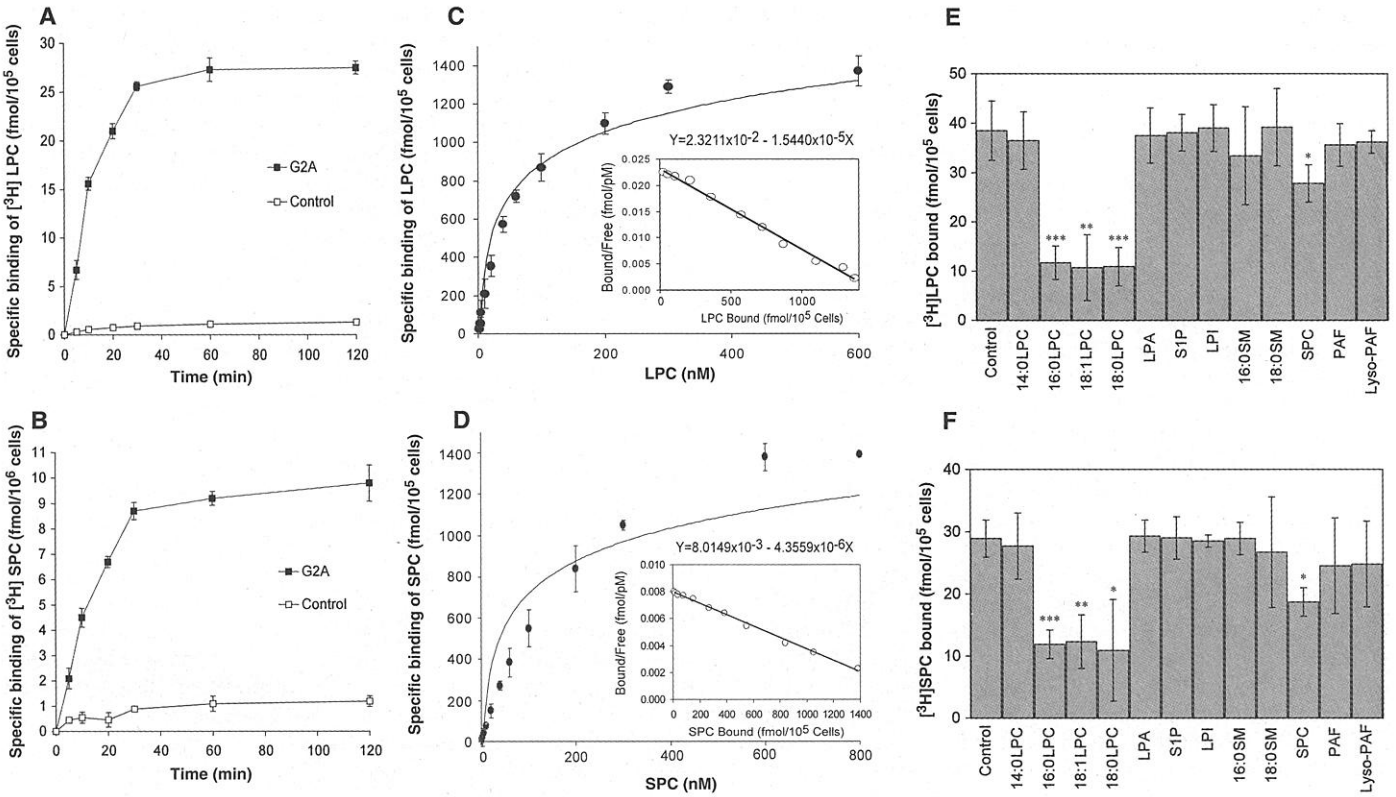
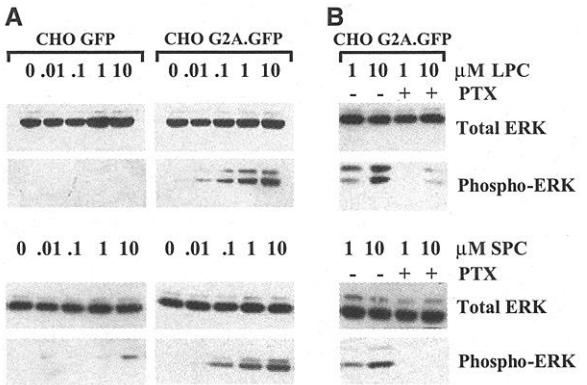


Fig. 2. LPC and SPC bind to G2A. (**A** and **B**) Time dependence of [^{3}H]LPC and [^{3}H]SPC binding. Cell homogenates from HEK 293 GFP or HEK 293 G2A.GFP cells (>90% GFP-positive) were incubated with [^{3}H]–16:0 LPC (1 nM) or [^{3}H]SPC (1 nM) for the indicated times. Specific binding is presented. (**C** and **D**) Saturation isotherms of [^{3}H]LPC and [^{3}H]SPC binding to HEK293 G2A.GFP cells. Cell homogenates were incubated with the indicated concentrations of [^{3}H]–16:0 LPC or [^{3}H]SPC and specific binding was measured. (Insets) Scatchard analyses of [^{3}H]–16:0 LPC and [^{3}H]SPC binding. (**E** and **F**) Structural specificity of [^{3}H]–16:0 LPC and [^{3}H]SPC binding to G2A. HEK 293 G2A.GFP homogenates were incubated with [^{3}H]–16:0 LPC (1 nM) or [^{3}H]SPC (1 nM) in the presence or absence of unlabeled lipids (100 nM). Total binding is presented. Data are means ± SD representing three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, compared to control (Student's t test).

Fig. 3. LPC and SPC activate ERK MAP kinase in G2A-expressing CHO cells. (**A**) Serum-starved CHO GFP and CHO G2A.GFP cells were stimulated with the indicated concentrations of agonist for 10 min. Total lysates were Western blotted with antibodies against ERK MAP kinase or phospho-ERK MAP kinase. (**B**) Pretreatment of CHO G2A.GFP cells with PTX (100 ng/ml, 16 hours) inhibits ERK MAP kinase activation induced by LPC and SPC.



defined. A related receptor, TDAG8, responds to the glycolipid psychosine (*28*), suggesting the possibility that this GPCR subfamily (OGR1, G2A, TDAG8, and GPR4) responds to a structurally diverse set of lipids.

G2A may be a hitherto unrecognized etiological factor in the chronic inflammatory diseases SLE and atherosclerosis. The receptor may play a role as a sensor of LPC levels at sites of inflammation to limit expansion of tissue-infiltrating cells and progression to overt autoimmune disease. An immunosuppressive action of LPC on T cell proliferation has been reported (*11*), and T cells from G2A-deficient mice exhibit hyperproliferative responses to antigen receptor stimulation in vitro (*16*). LPC may also influence homing and/or localization of lymphocytes through G2A to modulate T-dependent immune responses and atherogenesis. The effects of the physiologically high concentrations of LPC in body fluids and serum, as well as possible functional redundancy with G2A receptor analogs, may determine the suitability of these GPCRs in the treatment of disease.

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17. MCF10A, HEK 293, and CHO cells do not express G2A as determined by reverse transcriptase polymerase chain reaction (*29*).

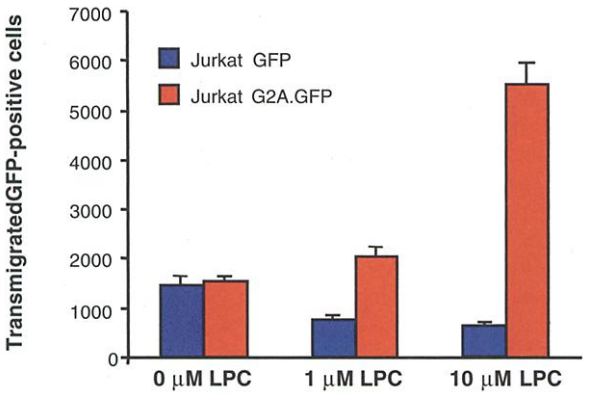

Fig. 4. LPC stimulates migration in G2A-expressing Jurkat T cells. A total of 10^5 Jurkat GFP or Jurkat G2A.GFP cells (both populations 20% GFP-positive) were allowed to transmigrate through 5-μm pore-size membranes toward the indicated concentrations of LPC for 1 hour. GFP-positive fractions (%) and cell numbers of transmigrated populations were measured by flow cytometry. Results are presented as numbers of transmigrated GFP-positive cells. Assays were performed in triplicate and results shown are representative of three independent experiments.

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19. Calcium assays were performed as described (*12*). MCF10A cells were loaded with Fura-2/AM (Molecular Probes) and $[Ca^{2+}]_i$ increases were measured in single transfected (GFP-positive) cells with a dual-wavelength spectrofluorometer (RFK-6002, Photon Technology, Brunswick, NJ) coupled to an inverted fluorescence microscope (Olympus, IX-70, Lake Success, NY).
20. HEK 293 GFP or HEK 293 G2A.GFP cells were serum-starved for 20 hours and collected in phosphate-buffered saline (PBS)/EDTA. Pelleted cells were stored at −80°C until use. Frozen cells were homogenized in "binding buffer" (10^6 cells/ml) (*13*). Assays were performed in 96-well plates in triplicate with 100-μl cell homogenate. [^{3}H]–16:0 LPC or [^{3}H]SPC were added to cell homogenates in 50 μl of binding buffer in the presence or absence of cold 16:0 LPC or SPC, or other competitors. Plates were incubated at 4°C for 2 hours, or for the indicated times. Cell-bound [^{3}H]LPC or [^{3}H]SPC was collected onto a filter (Printed Filtermat A, Wallac, Gaithersburg, MD) with an automated cell harvester (Harvester 96, Tomtec, Orange, CT). Specific binding was calculated by subtraction of nonspecific binding (in the presence of 100-fold excess unlabeled lipid) from total binding. [^{3}H]–18:0 LPC and [^{3}H]SPC were from Amersham Pharmacia Biotech (Buckinghamshire, England) (102 Ci/mmol, 1 mCi/ml for [^{3}H]–18:0 LPC, and 68 Ci/mmol, 1 mCi/ml for [^{3}H]SPC). [^{3}H] 16:0–LPC (60 Ci/mmol) was from American Radiolabelled Chemicals (St Louis, MO). LPCs (14:0, 16:0, 18:0, and 18:1), LPI, LPA, C16-PAF, and C16-lysoPAF were from Avanti Polar Lipids, (Alabaster, AL). Sphingomyelins (16:0 and 18:0), S1P, and SPC were from Toronto Research Chemicals (Toronto, ON) or Matreya (Pleasant Gap, PA).
21. Supplementary Web material is available on *Science* Online at www.sciencemag.org/cgi/content/full/293/5530/702/DC1.
22. HEK 293 G2A.GFP cells seeded onto glass cover slips were serum-starved for 18 hours before treatment with agonists for 2 hours. Cover slips were washed with PBS and fixed with PBS–4% paraformaldehyde. Subcellular localization of G2A.GFP was visualized under a confocal fluorescence microscope with an oil immersion lens (magnification, ×60).
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24. CHO GFP or CHO G2A.GFP cells were serum-starved for 18 hours before treatment with agonist for 10 min at 37°C. Western blotting was performed to detect total ERK MAP kinase with a polyclonal antibody to ERK2, and activated p44/42 ERK MAP kinase with a specific antibody to phospho-ERK (Santa Cruz Biotechnology).
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26. Although G2A is expressed in Jurkat cells, our experimental strategy was based on the hypothesis that increased expression in a physiologically relevant cell type may elicit a biological response. For transmigration assays, Jurkat GFP and Jurkat G2A.GFP cells were derived by retroviral infection and assayed 48 hours later. GFP-positive fractions of Jurkat GFP and Jurkat G2A.GFP populations were adjusted to 20% by the addition of appropriate numbers of parental Jurkat cells. Cells were washed three times in RPMI containing 0.25% bovine serum albumin (BSA) and finally resuspended in RPMI–0.25% BSA at 2×10^6 cells/ml. One hundred microliters of this cell suspension (10^5 cells) was applied to the upper chamber of a 6.5-mm diameter transwell cell culture insert comprising a 5-μm pore-size polycarbonate membrane (Corning Costar Corporation, Cambridge, MA) and containing 600 μl RPMI–0.25% BSA with or without agonist in the lower chamber. After incubation at 37°C for 1 hour, transmigrated cells were collected and analyzed by flow cytometry for GFP expression and cell number.
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Role of Inorganic Polyphosphate in Promoting Ribosomal Protein Degradation by the Lon Protease in *E. coli*

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Inorganic polyphosphate (polyP), a polymer of hundreds of phosphate (P_i) residues, accumulates in *Escherichia coli* in response to stresses, including amino acid starvation. Here we show that the adenosine 5′-triphosphate–dependent protease Lon formed a complex with polyP and degraded most of the ribosomal proteins, including S2, L9, and L13. Purified S2 also bound to polyP and formed a complex with Lon in the presence of polyP. Thus, polyP may promote ribosomal protein degradation by the Lon protease, thereby supplying the amino acids needed to respond to starvation.

PolyP is found in all microbes, fungi, plants, and animals (*1*). In *Escherichia coli*, levels of polyP are low in the exponential phase of growth, but increase more than 100-fold in response to acute stresses such as amino acid starvation and the multiple stresses in stationary phase (*2*, *3*). An *E. coli* mutant deficient in polyphosphate kinase (PPK), the principal enzyme for the synthesis of polyP, shows an extended lag in growth when shifted from a rich to a minimal medium (downshift); addition of amino acids abolishes the growth lag (*4*). Degradation of intracellular proteins is important in providing amino acids for use in the synthesis of the enzymes required for adaptations to starvation (*4*, *5*). The mutant fails to increase protein turnover after the downshift and thus extends the lag (*4*). In yeast and animal cells, the bulk degradation of proteins in response to starvation and cellular differentiation occurs by a ubiquitin-style conjugation system (*6*). However, in bacteria, the mechanisms underlying the regulation of intracellular protein degradation during amino acid starvation remain unknown (*5*).

In *E. coli*, more than 90% of cytoplasmic

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