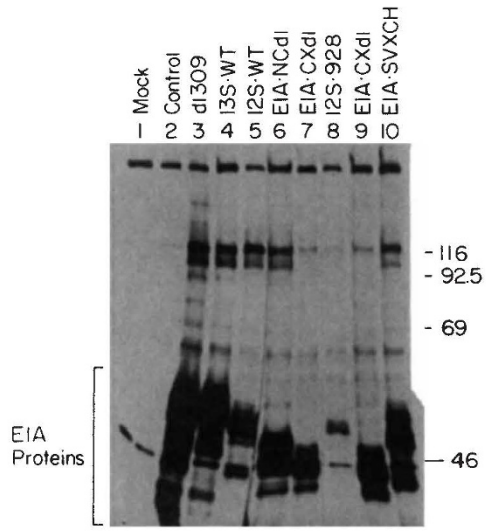

Fig. 3 Immunoprecipitation of E1A variant products in virus-infected HeLa cells. Protein extracts from HeLa cells mock-infected, or infected at a multiplicity of 100 PFU per cell with wild-type or mutant viruses as indicated in the figure, and described in the text, were immunoprecipitated with E1A fusion protein specific mouse monoclonal antibodies, series 73 as described[27] and separated on 10% SDS-polyacrylamide gels. Numbers on the right indicate the position of molecular weight markers (K). The region where the heterogeneous products of the E1A gene run is indicated on the left. The control lane contains a protein extract from Ad5dl309 infected cells, immunoprecipitated with a control mouse monoclonal antibody, Pab416, which does not recognize the E1A products.

interactions are significant for the cell cycle regulating functions of the E1A products.

The SV40 T antigen and Ad5 E1A regions compared here share less than 50% amino acid identity (Fig. 1), yet the chimaeric protein is biologically active. In contrast, a single amino acid substitution in one of the conserved residues (pm928 : $cys_{124} \rightarrow gly$) in Ad5 E1A is nearly as defective as a complete deletion of domain 2. The present demonstration that a functional substitution can be made between this region of T antigen and one of the transformation domains of the E1A gene products, suggests that similar protein structure and biochemical mechanisms underlie the transforming functions common to both proteins. Very strong support for this suggestion comes from the recent finding that T antigen binds the retinoblastoma gene product in CV-1 cells[29]. Computer analyses also support the prediction that the E1A product and T antigen regions compared here have similar secondary structures[22].

Further genetic evidence supporting the hypothesis that E1A domain 2 represents a functional motif used repeatedly, at least among the DNA tumour viruses, comes from the recent report that the deduced amino acid sequence of the product encoded by the human papillomavirus type 16 (HPV-16) E-7 gene, which encodes transformation functions similar to adenovirus E1A, contains a region which exhibits a striking degree of identity with the conserved residues in the transforming domains of the E1A products, particularly domain 2, and which includes the DLXCXE motif[30]. Although the functional significance of this region in the HPV-16-E-7 gene product has not yet been determined, the convergence of these results makes it tempting to speculate that similar functional motifs and mechanisms underlie the activity of transforming genes from different classes of DNA tumour viruses which did not appear to be structurally related until the significance of specific small regions in the products of the E1A gene was appreciated.

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Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation

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A new Ca^{2+}/calmodulin-dependent protein kinase has been recently discovered in mammalian cells[1,2]. The major substrate of this kinase, a protein of relative molecular mass (M_r) ≈100,000 (100K), has been identified as elongation factor 2 (EF-2)[3,4], which participates in protein synthesis. The *in vivo* activity of the EF-2 kinase depends upon growth factors and other agents affecting the level of Ca^{2+} and cAMP[5,6]. Its effect on EF-2 activity, however, remained obscure. This work shows that the phosphorylation of EF-2 by the EF-2 kinase results in a drastic inhibition of polyphenylalanine synthesis in poly(U)-directed translation. Phosphorylated EF-2 is completely inactive in translation and, moreover, inhibits the activity of non-phosphorylated EF-2. Dephosphorylation of EF-2 by phosphatase restores its activity. Hence, the phosphorylation of EF-2 directly affects the elongation stage of translation and thus represents a novel mechanism of translational control.

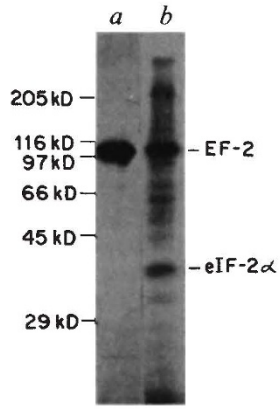

Fig. 1 Endogenously phosphorylated proteins in the ribosome-free extract (*a*) and crude cell-free translation system (*b*) from rabbit reticulocytes. A radioautograph of gels after SDS electrophoresis is shown.
Methods. The ribosome-free extract and crude lysate of rabbit reticulocytes were obtained as described[3,8]. The phosphorylation reaction in *a* was carried out in 50 mM HEPES-KOH buffer, *p*H 7.6, containing 10 mM magnesium acetate, 5 mM DTT, 50 μM [γ-^{32}P]ATP (1,500 c.p.m. pmol^{-1}) and 4 μl of the ribosome-free extract in a final volume of 40 μl. The sample was incubated for 2 min at 30 °C. The reaction was stopped by the addition of 10 μl of SDS-containing electrophoresis buffer and 20 μl was loaded on the gel. The phosphorylation reaction in *b* was in 20 mM HEPES-KOH buffer, *p*H 7.6, containing 150 mM potassium acetate, 1.2 mM $MgCl_2$, 10 mM creatine phosphate, 1 mM [γ-^{32}P]ATP (1,000 c.p.m. pmol^{-1}), 0.2 mM GTP, 0.5 mM spermidine, 1.0 mM DTT, a mixture of 20 amino acids (40 μM each) and 22 μl of reticulocyte lysate in a final volume of 30 μl. The sample was incubated for 10 min at 37°C. The reaction was stopped by the addition of SDS-containing electrophoresis buffer and 2.5 μl was loaded on the gel. Electrophoresis was as described[3]. Dried gels were exposed on the Kodak film at −70°C for 48 h.

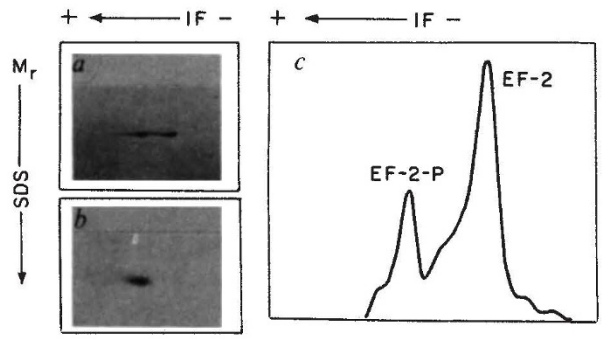

Fig. 2 Separation of phosphorylated and non-phosphorylated forms of EF-2 by isoelectrofocusing. *a*, Coomassie G-250-stained gel after two-dimensional separation[12] of the EF-2 preparation incubated with EF-2 kinase and 50 μM [γ-^{32}P]ATP (500 c.p.m. pmol^{-1}). *b*, Radioautograph of this gel (12-h exposure). *c*, Result of gel scanning after isoelectrofocusing. The gel column was stained with 0.05% Coomassie R-250, 0.1% $CuSO_4$ in a 3:1:6 (v/v/v) ethanol : acetic acid : water mixture for 3 h and the gels were washed in the ethanol : acetic acid : water solution for 4 h. The gel was scanned using a 2202 Ultrascan laser densitometer (LKB, Sweden). **Methods.** EF-2 (2 μg) was incubated for 2 min at 30 °C with 1 μg of partially purified EF-2 kinase and with [γ-^{32}P]ATP. The EF-2 kinase was prepared as follows. The rabbit reticulocyte ribosome-free extract was layered on a column with DEAE-cellulose (DE-52, Whatman) equilibrated with buffer A (10 mM Tris-HCl, *p*H 7.6, 1 mM $MgCl_2$, 50 mM KCl, 7 mM β-mercaptoethanol and 10% glycerol). EF-2 kinase was eluted by buffer A with 600 mM KCl. The eluate was diluted threefold with the same buffer and layered on a Mono Q column (Pharmacia) equilibrated with buffer A containing 200 mM KCl; EF-2 kinase was eluted by the KCl linear gradient (200–600 mM). Fractions with active EF-2 kinase, eluted at about 450 mM KCl, were frozen in liquid nitrogen and stored at −70 °C. The conditions of phosphorylation reaction were described previously[3,4].

Reversible phosphorylation of proteins is considered to be one of the main ways by which protein biosynthesis is regulated. Thus a study of phosphorylation of the translation initiation and elongation factors might provide insights into mechanisms of translational control. To date, eukaryotic initiation factor 2 (eIF-2) was the only protein participating in translation phosphorylation which had been demonstrated to affect its functional activity (see ref. 7 for recent review). In contrast, phosphorylation of the elongation factors with consequent changes in their activity has not been described.

We recently reported phosphorylation of EF-2 (ref. 3) in a rabbit reticulocyte ribosome-free extract, as well as in a crude lysate containing all the components sufficient for translation[3,4,8] (see Fig. 1). The protein kinase responsible for EF-2 phosphorylation, called EF-2 kinase[4], has been identified and partially purified from rabbit reticulocytes[4]. It has a M_r of ≈140K, as determined by gel filtration, and phosphorylates threonine residues in EF-2 (ref. 4). The EF-2 kinase is active only in the presence of Ca^{2+} and calmodulin, but it differs by a number of criteria from all known types of Ca^{2+}/calmodulin-dependent protein kinases[4]. It does, however, have the same properties as Ca^{2+}/calmodulin-dependent protein kinase III discovered in various mammalian tissues[1,2]. EF-2 kinase shows strong substrate specificity for EF-2 as other known protein kinases are unable to phosphorylate EF-2 to a significant extent, and EF-2 kinase does not phosphorylate substrates other than EF-2 (ref. 2 and our unpublished observations).

Reticulocyte lysate has been shown to contain a significant proportion of phosphorylated EF-2, sometimes up to 50% of the total EF-2 (refs 4, 8). Endogenously phosphorylated EF-2 could be separated from non-phosphorylated EF-2 by ion exchange chromatography[9], enabling the effect of EF-2 phosphorylation on its activity to be studied. Testing different ratios of phosphorylated and non-phosphorylated EF-2 in poly(U)-directed polyphenylalanine synthesis has revealed that the phosphorylated EF-2 is inactive in translation[9]. These investigations lacked evidence that it was the Ca^{2+}/calmodulin-dependent EF-2 kinase that phosphorylated EF-2, however, and so we have carried out a direct experiment using a partially purified preparation of the kinase.

EF-2 was purified from the ribosome-free extract of rabbit reticulocytes by successive chromatographies on RNA-Sepharose, hydroxyapatite and Mono Q (ref. 9). The EF-2 preparation was homogeneous according to electrophoresis with sodium dodecyl sulphate. Isoelectrofocusing, however, divided it into two bands corresponding to phosphorylated and non-phosphorylated EF-2 (see Fig. 2).

EF-2 kinase was also purified from the ribosome-free extract of rabbit reticulocytes (see legend to Fig. 2). The kinase activity was strictly dependent upon Ca^{2+} and calmodulin. Incubation of EF-2 with EF-2 kinase resulted in the phosphorylation of a portion of the factor as reflected in the increase of the band with a more acidic isoelectric point.

Figure 3 shows the kinetics of polyphenylalanine synthesis in the poly(U)-directed translation system on addition of EF-2 preparations containing different proportions of the phosphorylated form. The decrease of the non-phosphorylated EF-2 from 70% to 50% resulted in the threefold reduction of the rate of polyphenylalanine synthesis, and a preparation containing only 30% non-phosphorylated EF-2 was completely inactive. The inhibition of polyphenylalanine synthesis was not caused by any impurities in the kinase preparation, as kinase inactivated by the calmodulin antagonist, trifluoperazine (150 μM) did not inhibit translation (in fact it was slightly stimulated).

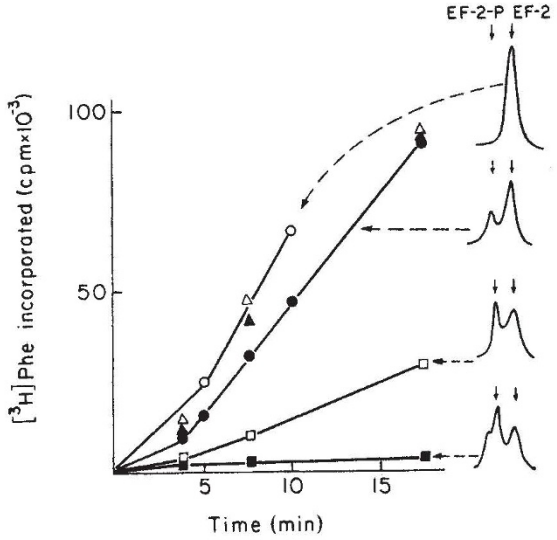

Fig. 3 Dependence of polyphenylalanine synthesis kinetics in the poly(U)-directed translation system on the addition of the EF-2 preparations with different proportions of the phosphorylated and non-phosphorylated forms of EF-2. ((●) 30:70, (□) 50:50; (▲) 30:70; (Δ) 30:70; (■) 70:30; (○) 0:100; see below for details.) The results of scanning the stained gels after isoelectrofocusing of the EF-2 preparations used in the given experiments are shown at the right.

Methods. In all experiments 0.4 μg of EF-2 was used and other components were in excess. The incubation mixture (100 μl) contained 3 pmol of ribosomes (an equimolar mixture of 40S and 60S subunits from rabbit reticulocytes), 10 μg of poly(U) (Boehringer), 3 μg of EF-1 from rabbit reticulocytes, 50 μg of crude *Escherichia coli* transfer RNA aminoacylated with [^{3}H]phenylalanine (2,000 c.p.m. per 1 μg of tRNA) in 20 mM Tris-HCl buffer, *p*H 7.6 with 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT and 1.2 mM GTP. Incubation was carried out at 37 °C and stopped by the addition of 5% trichloroacetic acid. The mixture was boiled for 10 min and filtered through GF/C glass filters (Whatman). The radioactivity of dry filters was determined by liquid-scintillation counting. Every point corresponds to the mean value obtained in two independent experiments. Separate treatments were as follows. (●) Original EF-2 preparation (8 μg) incubated for 20 min at 30 °C in buffer B (50 mM HEPES KOH, 5 mM Tris-HCl *p*H 7.6, 10 mM MgAc, 60 mM KCl, 150 μM $CaCl_2$, 0.5 mM ATP, 10 μg ml^{-1} bovine brain calmodulin, 6 mM DTT, 5% glycerol), final volume 180 μl. (□) Original EF-2 preparation (8 μg) was incubated with 3 μg of EF-2 kinase for 20 min at 30 °C in buffer B, final volume 180 μl. (▲) Original EF-2 preparation (8 μg) was incubated with 3 μg of EF-2 kinase as above, except that 150 μM trifluoperazine was added. (Δ) Original EF-2 preparation (8 μg) incubated in buffer B in 180 μl with 150 μM trifluoperazine for 20 min at 30 °C. (■) Original EF-2 preparation (8 μg) incubated with 7 μg of EF-2 kinase for 20 min at 30 °C in buffer B in 180 μl. (○) Original EF-2 preparation (2 μg) incubated for 25 min at 30 °C with 25 μg of alkaline phosphatase from calf intestine (Boehringer, grade II) in buffer C (20 mM Tris-HCl *p*H 7.6, 10 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol), final volume 88 μl.

Calmodulin, Ca^{2+} and ATP at the concentrations used in this study have no influence on the rate of polyphenylalanine synthesis (data not shown). Incubation of the EF-2 preparation with alkaline phosphatase stimulated EF-2 activity, and was accompanied by the disappearance of the phosphorylated EF-2 form (see Fig. 3).

When EF-2 pre-incubated with EF-2 kinase was subsequently incubated with alkaline phosphatase, EF-2 activity was restored to that of completely dephosphorylated EF-2 (Table 1). Thus, the dephosphorylation of EF-2 restores its activity.

All these results demonstrate that EF-2, when phosphorylated by the EF-2 kinase, is inactive in translation. Moreover, Fig. 3 shows that polyphenylalanine synthesis is inhibited to a greater extent than the decrease in non-phosphorylated EF-2. It is thus probable that the phosphorylated EF-2 is not only inactive in translation, but also inhibits it.

Table 1 Restoration of the activity of phosphorylated EF-2 after incubation with alkaline phosphatase

	[^{3}H]Phe incorporated c.p.m. 5 min	10 min
Original EF-2 preparation	11,646	34,677
EF-2 + EF-2 kinase	8,871	25,627
EF-2 + EF-2 kinase + alkaline phosphatase	26,666	64,495

The original EF-2 preparation used in this experiment contained 35% phosphorylated and 65% non-phosphorylated EF-2 forms. The preparation (2 μg) was incubated with 2 μg of EF-2 kinase at 30 °C for 4 min in buffer D (30 mM HEPES KOH, *p*H 7.6, 10 mM MgAc, 150 μM $CaCl_2$, 0.5 mM ATP, 10 μg ml^{-1} bovine brain calmodulin, 5 mM dithiothreitol (DTT)) and for 10 min in buffer E (15 mM HEPES KOH, 20 mM Tris-HCl, *p*H 7.6, 50 μM $CaCl_2$, 10 mM $MgCl_2$, 3 mM MgAc, 120 mM KCl, 4 mM DTT, 60 μM trifluoperazine (the second row)). The EF-2 preparation preincubated with EF-2 kinase at 30 °C for 4 min in buffer D was incubated with 70 μg of alkaline phosphatase in buffer E for 10 min at 30 °C (the third row). Original EF-2 preparation (2 μg) was incubated in buffer D for 4 min and buffer E for 10 min (the first row). The final volume in all the incubations was 164 μl. An aliquot (0.4 μg) of EF-2 was added to the translation system in each experiment. The reaction was carried out as described in Fig. 3.

Table 2 Inhibition of the activity of non-phosphorylated EF-2 by phosphorylated EF-2

	[^{3}H]Phe incorporated c.p.m. 7 min
Original EF-2 preparation (0.25 μg)	14,794
Original EF-2 preparation (0.25 μg) + phosphorylated EF-2 (1 μg)	10,596
Original EF-2 preparation (0.25 μg) + phosphorylated EF-2 (2 μg)	8,634

The original EF-2 preparation used in this experiment was completely dephosphorylated. This preparation was mixed with various amounts of fully phosphorylated EF-2. Phosphorylated EF-2 was obtained by incubating 50 μg of EF-2 with 5 μg of EF-2 kinase at 30 °C for 40 min in buffer D in a final volume of 250 μl. The translation assay was carried out as described in Fig. 3.

To test this directly, we studied the effect of fully phosphorylated EF-2 on the activity of non-phosphorylated EF-2 and found that the addition of increasing amounts of phosphorylated EF-2 markedly suppressed EF-2 activity (Table 2). This phenomenon may have important physiological implications, as it is not necessary to phosphorylate EF-2 completely to shut off translation.

Why does EF-2 become inactive after phosphorylation? This question is not easy to answer as the mechanism of EF-2 function is intricate and poorly understood. Our working hypothesis is that phosphorylation of EF-2 alters its interaction with the ribosome and makes it unable to catalyse translocation. If this is true, the inhibition of non-phosphorylated EF-2 by its phosphorylated form could be due to the competition between these two EF-2 forms for a common binding site on the ribosome.

Reversible EF-2 phosphorylation is undoubtedly a physiologically relevant mechanism. As has been found earlier[8], and as shown in Fig. 1, intensive phosphorylation of EF-2 is observed in a highly active non-fractionated translation system from rabbit reticulocytes. Moreover, the addition of 5 mM cAMP to such a system led to a three- to fivefold activation of endogenous protein synthesis, which correlates with the drastic dephosphorylation of EF-2 (ref. 8). This observation seems to reflect the *in vivo* situation as recently it has been shown[6,10] that treatment of PC-12 cells with a nerve growth factor, or with

agents that increase the intracellular cAMP concentration, leads to dephosphorylation of EF-2.

Another example of an *in vivo* system where reversible EF-2 phosphorylation is implicated is the mitogenic stimulation of cells. A dramatic transient increase of EF-2 phosphorylation has been observed[5] after mitogenic stimulation of fibroblasts, which ran parallel to but lagged slightly behind the intracellular Ca^{2+} transient.

Parallel studies of the elongation rate changes were not done in any of the above examples. Nevertheless, these results, together with those reported here, strongly indicate that the reversible EF-2 phosphorylation may serve as a link between intracellular second messengers such as cAMP and the rate of translation.

Finally we would like to note that, after this paper was submitted, a communication by Nairn and Palfrey[11] reported that EF-2 from rat pancreas, that has been phosphorylated by EF-2 kinase (Ca^{2+}/calmodulin-dependent protein kinase III) from the same source, is almost completely inactive in poly(U)-directed translation.

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Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes

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Newly synthesized proteins to be exported out of the cytoplasm of bacterial cells have to pass across the inner membrane. In Gram-negative bacteria ATP[1,2], a membrane potential[3,4], the products of the *sec* genes[5] and leader peptidases[6,7] (enzymes which cleave the N-terminal signal peptides of the precursor proteins) are required. The mechanism of translocation, however, remains elusive. Important additional roles for membrane lipids have been repeatedly suggested both on theoretical grounds[8–11] and on the basis of experiments with model systems[12–14] but no direct evidence had been obtained. We demonstrate here, using mutants of *Escherichia coli* defective in the synthesis of the major anionic membrane phospholipids, that phosphatidylglycerol is involved in the translocation of newly synthesized outer-membrane proteins across the inner membrane.

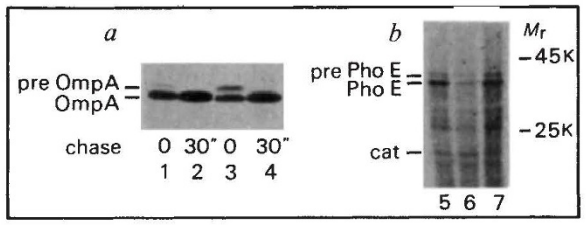

Fig. 1 Effect of PG and CL content on the *in vivo* synthesis and translocation of *E. coli* outer membrane proteins. *a*, Plasmid pHD102 containing cells of wild-type strain CE1224 (lanes 1 and 2, high PG and CL) and *pgsA3*-containing strain CE1250 (lanes 3 and 4, low PG and CL) were grown in a synthetic medium[27] at 43° C, until a low PG content was reached in CE1250. Cells were then pulse-labelled with [^{35}S]methionine at 37° C for 30 s (ref. 28), followed by a chase with unlabelled methionine for the indicated time period. After trichloracetic acid precipitation the samples were immunoprecipitated using rabbit antiserum against OmpA protein[29] and protein A coupled to Sepharose[30]. *b*, Strains SD12 (lane 5, wild-type), HD3122 (*pgsA3*) (lane 6, low PG and CL) and SD11 (lane 7, low CL), each containing plasmid pJP29 which carries the *phoE* gene[28], were induced for PhoE protein synthesis by growth at 37° C in low phosphate medium[31], supplemented with chloramphenicol (25 μg ml^{-1}). Whole cells were pulse-labelled with [^{35}S]methionine for 10 s at 30° C. Strain CE1250 was derived from *E. coli* K12 strain CE1224[32], containing pHD102, by P1 transduction[33] using strain HD3122 which carries a *Tn10* insertion close to the *pgsA3* allele as donor. A tetracycline-resistant transformant with a low PG content after growth at 43° C was designated CE1250. Curing plasmid pHD102 from this strain followed by plating resulted in pinpoint colonies, indicating that loss of an intact *pgsA* allele is harmful in this genetic background. All samples were analysed by SDS-polyacrylamide gel electrophoresis[34] and the dried gels were subjected to fluorography. The position of some relative molecular mass (M_r) standard proteins are shown on the right. For determination of the phospholipid composition, phospholipids were isolated from whole cells or membrane vesicles by solvent extraction[35] and separated by thin-layer chromatography on silica gel using chloroform:methanol:acetic acid (65:25:10) as solvent one and chloroform:pyridine:formic acid (60:30:7) as solvent two. The phosphate content in each spot was measured after perchloric acid destruction[36].

Phosphatidylglycerol phosphate synthase is a key enzyme in the biosynthesis of phosphatidylglycerol (PG), the major acidic phospholipid in *E. coli*. A mutation in the *pgsA* locus of the *E. coli* genome was described[15], which resulted in undetectable levels of activity of this enzyme and consequently very low levels of PG and cardiolipin (CL), which is derived from PG. Several *E. coli* strains carrying this *pgsA3* allele required the presence of a plasmid (such as pDH102) carrying a functional *pgsA* gene for growth[16], suggesting that these lipids are very important. We used such *pgsA3* *E. coli* strains to study the role of the anionic lipids in protein translocation.

Cells of the *pgsA3* *E. coli* strain CE1250 were cured of plasmid pHD102 by growth at the restrictive temperature for plasmid replication (43° C). When the PG content dropped to 1–3 mol% of the total phospholipids (analysed as described in Fig. 1), the cells were pulse-labelled with [^{35}S]methionine to follow the biosynthesis of the outer membrane protein OmpA. Immunoprecipitation showed that significantly more precursor (pre-OmpA) was present, compared to the wild-type strain with normal PG levels (Fig. 1*a*, lanes 1 and 3). In both cases the precursor disappeared during a 30-second chase (Fig. 1*a*, lanes 2 and 4). This is the first indication that the rate of translocation of an outer membrane protein across the inner membrane is decreased by reduced levels of anionic phospholipids.

The *E. coli* strain SD12 carrying the *pgsA3* allele is able to grow normally[16] (strain HD3122) although in agreement with previous reports, the mutation caused a reduction in PG and CL from 11 to 1.0 and 3–4 to 0.5 mol% of total phospholipid respectively. This drop in total level of anionic phospholipids