galactose of the complemented Δsss1 strain was approximately equal to that of the wild type.

We also tested whether the translocation defect, which occurs upon depletion of SSS1p from yeast cells and is indicated by the accumulation of precursors of exported proteins[12], can be reversed by expression of Sec61-γ. Little accumulation of precursors to α-factor or Kar2p (BiP) was observed if the expression of Sec61-γ was induced in the presence of galactose (Fig. 3b, lane 1), indicating that the export of these proteins from the cytoplasm was close to normal. After shift to glucose, a severe defect of translocation developed (Fig. 3b, lanes 2–4), concomitant with the depletion of Sec61-γ from the cells (Fig. 3c, lanes 4, 6, 8). After 6 h, the cells ceased to grow (data not shown).

Signal sequences are similarly structured and exchangeable[14] and one mechanism by which they are recognized—that involving the signal recognition particle—exists in all organisms[15-19]. It is now clear that the component mediating the actual membrane passage of a polypeptide, the Sec61/SecYp complex, is also ubiquitous: two of its subunits, Sec61-α/SecYp and Sec61-γ/ SSS1/SecEp, have been found in mammals, yeast and bacteria and, where tested, they are associated with each other. It remains to be seen whether Sec61-β, which so far has been found only in plants, also exists in yeast and whether it is related to the band-1 protein of the E. coli SecYp complex.

It is likely that Sec61-α/SecYp forms a channel that guides polypeptides across the membrane[5,20]. Such a channel would be gated in two dimensions: perpendicular to the plane of the membrane to let polypeptides across, and horizontally within the membrane to release membrane-spanning regions of membrane proteins into the phospholipid bilayer and perhaps to let signal sequences in. Although the function of Sec61-γ/SSS1/SecEp is unknown, it may be involved in the second gating mechanism. Such a function would be consistent with the fact that this essential polypeptide, containing little more than a membrane-spanning region and a few adjacent residues, has remained separate from its multi-spanning partner during evolution. □

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Interaction of E. coli Ffh/4.5S ribonucleoprotein and FtsY mimics that of mammalian signal recognition particle and its receptor

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THE mechanism of protein translocation across the endoplasmic reticulum membrane of eukaryotic cells and the plasma membrane of prokaryotic cells are thought to be evolutionarily related[1-7]. Protein targeting to the eukaryotic translocation apparatus is mediated by the signal recognition particle (SRP), a cytosolic ribonucleoprotein, and the SRP receptor, an endoplasmic reticulum membrane protein[8,9]. During targeting, the 54K SRP subunit (M_r 54,000; SRP54), a GTP-binding protein[10-12], binds to signal sequences[13,14] and then interacts with the α-subunit of the SRP receptor (SRα), another GTP-binding protein[12,15]. Two proteins from Escherichia coli, Ffh and FtsY, structurally resemble SRP54 and SRα[10,11,16]. Like SRP54, Ffh is a subunit of a cytosolic ribonucleoprotein that also contains the E. coli 4.5S RNA[17,18]. Although there is genetic and biochemical evidence that the E. coli Ffh/ 4.5S ribonucleoprotein has an SRP-like function[19-21], there is no evidence for an SRα-like role for FtsY. Here we show that the Ffh/ 4.5S ribonucleoprotein binds tightly to FtsY in a GTP-dependent manner. This interaction results in the stimulation of GTP hydrolysis which can be inhibited by synthetic signal peptides. These properties mimic those of mammalian SRP and its receptor, suggesting that the E. coli Ffh/4.5S ribonucleoprotein and FtsY have functions in protein targeting that are similar to those of their mammalian counterparts.
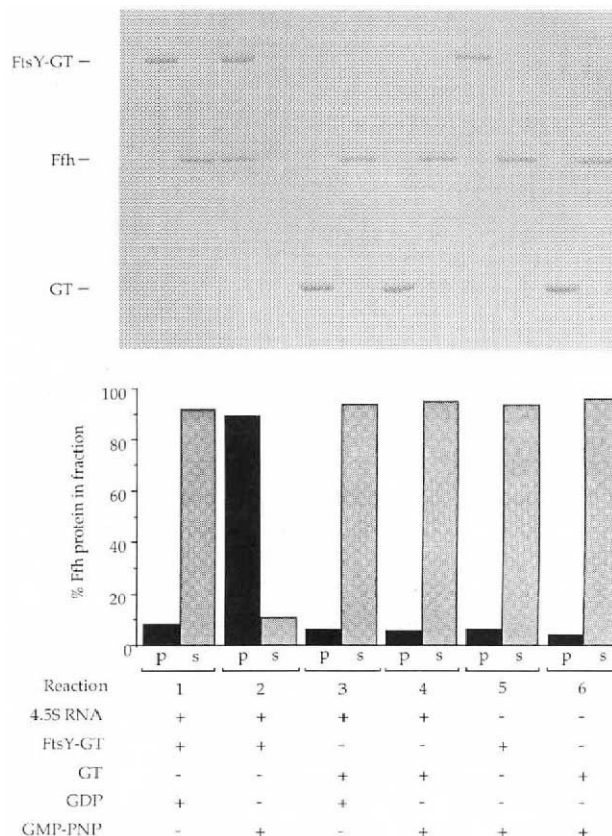
To test for an interaction between the Ffh/4.5S ribonucleoprotein (RNP) and FtsY, Ffh and 4.5S RNA were purified from overproducing strains and reconstituted into an RNP. FtsY was purified as a fusion protein with glutathione S-transferase (FtsY–GST) and immobilized on a glutathione-affinity resin, which was then incubated with Ffh/4.5S RNP in the presence of either GDP or the non-hydrolysable GTP analogue GMP-PNP. Nucleotide was included in these reactions because both Ffh and FtsY contain GTP-binding domains which might regulate their interaction. More than 90% of the Ffh co-fractionated with the FtsY–GST resin in the presence of GMP-PNP; most of the Ffh was recovered in the supernatant fraction in the presence of GDP (Fig. 1). Similarly, most of the Ffh was recovered in the supernatant fraction in the presence of the non-hydrolysable ATP analogue AMP-PNP (not shown), indicating that the interaction requires guanosine triphosphate. Free Ffh protein failed to bind to FtsY–GST resin even in the presence of GMP-PNP (Fig. 1), indicating that FtsY binding requires both Ffh and 4.5S RNA. Neither Ffh/4.5S RNP nor free Ffh bound to a control resin containing immobilized GST, indicating that binding is specific for FtsY (Fig. 1).

Interestingly, no binding was observed when the reaction shown in Fig. 1 was performed with GTP instead of GMP-PNP (not shown), indicating that GTP might be hydrolysed during the reaction. We therefore analysed the ability of these components to hydrolyse GTP. As shown in Fig. 2, purified Ffh hydro-

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FIG. 1 Ffh/4.5S binds to immobilized FtsY–GST in the presence of GMP-PNP. Purified Ffh protein and Ffh/4.5S RNP were assayed for their ability to bind to FtsY-GST or GST immobilized on glutathione–agarose beads in the presence of either GDP or GMP-PNP. After binding, the beads were collected by centrifugation, and the proteins recovered in the pellet (p) and supernatant (s) fractions were analysed on SDS–polyacrylamide gels and visualized by staining with Coomassie blue. The amount of Ffh protein in each lane is expressed as a percentage of the total Ffh protein recovered in pellet and supernatant fractions. METHODS. Ffh protein and 4.5S RNA were prepared as described[17,21]. The FtsY–GST fusion protein was produced by fusing amino acids 48–494 of FtsY in-frame to the C terminus of GST in the pGEX1 vector[27]. The region of strong homology between FtsY and SRα encompasses amino acids 197–494 and includes the GTP-binding domain. Purified FtsY–GST fusion protein (10 pmol) or GST protein was incubated under constant mixing with 50 µl of a 50% suspension of glutathione–agarose beads (Sigma) in phosphate-buffered saline for 1 h at room temperature. The resin was washed with buffer A containing 50 mM triethanolamine/acetic acid (TEA), pH 7.5, 25 mM potassium acetate, 2.5 mM $Mg(OOC \cdot CH_3)_2$, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% Nikkol detergent (Nikko Chemicals, Tokyo) and then added to 100 µl of the same buffer containing 5 pmol of Ffh or Ffh/4.5S RNP. GDP or GMP-PNP was added to a final concentration of 1 mM where indicated. Reactions were incubated with constant mixing at room temperature for 30 min. The resin was pelleted by centrifugation in a microfuge for 30 s and the supernatant removed. Proteins in the supernatant were precipitated with an equal volume of 30% trichloroacetic acid and solubilized in SDS sample buffer. The resin was washed with buffer A containing either GDP or GMP-PNP at 1 mM, and bound proteins were then solubilized by boiling the beads in SDS sample buffer. Quantification was by laser scanning desitometry of the stained gel. The Ffh/4.5S RNP was prepared by incubating a solution containing 10 µM Ffh and 20 µM 4.5S RNA in buffer B containing 50 mM TEA, 500 mM potassium acetate, 5 mM $Mg(OOC \cdot CH_3)_2$, 1 mM DTT, 0.01% Nikkol and 10% glycerol for 10 min on ice followed by 10 min at 37 °C.

Reaction	1		2		3		4		5		6	
	p	s	p	s	p	s	p	s	p	s	p	s
4.5S RNA	+		+		+		+		-		-	
FtsY-GT	+		+		-		-		+		-	
GT	-		-		+		+		-		+	
GDP	+		-		+		-		-		-	
GMP-PNP	-		+		-		+		+		+	

FIG. 2 Enhanced GTP hydrolysis results from interaction of Ffh/4.5S RNP and FtsY. Purified Ffh/4.5S RNP and purified Ffh protein were assayed for the ability to hydrolyse GTP in the presence or absence of FtsY–GST or GST.
METHODS. Reactions (100 µl) were incubated in buffer A containing 10% glycerol. Ffh/4.5S RNP complexes were formed as described in Fig. 1 legend. Ffh was present at 5 nM, 4.5S RNA at 10 nM, FtsY–GST and GST at 150 nM. $[\gamma\text{-}^{32}P]GTP$ (0.5 µCi; ICN) was added at 0.5 µM. After a 20-min incubation at 25 °C an 8-µl aliquot of the reaction was mixed wth 200 µl of a 5% suspension of activated charcoal (Sigma) in 20 mM phosphoric acid, incubated on ice for 10 min and then centrifuged for 10 min at maximum speed in a microfuge to pellet the charcoal and bound nucleotide. A 100-µl aliquot of the supernatant fraction containing the phosphate liberated in the reaction was analysed by Cerenkov counting in a liquid scintillation counter and corrected for background counts from a reaction performed with buffer only. The Michaelis constant of the Ffh/4.5S RNP for GTP hydrolysis was 0.4 µM; the rate of hydrolysis was linear during the 20-min incubation.

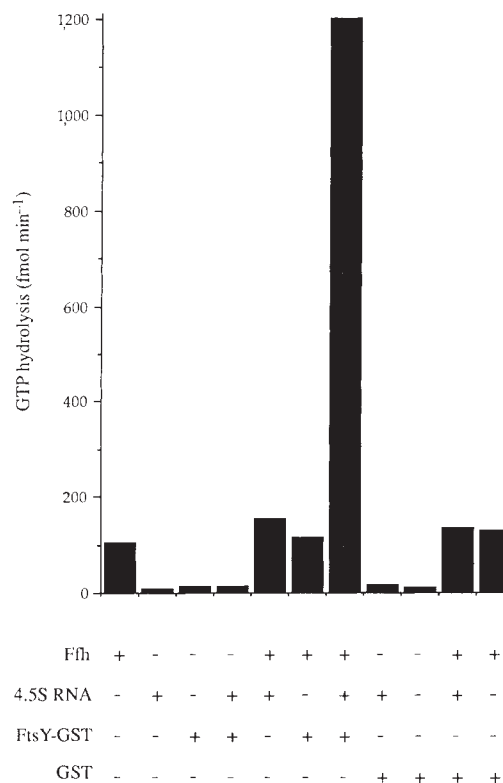| Ffh | + | - | - | - | + | + | + | - | - | + | + |
4.5S RNA	-	+	-	+	+	-	+	+	-	+	-
FtsY-GST	-	-	+	+	-	+	+	-	-	-	-
GST	-	-	-	-	-	-	-	+	+	+	+

FIG. 3 Synthetic signal peptides inhibit GTP hydrolysis. a, Effect of titrating synthetic signal peptides into GTP hydrolysis reactions containing Ffh/4.5S RNP and FtsY. b, Effect of titrating signal peptide into GTP hydrolysis reactions containing Ffh/4.5S RNP in the absence of FtsY. Reactions are described in Fig. 2 legend, except that reaction mixtures were preincubated for 30 min at 25 °C with the appropriate synthetic signal peptide before addition of GTP. Four different peptides were used: wild-type peptide (□; sequence MMITLRKLPLA-VAVAAGVMSAQAMA), corresponding to the signal sequence of LamB, a bacterial outer-membrane protein; deletion mutant peptide (◆), corresponding to the Lam B signal sequence with residues L10–A13 deleted; r1 (▲) (corresponding to the deletion mutant peptide with a G17C change) and r2 (△) (corresponding to the deletion mutant peptide with a P9L change) peptides, which correspond to second-site, single-amino-acid revertants of the deletion mutant. The synthetic wild-type peptide can readily adopt an α-helical conformation as analysed by circular dichroism spectroscopy[24]. The missing four amino acids in the deletion mutant peptide cause a proline and a glycine residue to be brought closer so
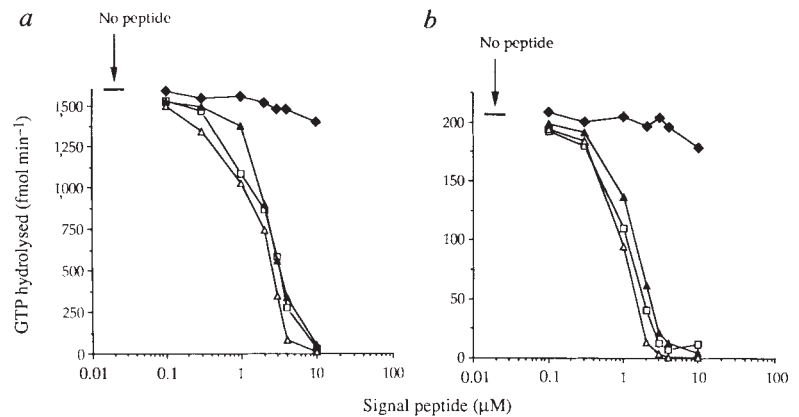
that these two residues function as helix breakers. The synthetic deletion mutant peptide does not form an α-helix[24], and does not function as a signal peptide in vivo[23]. Both r1 and r2 peptides regain the ability to form an α-helix and function as signal sequences in vivo[23,24].

lysed GTP, and the rate of hydrolysis was independent of the presence of 4.5S RNA. The enzymatic properties of this reaction closely resemble those previously described for an Ffh homologue from *Mycoplasma mycoides*[22]. In contrast, FtsY–GST caused no significant GTP hydrolysis (Fig. 2). When the Ffh/4.5S RNP and FtsY–GST were combined, however, GTP hydrolysis was significantly stimulated. The stimulation requires that Ffh be complexed to 4.5S RNA; in the absence of the RNA only the basal, Ffh-catalysed hydrolysis rate was observed. No stimulation of GTP hydrolysis was observed when GST was incubated with Ffh/4.5S RNP, indicating that the activity is specific for FtsY (Fig. 2). Together with the binding data shown in Fig. 1, these results provide strong evidence for a functional interaction between Ffh/4.5S RNP and FtsY.

As signal sequences are possible physiological ligands of the Ffh/4.5S RNP[20,21], we tested whether the binding of synthetic signal peptides could modulate GTP hydrolysis. We assayed the effects of peptides derived from the signal sequence of LamB, a bacterial outer-membrane protein that exhibits a translocation defect in Ffh-depleted cells[19]. Four different peptides were used: a peptide corresponding to the wild-type signal sequence, a deletion mutant that is inactive *in vivo*, and two different single-amino-acid, second-site revertants of the LamB deletion mutant (r1 and r2), both of which restore signal-sequence function[23,24]. The wild-type, r1 and r2 synthetic peptides, but not the deletion mutant peptide, inhibit *in vitro* protein translocation into *E. coli* inverted plasma membrane vesicles[25].

The data shown in Fig. 3a indicate that the wild-type signal peptide potently inhibits the FtsY–GST-stimulated GTP hydrolysis reaction with a half-maximal inhibitory concentration (IC$_{50}$) of ~2 µM. In contrast, the deletion mutant peptide had little effect on GTP hydrolysis even at concentrations at which the wild-type peptide inhibited completely. Both reversion mutations, r1 and r2, restored the inhibitory activity to the deletion mutant peptide to about the same level as that exerted by the wild-type peptide. Therefore only peptides corresponding to functional signal sequences (namely wild-type, r1 and r2) inhibit GTP hydrolysis.

Examination of the inhibition curves reveals that the functional signal peptides block GTP hydrolysis completely, so FtsY-stimulated hydrolysis and basal hydrolysis catalysed by Ffh alone were both blocked. This was confirmed in experiments shown in Fig. 3b, in which the same set of peptides were titrated into a reaction containing only Ffh. The functional peptides inhibited the GTPase activity of Ffh with a concentration dependence similar to that of the FtsY-stimulated reaction (Fig.

3b). Whether Ffh, FtsY or both hydrolyse GTP in the FtsY-stimulated reaction remains to be determined.

These results provide evidence that the SRP receptor-like FtsY protein interacts directly with the SRP-like Ffh/4.5S RNP. The interaction results in enhanced GTP hydrolysis that can be modulated by signal sequences. The biochemistry of the interaction of the Ffh/4.5S RNP with FtsY and with two other ligands, signal sequences and GTP, is remarkably similar to that found for the corresponding mammalian components. In particular, SRP and its receptor form a stable complex in the presence of GMP-PNP[26]. Furthermore, SRP receptor and SRP54 interact to hydrolyse GTP in an SRP RNA-dependent manner, and functional signal sequences inhibit this reaction with comparable IC$_{50}$s (ref. 12). This striking similarity supports the hypothesis that bacteria have a signal-recognition and protein-targeting apparatus composed of the Ffh/4.5S RNP and FtsY which resembles the SRP and SRP receptor system in eukaryotic cells. □

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