Molecular Biology of the Cell
Vol. 18, 2636–2645, July 2007

Efficient Interaction between Two GTPases Allows the Chloroplast SRP Pathway to Bypass the Requirement for an SRP RNA[D]

Peera Jaru-Ampornpan, Sowmya Chandrasekar, and Shu-ou Shan

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

Submitted January 17, 2007; Revised April 18, 2007; Accepted April 20, 2007
Monitoring Editor: Reid Gilmore

Cotranslational protein targeting to membranes is regulated by two GTPases in the signal recognition particle (SRP) and the SRP receptor; association between the two GTPases is slow and is accelerated 400-fold by the SRP RNA. Intriguingly, the otherwise universally conserved SRP RNA is missing in a novel chloroplast SRP pathway. We found that even in the absence of an SRP RNA, the chloroplast SRP and receptor GTPases can interact efficiently with one another; the kinetics of interaction between the chloroplast GTPases is 400-fold faster than their bacterial homologues, and matches the rate at which the bacterial SRP and receptor interact with the help of SRP RNA. Biochemical analyses further suggest that the chloroplast SRP receptor is pre-organized in a conformation that allows optimal interaction with its binding partner, so that conformational changes during complex formation are minimized. Our results highlight intriguing differences between the classical and chloroplast SRP and SRP receptor GTPases, and help explain how the chloroplast SRP pathway can mediate efficient targeting of proteins to the thylakoid membrane in the absence of the SRP RNA, which plays an indispensable role in all the other SRP pathways.

INTRODUCTION

Signal recognition particle (SRP) and SRP receptor (SR) comprise the major cellular machinery that delivers nascent proteins to the eukaryotic endoplasmic reticulum membrane or the bacterial plasma membrane (Walter and Johnson, 1994; Keenan *et al*., 2001). The functional core of SRP is the SRP54 protein (called Ffh in bacteria) in complex with an SRP RNA, which recognizes the cargo protein and interacts with the SR (called FtsY in bacteria). The protein targeting reaction is regulated by the guanosine-5′-triphosphate (GTP)-binding domains in both SRP54 and SR. SRP recognizes the signal sequence on nascent polypeptides that emerge from a translating ribosome (Walter *et al*., 1981). The ribosome•nascent chain complex is delivered to the membrane via the interaction of SRP with SR when both proteins are bound with GTP (Gilmore *et al*., 1982a,b). Upon arrival at the membrane, SRP releases the cargo protein to a protein conducting channel embedded in the membrane (Simon and Blobel, 1991; Gorlich *et al*., 1992), where the nascent protein is either integrated into the membrane or translocated across the membrane to enter the secretory pathway. GTP hydrolysis is stimulated in the SRP•SR complex, which then drives disassembly and recycling of SRP and SR (Connolly *et al*., 1991).

The SRP and SR GTPases comprise a unique subgroup in the GTPase superfamily (Keenan *et al*., 2001). Both proteins have a GTPase, "G" domain that shares homology with the classical *Ras* GTPase fold (Freymann *et al*., 1997; Montoya *et al*., 1997). In addition, the SRP-type GTPases contain an N-terminal four-helix bundle, the "N" domain, that packs tightly against the G domain. The G and N domains form a structural and functional unit called the NG domain. Unlike classical signaling GTPases that undergo large conformational changes depending on whether GTP or guanosine-5′-diphosphate (GDP) is bound, the structures of these GTPases are similar regardless of which nucleotide is bound (Freymann *et al*., 1999; Montoya *et al*., 1997; Padmanabhan and Freymann, 2001; Gawronski-Salerno *et al*., 2006; Reyes and Stroud, unpublished data). Substantial conformational changes occur only when the two GTPases form a complex with one another (Egea *et al*., 2004; Focia *et al*., 2004). Most notably, the G and N domains readjust their relative positions such that the N domains of both proteins move closer to the dimer interface and form additional interface contacts to stabilize the complex.

The importance of this N–G domain rearrangement is supported by biochemical analyses. Many mutations at this interface disrupt SRP–SR complex formation and protein targeting (Lu *et al*., 2001). Interestingly, unlike classical GTPases, free FtsY displays little discrimination between GTP and noncognate nucleotides. In contrast, FtsY acquires substantial nucleotide specificity only when it binds SRP. These results have led to the proposal that during complex formation, FtsY changes from a nondiscriminative, "open" state to a "closed" state in which specific interactions between GTP and active site residues are established (Shan and Walter, 2003). Consistent with these observations, the

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–01–0037) on May 2, 2007.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

Address correspondence to: Shu-ou Shan (sshan@caltech.edu).

Abbreviations used: GDP, guanosine-5′-diphosphate; GppNHp, 5′-guanylylimido-diphosphate; GTP, guanosine-5′-triphosphate; LHCP, light-harvesting chlorophyll-binding proteins; mant, *N*-methyl-anthraniloyl; SR, signal recognition particle receptor; SRP, signal recognition particle; XDP, xanthosine-5′-diphosphate; XppNHp, 5′-xanthylylimido-diphosphate; XTP, xanthosine-5′-triphosphate.

crystal structure showed that, upon complex formation, the rearrangement at the N–G domain interface brings the nucleotide specificity determinant Asp449 closer to the bound GTP and within hydrogen bonding distance with the amino groups of the guanine ring (Egea *et al*., 2004). Thus, the N–G domain rearrangement is primarily responsible for the open → closed conformational change that occurs during SRP–SR complex formation and precisely aligns active site residues with respect to the bound GTP.

The unique structural features of the SRP subgroup of GTPases confer upon them many characteristics that are distinct from canonical GTPases. Most importantly, SRP-type GTPases bind nucleotides much weaker than signaling GTPases and release nucleotides quickly (Moser *et al*., 1997; Jagath *et al*., 1998, 2000; Peluso *et al*., 2001). Therefore, they do not use nucleotide exchange factors to facilitate the conversion from the GDP- to the GTP-bound form. These GTPases also do not use external GTPase-activating proteins; instead, SRP and SR reciprocally activate one another upon complex formation (Powers and Walter, 1995).

A third unique feature of the GTPases engaged in the SRP pathway is the requirement for a universally conserved SRP RNA. Mammalian SRP is a cytosolic ribonucleoprotein complex that consists of six polypeptides and a 7S SRP RNA molecule. Besides SRP54, the other protein components are not conserved, whereas the SRP RNA has been shown to play an indispensable role in protein targeting in all three kingdoms of life. In early biochemical studies on the mammalian SRP, the SRP RNA seemed to be nothing more than a scaffold that holds all the SRP proteins together in a complex (Walter and Blobel, 1982, 1983). The finding that bacteria contain a much simpler SRP, made up solely of a complex of Ffh and the 4.5S SRP RNA, was therefore intriguing. This smaller RNA contains the most phylogenetically conserved region of the SRP RNA, domain IV, which is likely to have been maintained for functional purposes (Poritz *et al*., 1988; Struck *et al*., 1988). Subsequently, kinetic analyses of the role of the 4.5S SRP RNA on the GTPase cycles of Ffh and FtsY showed that a major role of this RNA is to accelerate complex formation between the two GTPases. In the absence of the SRP RNA, Ffh–FtsY association is extremely slow, with a rate constant of $5 \times 10^3\ M^{-1}\ s^{-1}$. The SRP RNA accelerates their association kinetics by 400-fold, to a rate that can allow the SRP and SRP receptor to adequately carry out their biological functions, thus accounting for the indispensable role of the SRP RNA in the bacterial, archeal, and eukaryotic SRP pathways.

A novel SRP targeting pathway was discovered in the chloroplast (Schuenemann *et al*., 1998). cpSRP54 and cpFtsY are the chloroplast homologues of SRP and SR GTPases, respectively (Franklin and Hoffman, 1993; Li *et al*., 1995; Tu *et al*., 1999). cpSRP54 recognizes its cargo, the light-harvesting chlorophyll-binding proteins (LHCP), via a protein adaptor cpSRP43 (Tu *et al*., 2000). Together, cpSRP54 and cpSRP43 deliver the cargo protein from the stroma to the thylakoid membrane via the GTP-dependent interaction between cpSRP54 and cpFtsY (Tu *et al*., 1999). Surprisingly, the otherwise universally conserved SRP RNA has not been found to date in the chloroplast SRP system. To rationalize the absence of the SRP RNA, we characterized the kinetic and thermodynamic features of the GTPase cycles of cpSRP54 and cpFtsY. We found that, unlike their bacterial and mammalian homologues, the chloroplast SRP and SR GTPases can efficiently interact with one another by themselves. This helps explain why the cpSRP pathway could bypass the requirement for an SRP RNA.

MATERIALS AND METHODS

Protein Expression and Purification

cpSRP54 from *Arabidopsis thaliana* was expressed from baculovirus at the Protein Expression Facility at California Institute of Technology (Pasadena, CA). Recombinant cpSRP54 is purified by affinity chromatography using nickel-nitrilotriacetic acid (QIAGEN, Valenica, CA) and cation exchange over a MonoS column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using a linear gradient of 150 to 600 mM NaCl. cpFtsY from *A. thaliana* was expressed and purified as described previously (Yuan *et al*., 2002). Two additional chromatographic steps (Superdex 75 and monoQ [GE Healthcare]) were added to remove contaminating GTPases. Mutant cpFtsY(D283N) was constructed using the QuikChange procedure (Stratagene, La Jolla, CA), and it was expressed and purified by the same procedure as that for wild-type cpFtsY.

Kinetics

All reactions were carried out at 25°C in assay buffer [50 mM KHEPES, pH 7.5, 150 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM dithiothreitol, and 0.01% Nikkol]. GTP hydrolysis reactions were followed and analyzed as described previously (Peluso *et al*., 2001). The general procedures for characterizing the basal and stimulated GTPase reactions between SRP and SR have been described in detail previously (Peluso *et al*., 2001; Shan and Walter, 2003, 2005), and they are summarized briefly here. The justification for how each microscopic rate constant was derived from these measurements is provided in Supplemental Material.

Basal GTPase or XTPase activities of cpSRP54, cpFtsY, and cpFtsY(D283N) were measured in single turnover reactions as described previously ([GTP] ≪ [E]; Peluso *et al*., 2001). The dependence of the observed rate constant (k_{obsd}) on protein concentration were fit to Eq. 1,

$$k_{obsd} = k_{max} \times \frac{[protein]}{K_{1/2} + [protein]} \quad (1)$$

in which k_{max} is the maximal rate constant at saturating protein concentrations, and $K_{1/2}$ is the protein concentration required to reach half the maximal rate.

The nucleotide affinities of the GTPases were determined using several independent methods. The GTP affinities for cpSRP54 and cpFtsY and the XTP (xanthosine-5′-triphosphate) affinity for cpFtsY(D283N) were obtained from the $K_{1/2}$ values from fits of the basal GTPase or XTPase reactions to Eq. 1. Because the chemical step is rate limiting for the basal GTPase and XTPase reactions, $K_{1/2}$ is equal to K_d, the dissociation constant of the nucleotide. The affinities of GDP, 5′-guanylylimido-diphosphate (GppNHp), xanthosine-5′-diphosphate (XDP), and 5′-xanthylylimido-diphosphate (XppNHp) were determined using these nucleotides as inhibitors of the basal GTPase or XTPase reactions (Peluso *et al*., 2001). With subsaturating protein, the inhibition constant, K_i, is equal to K_d. Finally, the binding of nucleotides to the GTPases was determined directly by using fluorescent *N*-methyl-anthraniloyl (mant) derivatives of GTP, GDP, and XTP, as described below.

The reciprocally stimulated GTPase reaction between cpSRP54 and cpFtsY was determined in multiple turnover reactions ([GTP] ≫ [E]) in the presence of a small, fixed amount of cpSRP54 and varying concentrations of cpFtsY, using a GTP concentration that saturates both GTPase sites. The concentration dependence of the observed rate constant (k_{obsd}) is fit to Eq. 2,

$$k_{obsd} = k_{cat} \times \frac{[cpFtsY]}{K_m + [cpFtsY]} \quad (2)$$

in which k_{cat} is the rate constant at saturating cpFtsY concentrations, and K_m is the concentration of cpFtsY that gives half the maximal rate. The stimulated GTPase reaction between cpSRP54 and GTP-bound cpFtsY(D283N) was determined using the same experimental setup. The stimulated GTPase reaction of cpSRP54 by XTP-bound cpFtsY(D283N) was determined analogously, except that the concentration of GTP and XTP were adjusted such that cpSRP54 was predominantly occupied by GTP whereas cpFtsY(D283N) was predominantly occupied by XTP.

The cpFtsY(D283N)-stimulated GTP hydrolysis from cpSRP54 was also determined in single turnover experiments. The hydrolysis of trace GTP* was monitored in the presence of subsaturating cpSRP54 and varying amounts of cpFtsY(D283N), with 25 μM XTP present to selectively occupy the active site of cpFtsY(D283N). Under these conditions, the third-order reaction GTP* + cpSRP54 + $cpFtsY(D283N)^{\cdot XTP}$ → products was followed. The reciprocal reaction, XTP* + cpFtsY(D283N) + $cpSRP54^{\cdot GTP}$ → products was determined using an analogous setup, except that the concentration of cpSRP54 was varied, and 25 μM GTP was present to selectively occupy the active site of cpSRP54. The data were fit to Eq. 1 above. Finally, first-order rate constants of the stimulated GTP and XTP hydrolysis reactions from the ^{*GTP}cpSRP54•cpFtsY(D283N)$^{\cdot XTP*}$ complex were determined using high concentrations of both proteins (20–80 μM) in the presence of stoichiometric amounts of their respective nucleotides. The reaction time courses were

monitored in a quench flow apparatus (Kintek, Pittsford, NY) and fit to a single exponential rate equation to obtain the first-order rate constants.

The effect of XTP on the reaction ^{*GTP}cpSRP54 + cpFtsY(D283N)^{GTP*} → products was determined in the presence of subsaturating concentrations of both proteins and a high concentration of GTP (200 μM) to saturate both active sites. The XTP concentration dependence was fit to Eq. 3,

$$k_{obsd} = k_0 \times \frac{K_i^{app}}{K_i^{app} + [XTP]} + k_1 \times \frac{[XTP]}{[XTP] + K_i^{app}} \quad (3)$$

in which k_0 is the rate constant in the absence of any inhibitor, k_1 is the rate constant at infinite XTP concentrations, and K_i^{app} is the apparent inhibition constant of XTP determined from this experiment. K_i^{app} is related to the dissociation constant of XTP by Eq. 4,

$$K_i^{app} = K_d^{XTP} \times \left(1 + \frac{[GTP]}{K_d^{GTP}}\right) \quad (4)$$

in which K_d^{XTP} and K_d^{GTP} are the dissociation constants of XTP and GTP for cpFtsY(D283N), respectively.

Fluorescence

All fluorescence measurements were conducted at 25°C using the single photon counting Fluorolog 3–22 spectrofluorometer (Jobin Yvon, Edison, NJ). Fluorescence emission spectra of mant-derivatives of GTP, GDP, and XTP were acquired using an excitation wavelength of 356 nm. Nucleotide binding affinities were determined by recording the change in fluorescence intensity at 445 nm in the presence of 0.4 to 1 μM mant-nucleotides and increasing concentrations of cpSRP54, cpFtsY, or cpFtsY(D283N). The data were fit to Eq. 5,

$$F_{obsd} = (F_{max} - F_0) \times \frac{[protein]}{K_d + [protein]} + F_0 \quad (5)$$

in which F_{max} is the fluorescence at saturating protein concentrations, F_0 is the fluorescence in the absence of any protein, and K_d is the dissociation constant of the mant-nucleotide.

The rate constants for dissociation of mant-GTP and mant-GDP were determined using a pulse-chase experiment as described previously (Jagath *et al.*, 1998). The time course for decay of fluorescence was followed in a stopped flow apparatus (Applied Photophysics, Surrey, United Kingdom) and fit to single exponential functions to obtain the dissociation rate constants.

RESULTS

To understand why and how the cpSRP pathway bypasses the requirement for an SRP RNA, which plays a critical role in facilitating the interaction between the SRP and SR GTPases in all the other SRP pathways, we characterized the rate and equilibrium of the individual steps in the GTPase cycles of cpSRP54 and cpFtsY and their GTP-dependent interaction with one another (Figure 1). Each protein can bind and hydrolyze GTP by itself (steps 1–3 for cpSRP54 and 1′–3′ for cpFtsY). cpSRP54 forms a stable complex with cpFtsY when both proteins are bound with GTP (step 4). Both GTP molecules are rapidly hydrolyzed from the complex (step 5). GTP hydrolysis destabilizes the complex and drives its dissociation (step 6). The rate and equilibrium constants for each step are summarized in Table 1. For simplicity, additional possibilities such as hydrolysis of one of the GTPs followed by complex disassembly are not shown; these possibilities are presented in the *Discussion*.

Basal GTPase Cycles of cpSRP54 and cpFtsY

We first determined the basal GTP hydrolysis activities of the individual GTPases. Both proteins hydrolyze GTP slowly, with maximal hydrolysis rates of 0.017 and 0.0045 min^{-1} at saturating protein concentrations for cpSRP54 and cpFtsY, respectively (Figure 2). The protein concentration dependence of the hydrolysis rate gives the affinity of each protein for GTP. Both GTPases bind their substrates weakly, with dissociation constants of 2.8 and 2.1 μM for cpSRP54 and cpFtsY, respectively (Figure 2). We also determined the affinities of cpSRP54 and cpFtsY for GDP and the nonhydrolyzable GTP analogue GppNHp by using these nucleotides

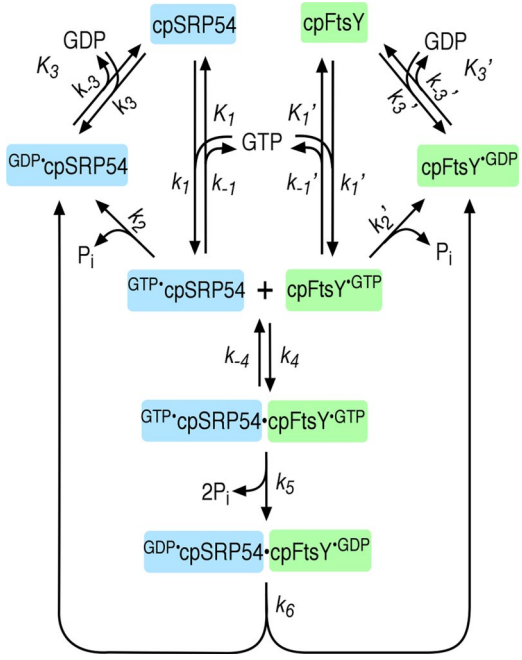

Figure 1. GTPase cycles of cpSRP54 (blue) and cpFtsY (green). The triangular cycles on the top left and right depict the basal GTPase cycles of cpSRP54 and cpFtsY, respectively. Binding of GTP to cpSRP54 (or cpFtsY) is characterized by the association rate constant k_1 (or k_1′), dissociation rate constant k_{-1} (or k_{-1}′), and equilibrium dissociation constant K_1 (or K_1′). GTP hydrolyzes from cpSRP54 and cpFtsY with rate constants of k_2 and k_2′, respectively. Binding of GDP to cpSRP54 (or cpFtsY) is characterized by the association rate constant k_3 (or k_3′), dissociation rate constant k_{-3} (or k_{-3}′), and equilibrium dissociation constant K_3 (or K_3′). Complex formation between cpSRP54 and cpFtsY is characterized by the association rate constant k_4 and dissociation rate constant k_{-4}. The two bound GTPs are hydrolyzed from the ^{GTP}cpSRP54•cpFtsY^{GTP} complex, represented collectively by the rate constant k_5. The ^{GDP}cpSRP54•cpFtsY^{GDP} complex then dissociates with a rate constant k_6.

Table 1. Rate and equilibrium constants for the GTPase cycle of cpSRP54 and cpFtsY

Equilibrium or rate constant[a]	Value determined
k_1	3.7×10^6 M^{-1} s^{-1}
k_{-1}	10.4 ± 0.8 s^{-1}
K_1	2.8 ± 0.4 μM
k_2	0.017 ± 0.002 min^{-1}
k_3	2.9×10^6 M^{-1} s^{-1}
k_{-3}	32 ± 2 s^{-1}
K_3	5.1 ± 0.3 μM
k_1′	2.6×10^6 M^{-1} s^{-1}
k_{-1}′	5.4 ± 0.3 s^{-1}
K_1′	2.1 ± 0.2 μM
k_2′	0.0045 ± 0.002 min^{-1}
k_3′	2.6×10^6 M^{-1} s^{-1}
k_{-3}′	8.1 ± 0.2 s^{-1}
K_3′	3.1 ± 0.2 μM
k_4	≥8.6 (± 0.3) × 10^5 M^{-1} s^{-1}
k_{-4}	N.D.
k_5	0.83 ± 0.04 s^{-1}
k_6	≥0.83 s^{-1}

N.D., not determined.

[a] The rate and equilibrium constants are defined in Figure 1 and were determined as described in *Materials and Methods*.

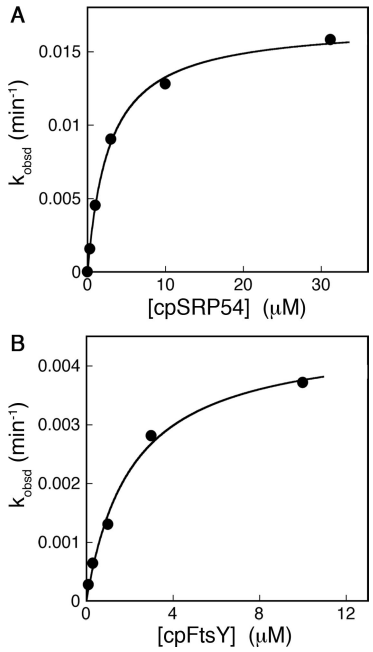

Figure 2. Basal GTPase reactions of cpSRP54 (A) and cpFtsY (B). The data were fit to Eq. 1 in *Materials and Methods* and gave a k_{max} of 0.017 min^{-1} and $K_{1/2}$ of 2.8 μM for cpSRP54, and a k_{max} of 0.0045 min^{-1} and $K_{1/2}$ of 2.1 μM for cpFtsY.

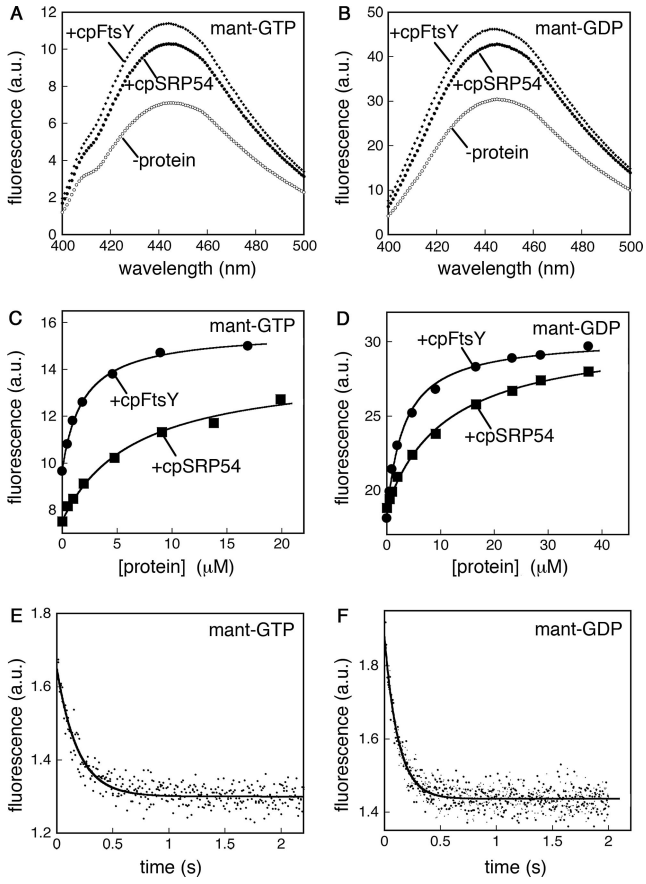

Figure 3. Interaction of nucleotides with cpSRP54 and cpFtsY. (A and B) Fluorescence emission spectra of mant-GTP (A) or mant-GDP (B) in the absence of protein (○) and in the presence of 5 μM cpSRP54 (●) or cpFtsY(◆). (C and D) Titration of the fluorescence changes of mant-GTP (C) and mant-GDP (D) in the presence of cpSRP54 (■) or cpFtsY (●). The data were fit to Eq. 5 in *Materials and Methods*, and the K_d values are summarized in Table 2. (E and F) Dissociation of mant-GTP (E) and mant-GDP (F) from cpFtsY. The data were fit to single exponential rate equations and gave dissociation rate constants of 5.4 and 8.1 s^{-1} for mant-GTP and mant-GDP, respectively.

as competitive inhibitors of the basal GTPase reactions. Both proteins bind GDP and GppNHp weakly, with inhibition constants in the micromolar range (Table 2).

We directly measured the interaction of nucleotides with both GTPases using fluorescent mant-derivatives of GTP and GDP. Binding of both GTPases to mant-GTP or mant-GDP induces a 50–80% increase in fluorescence (Figure 3, A and B, respectively). Titration of this fluorescence change as a function of protein concentration gave dissociation constants of 6.5 and 11 μM for binding of mant-GTP and mant-GDP to cpSRP54, respectively, and 1.9 and 3.1 μM for binding of mant-GTP and mant-GDP to cpFtsY, respectively (Figure 3, C and D; Table 2). For cpFtsY, these affinities are the same, within error, as those of unmodified nucleotides determined using the GTPase assay. For cpSRP54, these affinities are only approximately twofold larger than those of unmodified GTP and GDP. Thus, the mant-group does not significantly perturb the binding of nucleotides.

A hallmark of the SRP subgroup of GTPases is the fast rate at which they release and exchange nucleotides. The weak nucleotide binding affinities of cpSRP54 and cpFtsY suggest that this is also the case for the chloroplast SRP GTPases. This was confirmed by directly measuring the dissociation rate constants of mant-GTP and mant-GDP. As expected, both cpSRP54 and cpFtsY release mant-GTP quickly, with

Table 2. Nucleotide affinities of cpSRP54, wild-type cpFtsY, and mutant cpFtsY(D283N)

	K_d (μM)					
Protein	GTP	GDP	GppNHp	XTP	XDP	XppNHp
cpSRP54	2.8[a]/6.5[c]	5.1[b]/11[c]	26[b]	50[b]	N.D.	N.D.
cpFtsY wild-type	2.1[a]/1.9[c]	3.1[c]	4.6[b]	510[b]	557[b]	970[b]
cpFtsY(D283N)	76[b]	180[b]	360[b]	2.2[a]/2.7[c]	6.5[c]	34[b]

N.D., not determined.

[a] Determined by cpFtsY concentration dependences as described in *Materials and Methods.*

[b] Determined by inhibition methods as described in *Materials and Methods.*

[c] Determined by fluorescence using mant-nucleotides as described in *Materials and Methods.*

dissociation rate constants of 10.4 and 5.4 s^{-1}, respectively (Figure 3E and Table 1). Similarly, mant-GDP is released quickly by both cpSRP54 and cpFtsY, with dissociation rate constants of 32 and 8.1 s^{-1}, respectively (Figure 3F and Table 1). Thus, analogous to their bacterial and mammalian homologues, the chloroplast SRP GTPases hydrolyze GTP slowly and can exchange nucleotides quickly, in contrast to classical signaling GTPases that release nucleotide slowly (on the order of 10^{-3} – 10^{-4} s^{-1}) and that require external exchange factors to facilitate nucleotide release.

Interaction between cpSRP54 and cpFtsY Is Much More Efficient than Classical SRP Systems

In classical SRP systems, complex formation between the SRP and SR GTPases is very slow, and is accelerated 400-fold by the SRP RNA (Peluso *et al*., 2000, 2001). Once a complex is formed, SRP and SR stimulate each other's GTPase activity, and the rate of this stimulated GTPase reaction within the complex is also accelerated 5- to 10-fold by the SRP RNA (Peluso *et al*., 2001). Because no SRP RNA has been found in the chloroplast SRP system, we asked whether and how efficiently cpSRP54 and cpFtsY can interact with and activate each other in the absence of an SRP RNA.

To this end, we determined the rate of stimulated GTP hydrolysis reaction in the presence of both cpSRP54 and cpFtsY; GTPase activation in the cpSRP54•cpFtsY complex provides a means to monitor complex formation between the two GTPases (Peluso *et al*., 2000, 2001). To our surprise, cpSRP54 and cpFtsY interact with each other efficiently even in the absence of an SRP RNA (Figure 4, ●). The slope of the initial linear portion of the protein concentration dependence, which represents the rate constant of the reaction ^{GTP}cpSRP54 + cpFtsY^{GTP} → products (k_{cat}/K_m), is ~400-fold faster than that of the corresponding reaction between the *Escherichia coli* GTPases in the absence of the SRP RNA (Figure 4, ● vs. ▲). Indeed, this rate constant matches that of the *E. coli* GTPases in the presence of the 4.5S SRP RNA (Figure 4, ▼). The rate constant at saturating protein concentrations, which represents the rate of GTP hydrolysis within the ^{GTP}cpSRP54•cpFtsY^{GTP} complex, is also identical between the chloroplast and the *E. coli* GTPases in the presence of the SRP RNA (● vs. ▼), and eightfold faster than that of the *E. coli* GTPases without the RNA bound (▲).

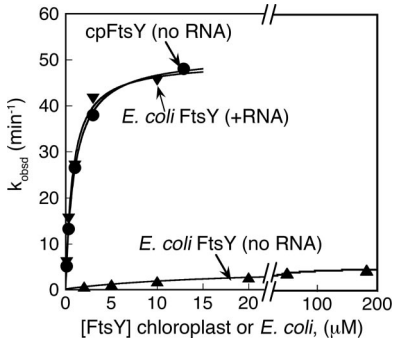

Figure 4. Interaction of cpSRP54 and cpFtsY is much more efficient than that of their *E. coli* homologues. Rates of the stimulated GTPase reaction were determined for 100 nM cpSRP54 and cpFtsY (●) or for 100 nM *E. coli* Ffh and *E. coli* FtsY with (▼) and without (▲) 4.5S SRP RNA. The data were fit to Eq. 2 in *Materials and Methods* and gave a k_{cat} value of 50 min^{-1} and a K_m value of 0.97 μM for the chloroplast GTPases, and k_{cat} values of 49 and 4.8 min^{-1} and K_m values of 0.76 and 18 μM for the *E. coli* GTPases with and without the SRP RNA, respectively.

In the *E. coli* SRP system, complex formation is rate limiting for the reaction ^{GTP}SRP + FtsY^{GTP} → products (both in the presence and absence of SRP RNA) (Peluso *et al*., 2001). Therefore, k_{cat}/K_m is equal to the association rate constant between the two GTPases. If this were also true for the cpSRP54 and cpFtsY, then the association between cpSRP54 and cpFtsY would be 400-fold faster than their *E. coli* homologues. Alternatively, k_{cat}/K_m is limited by the chemical step instead of complex formation for the chloroplast GTPases. If this were true, then the difference in association rates between the chloroplast and *E. coli* GTPases would be even greater. Thus, the results in Figure 4 demonstrate that complex formation between the chloroplast SRP and SR GTPases is much more efficient than that of their bacterial and mammalian homologues and therefore do not need the help from an SRP RNA.

cpFtsY Exhibits High Nucleotide Specificity

Association between bacterial SRP and SR GTPases is slow, presumably because significant domain rearrangements are required to form a stable complex, including a change from the open to the closed conformation that is manifested functionally as an increase in the nucleotide specificity of the *E. coli* FtsY (Shan and Walter, 2003; see *Introduction*). We hypothesized that the chloroplast SRP GTPases are preorganized in the closed conformation even in the absence of their binding partner, thus reducing the cost for the open → closed rearrangement and resulting in a faster rate of protein–protein interaction.

A prediction from this model is that cpFtsY can effectively discriminate between cognate and noncognate nucleotides by itself without the help from cpSRP54. To test this idea, we mutated the conserved specificity determinant Asp283 to an asparagine. This mutation converts many GTPases to XTP-specific proteins by swapping the hydrogen bond between the carboxylate oxygen of Asp and the exocyclic amino group of the guanine ring (Hwang and Miller, 1987; Weijland *et al*., 1994; Zhong *et al*., 1995; Bishop *et al*., 2000). As predicted, wild-type cpFtsY preferentially hydrolyzes GTP. The rate constant of the reaction: GTP* + FtsY → GDP + P_i* is 37-fold faster than that of mutant cpFtsY(D283N) (Figure 5A). Similarly, mutant cpFtsY(D283N) hydrolyzes XTP much faster than wild-type cpFtsY (Figure 5B). In contrast, *E. coli* FtsY exhibits no more than a fourfold difference between wild-type and mutant GTPases in the hydrolysis rates of either nucleotide (Shan and Walter, 2003).

We next asked whether cpFtsY can specifically bind its cognate nucleotide. Using both the GTPase assays (Figure 5, C and D) and fluorescent mant-nucleotides (Figure 5, E and F), we showed that wild-type cpFtsY preferentially binds guanine-based nucleotides, with affinities 40- to 70-fold higher than mutant cpFtsY(D283N) (Table 2). Analogously, mutant cpFtsY(D283N) preferentially binds xanthine-based nucleotides, with affinities 90- to 250-fold higher than wild-type cpFtsY (Table 2). In contrast, *E. coli* FtsY exhibits no more than a twofold discrimination between wild-type and mutant GTPases for any nucleotides (Shan and Walter, 2003). Together, the results in this section show that, unlike its bacterial homologue, the active site of cpFtsY can specifically recognize GTP even in the absence of cpSRP54. This is consistent with the notion that free cpFtsY is already in the closed conformation and pre-organized to interact with cpSRP54.

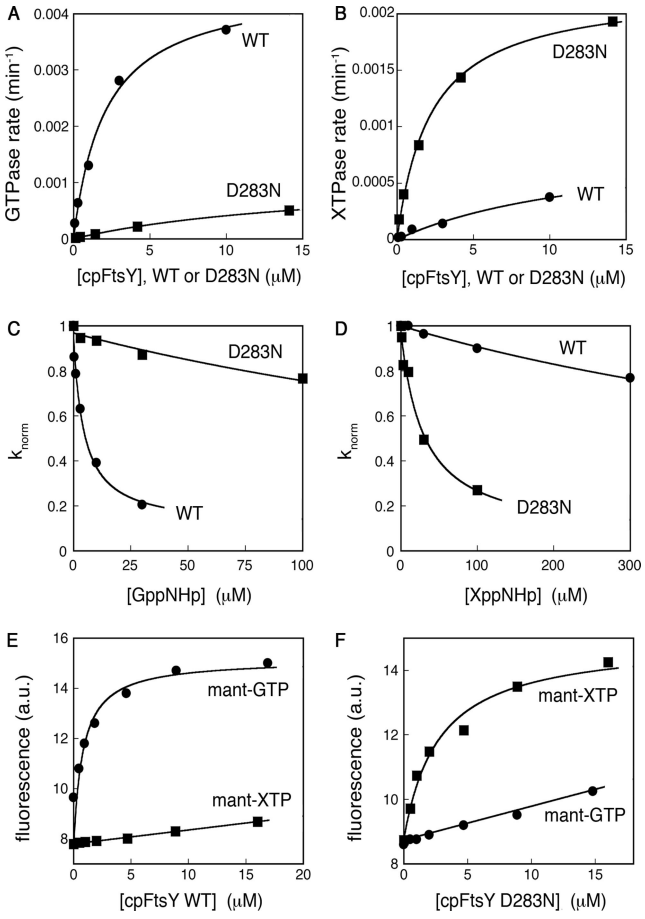

Figure 5. cpFtsY preferentially binds and hydrolyzes its cognate nucleotide. (A and B) Basal GTPase (A) and XTPase (B) reactions of wild-type cpFtsY (●) and mutant cpFtsY(D283N) (■). The data were fit to Eq. 1 and gave a k_{max} value of 0.0045 min^{-1} and a $K_{1/2}$ value of 2.1 μM for GTP hydrolysis by wild-type cpFtsY, and a k_{max} value of 0.0022 min^{-1} and a $K_{1/2}$ value of 2.2 μM for XTP hydrolysis by mutant cpFtsY(D283N). (C) GppNHp binds more strongly to wild-type cpFtsY (●) than to mutant cpFtsY(D283N) (■). (D) XppNHp binds more strongly to mutant cpFtsY(D283N) (■) than to wild-type cpFtsY (●). The K_i values are reported in Table 2. (E and F) Titration of the change in fluorescence of mant-GTP (●) and mant-XTP (■) upon binding to wild-type cpFtsY (E) and mutant cpFtsY(D283N) (F). The data were fit to Eq. 5, and the K_d values are summarized in Table 2.

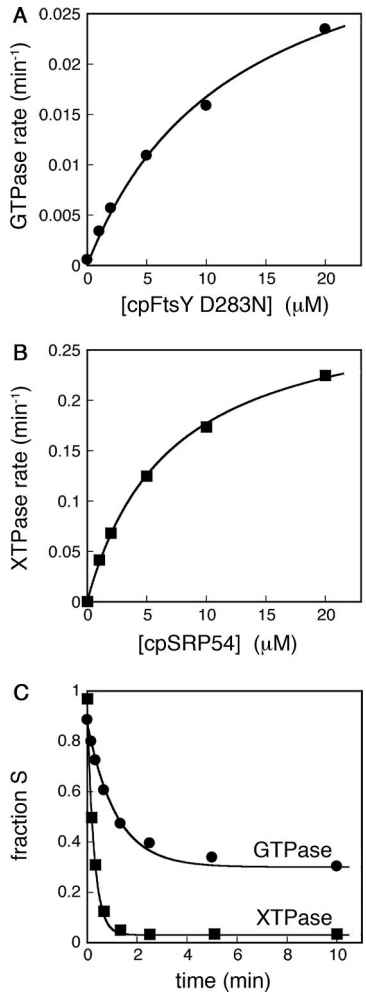

Figure 6. Nucleotide hydrolyses from the cpSRP54•cpFtsY(D283N) complex are asymmetric. (A) Stimulation of the GTPase reaction of cpSRP54 by cpFtsY(D283N), determined as described in *Materials and Methods* using 0.2 μM cpSRP54 and 20 μM XTP. The data were fit to Eq. 1 and gave a maximal rate constant of 0.037 min^{-1}. (B) Stimulation of the XTPase reaction of cpFtsY(D283N) by cpSRP54, determined as described in *Materials and Methods* using 0.2 μM cpFtsY(D283N) and 20 μM GTP. The data were fit to Eq. 1 and gave a maximal rate constant of 0.30 min^{-1}. (C) Time courses for GTP and XTP hydrolyses from the ^{GTP}•cpSRP54•cpFtsY(D283N)^{XTP} complex, determined as described in *Materials and Methods*. The data were fit to single exponential rate equations and gave rate constants of 0.86 and 3.7 min^{-1} for the GTPase (●) and XTPase (■) reactions, respectively.

GTPase Activation between cpSRP54 and cpFtsY Is Reciprocal but Asymmetric

The XTP-specific mutant cpFtsY(D283N) also allowed us to test whether cpSRP54 and cpFtsY reciprocally stimulate the GTPase activity of one another, as is the case for the bacterial system. If this were the case, XTP hydrolysis by cpFtsY(D283N) would be stimulated by cpSRP54, and, conversely, GTP hydrolysis by cpSRP54 would be stimulated by cpFtsY(D283N).

To examine the effect of cpFtsY(D283N) on GTP hydrolysis by cpSRP54, we measured the rate of GTP hydrolysis in the third-order reaction GTP* + cpSRP54 + D283N$^{\cdot XTP}$ → GDP + P_i*. As predicted, the rate of GTP hydrolysis is significantly stimulated by the presence of cpFtsY(D283N) (Figure 6A), consistent with the notion that cpFtsY acts as the activating protein for cpSRP54. Analogously, the reciprocal reaction, XTP hydrolysis by cpFtsY(D283N), is significantly stimulated by the presence of cpSRP54 (Figure 6B; the third-order reaction: XTP* + D283N + cpSRP54$^{\cdot GTP}$ → XDP + P_i* was followed).

Interestingly, the rate of stimulated GTP hydrolysis from cpSRP54 is ~10-fold slower than that of XTP hydrolysis from cpFtsY(D283N) (cf. rates in Figure 6, A and B), raising the possibility that nucleotide hydrolyses from the two GTPase sites in the complex are not symmetric. To test this possibility, we formed the ^{GTP}•cpSRP54•cpFtsY(D283N)$^{\cdot XTP}$ complex by using high concentrations of both proteins and stoichiometric amounts of GTP and XTP, and directly measured the rate constants for hydrolysis of both GTP and XTP from this complex. As shown in Figure 6C, the rate constant for XTP hydrolysis is 3.7 min^{-1} (squares), over fourfold faster than the rate constant of 0.87 min^{-1} for GTP hydrolysis (circles). This represents only a lower limit for the difference in hydrolysis rates between the two active sites, because cpFtsY(D283N) bound

with GTP is much more active in binding and activating cpSRP54 (see the next section), even though it preferentially binds XTP by itself. Thus, part of the GTP hydrolysis rate observed in Figure 6C (circles) is contributed by an alternative ^{GTP}cpSRP54•cpFtsY(D283N)^{GTP} complex. The actual difference between the hydrolysis rates from the two active sites is larger than that observed in Figure 6C and is closer to the ~10-fold difference observed in Figure 6, A and B. This is because the experiments in Figure 6, A and B, measure the rate of third-order reactions, which is determined by the affinities of free cpSRP54 and cpFtsY(D283N) for their respective nucleotides as well as the rates at which GTP and XTP are hydrolyzed from the respective active sites in the complex. Because cpSRP54 and cpFtsY(D283N) exhibit similar affinities for GTP and XTP, respectively (Table 2), the ~10-fold difference in the observed reaction rate (Figure 6, A and B) primarily reflects the difference in hydrolysis rate from the two active sites. Thus, like the classical SRP systems, cpSRP54 and cpFtsY act as reciprocal activating proteins for one another, yet unlike their bacterial homologues, nucleotide hydrolyses from the two active sites are asymmetric.

Mutant cpFtsY(D283N) Prefers GTP over XTP upon Complex Formation with cpSRP54

Another intriguing observation from the results in Figure 6C is that the rate constants of the stimulated GTPase and XTPase reactions from the ^{GTP}cpSRP54•cpFtsY(D283N)^{XTP} complex (0.87 and 3.7 min^{-1}, respectively) are over 10-fold slower than that from the wild-type ^{GTP}cpSRP54•cpFtsY^{GTP} complex (Figure 4), even accounting for the fact that two GTP molecules are hydrolyzed in the wild-type complex. Therefore, we suspected that the D283N mutation or the replacement of GTP with XTP renders cpFtsY less active in binding and activating cpSRP54. This is reminiscent of the behavior of an XTP-specific mutant of the *E. coli* SRP GTPase, SRP(D251N), which is deficient in binding and activating FtsY in its XTP-bound form. Instead, mutant SRP(D251N) can better bind and activate FtsY when bound to the noncognate GTP (Shan and Walter, 2005).

To test whether this is also the case for mutant cpFtsY(D283N), we measured the rate constant for GTP hydrolysis from the ^{GTP}cpSRP54•cpFtsY(D283N)^{GTP} complex when cpFtsY(D283N) is forced to bind its noncognate nucleotide by using a high GTP concentration. When mutant cpFtsY(D283N) is bound with the noncognate GTP, the rate of stimulated GTP hydrolysis is much faster than when it is bound with the cognate XTP (Figure 7A, diamonds vs. squares). The rate constant at saturating protein concentration, which represents the rate constant for GTP hydrolysis from the ^{GTP}cpSRP54•cpFtsY(D283N)^{GTP} complex, is comparable to that of the wild-type complex (Figure 7A; diamonds vs. circles), suggesting that the ^{GTP}cpSRP54•cpFtsY(D283N)^{GTP} complex achieves the same active conformation as the complex formed by the wild-type proteins. Because a fivefold higher concentration of mutant cpFtsY(D283N) than wild-type cpFtsY is required to reach saturation, complex formation is modestly compromised for GTP-bound cpFtsY(D283N) (Figure 7A; diamonds vs. circles). In contrast, no saturation is observed in the reaction with XTP-bound cpFtsY(D283N) up to 30 μM (squares), indicating that complex formation is significantly compromised when the mutant is bound with its cognate nucleotide. Thus, mutant cpFtsY(D283N) prefers the noncognate GTP over cognate XTP when it forms a complex with cpSRP54.

To provide independent evidence on this switch in nucleotide preference upon complex formation, we explored the effect of XTP on the rate of the reaction: ^{GTP}cpSRP54 + cpFtsY(D283N)^{GTP} → products. If cpFtsY(D283N) is less active in binding and activating the GTPase reaction of cpSRP54 when it is bound with cognate XTP than with noncognate GTP, then addition of XTP, which competes off the GTP bound at the active site of cpFtsY(D283N), should inhibit the stimulated GTPase reaction. As predicted, addition of XTP inhibits this stimulated reaction (Figure 7B). The observed inhibition constant for XTP is 9.0 μM, consistent with the expected value of 8.9 ± 0.9 μM given the affinities of mutant cpFtsY(D283N) for GTP and XTP and the GTP concentration used in this experiment (Eq. 4 in *Materials and Methods*). This strongly suggests that the binding of XTP to cpFtsY(D283N) is responsible for the observed inhibitory effect. Together, the results in this section show that although cpFtsY(D283N) by itself exhibits a specificity for XTP, this mutant prefers the noncognate GTP for interacting with and stimulating GTP hydrolysis from cpSRP54. Thus, Asp283 and/or the bound GTP play a much more important role than specifying the nucleotide preference of cpFtsY and likely participate in critical interface interactions with cpSRP54 in the cpSRP54•cpFtsY complex.

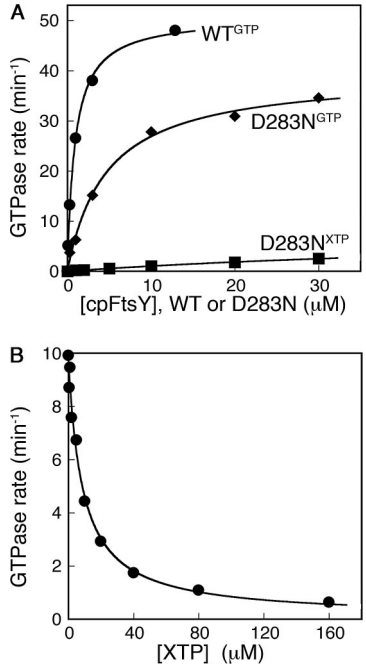

Figure 7. cpFtsY(D283N) prefers GTP over XTP when it forms a complex with cpSRP54. (A) GTP hydrolysis rates when 100–500 nM cpSRP54 interacts with wild-type cpFtsY (●), cpFtsY(D283N) bound to GTP (◆) and cpFtsY(D283N) bound to XTP (■). The following nucleotide concentrations were used: 100 μM GTP for reaction with wild-type cpFtsY, 200 μM GTP for reaction with cpFtsY(D283N) bound to GTP, and 20 μM GTP and 50 μM XTP for reaction with cpFtsY(D283N) bound to XTP. The data were fit to Eq. 2, which gave k_{cat} values of 50 (●) and 39 min^{-1} (◆). (B) XTP inhibits the ability of GTP-bound cpFtsY(D283N) to stimulate GTP hydrolysis by cpSRP54. Reactions were carried out in the presence of 500 nM cpSRP54, 2 μM cpFtsY(D283N) and 200 μM GTP, as described in *Materials and Methods*. The data were fit to Eq. 3 and gave an apparent inhibition constant of 9.0 μM.

DISCUSSION

The Chloroplast SRP and SR GTPases Are Preorganized to Efficiently Interact with Each Other

cpSRP54 and cpFtsY share 69.5 and 65.4% similarity with their *E. coli* homologues. All the essential motifs in the GTP

binding pocket are highly conserved. As expected from the high sequence conservation, both proteins share many biochemical features characteristic of the SRP subfamily of GTPases, including weak nucleotide affinities, fast nucleotide exchange rates, and the ability to reciprocally stimulate each other's GTPase reaction after they form a complex.

Given these similarities, it is surprising that the otherwise universally conserved SRP RNA, which plays a crucial role in eukaryotic and prokaryotic SRP protein targeting pathways, is missing in the chloroplast SRP pathway. In *E. coli*, association between the SRP and SR GTPases is extremely slow, with a rate constant of 5×10^3 M^{-1} s^{-1} (Peluso *et al*., 2001). This slow association rate does not seem to be caused by the extended A-domain of *E. coli* FtsY, because *Thermus aquaticus* FtsY, which lacks an extended A-domain, also interacts with its binding partner very slowly (Shepotinovskaya and Freymann, 2001). At this rate and the in vivo concentration of these GTPases (nanomolar range), the association between the two GTPases will take hours to complete. The protein targeting reaction, however, occurs on a much faster time scale. A recent report shows that thioredoxin, a small protein with only 105 amino acids, is targeted by the SRP when an appropriate signal sequence is attached (Huber *et al*., 2005). Because it takes only 3–5 s for complete synthesis of the thioredoxin chain, SRP-dependent protein targeting must occur in <3 s. Thus, the slow interaction kinetics between the SRP and SR GTPases is inappropriate for the targeting reaction. The SRP RNA overcomes this problem by accelerating the association between the two GTPases 400-fold (Peluso *et al*., 2000, 2001). Another contribution of the SRP RNA is to increase the rate of GTP hydrolysis in the SRP•SR complex by 5- to 10-fold (Peluso *et al*., 2001); GTP hydrolysis is known to drive disassembly and recycling of the SRP and SR (Wilson *et al*., 1988; Connolly *et al*., 1991). Here, we showed that cpSRP54 and cpFtsY can interact efficiently with each other even in the absence of an SRP RNA: their association rate is at least as fast as that of their *E. coli* homologues that contain the SRP RNA, and GTP hydrolysis from the cpSRP54•cpFtsY complex also occurs at the same rate as the *E. coli* GTPase complex in the presence of the RNA. This helps explain how the chloroplast SRP system can bypass the requirement for the SRP RNA.

Why is the protein–protein interaction so efficient between the chloroplast GTPases? Interaction between the bacterial SRP and SR GTPases is slow, presumably due to the requirement for extensive conformational changes during complex formation. One of the important rearrangements is a repositioning of the N–G domain interface, which led to a change of the GTPase site from a floppy, nonspecific open state to a closed state in which active site interactions with the bound nucleotide are established (Shan and Walter, 2003). Thus, one possibility is that cpSRP54 and cpFtsY are preorganized into the closed conformation that is ready to interact with each other. The results herein strongly suggest that this is the case at least for cpFtsY. Free cpFtsY can specifically recognize its cognate nucleotide, in contrast to *E. coli* FtsY, which acquires nucleotide specificity only when it forms a complex with SRP. Furthermore, cpFtsY exhibits higher affinities for GTP and GDP than its bacterial homologues, with dissociation constants of 2–3 μM instead of 19–30 μM for *E. coli* FtsY. These observations strongly support the notion that free cpFtsY is preorganized in a closed conformation, and thus can interact with cpSRP54 without paying substantial energetic penalty to rearrange the relative position of the N and G domains. It remains to be seen whether cpSRP54 is similarly preorganized into the closed conformation before interaction with cpFtsY.

It seems that the SRP RNA has been evolved to accelerate the very inefficient interaction between the SRP and SR GTPases in classical SRP pathways. Although models are abundant (Peluso *et al*., 2000; Buskiewicz *et al*., 2005; Spanggord *et al*., 2005), the molecular mechanism by which the SRP RNA acts as a catalyst to accelerate both the formation and disassembly of the SRP•SR complex is still poorly understood. It is possible that in the transition state for complex assembly, the SRP RNA may provide a transient tether that facilitates the rearrangement of one or both GTPases into the closed conformation; alternatively, the RNA and the chloroplast GTPases may use completely different mechanisms to attain a faster association kinetics.

Asymmetic Nucleotide Hydrolysis from the cpSRP54•cpFtsY Complex

The crystal structure of the *T. aquaticus* Ffh•FtsY complex shows that the two GMPPCP molecules are bound at a composite active site formed at the dimer interface (Egea *et al*., 2004; Focia *et al*., 2004). Consistent with the composite nature of the active site and the extensive degree of cross-talk between the two GTPase sites, the two nucleotides are hydrolyzed at the same rate from the *E. coli* SRP•FtsY complex. These observations have led to earlier proposals of concerted GTP hydrolysis in the SRP•SR complex (Powers and Walter, 1995). In contrast to this notion, we showed here that nucleotide hydrolysis in the cpSRP54•cpFtsY complex can be asymmetric, with the nucleotide hydrolyzing ~10-fold faster from the cpFtsY than the cpSRP54 active site. This observation argues against a concerted mechanism. Even in the *E. coli* system, mutant GTPases have been identified in which GTP is hydrolyzed much faster from the SRP than the FtsY active site (Shan *et al*., 2004). Furthermore, when either of the GTPases is bound with a nonhydrolyzable GTP analogue, it can still activate efficient GTP hydrolysis on its binding partner (Shan, S., unpublished results). Together, these results strongly suggest that hydrolyses of the two GTPs in the SRP•SR complex do not proceed through a concerted mechanism or an ordered pathway (i.e., one GTP must be hydrolyzed first before hydrolysis of the second GTP can occur). Rather, each active site can hydrolyze its bound GTP independently.

Even though the nucleotide is hydrolyzed ~10-fold slower from cpSRP54 than from cpFtsY(D283N), multiple rounds of XTP hydrolysis from the cpSRP54•cpFtsY(D283N) complex is not blocked and occur as efficiently as single turnover reactions (data not shown). Thus, disassembly of the complex must occur on a faster time scale than the second hydrolysis event, implying that SRP and SR can dissociate from one another even when only one of the nucleotides is hydrolyzed. A similar observation was made for the *E. coli* SRP•SR complex (Shan *et al*., 2004). Together, the data from the *E. coli* and chloroplast systems suggest that only one GTP hydrolysis event is required to drive disassembly of the SRP•SR complex. It remains to be clarified how many GTPs need to be hydrolyzed during each round of protein targeting, and what the precise role of each GTP hydrolysis event is.

The Nucleotide Specificity Determinant of cpFtsY, Asp283, Mediates Molecular Cross-Talk between the Two GTPases

Given the high specificity of cpFtsY(D283N) for XTP, it is surprising to find that this mutant prefers the noncognate GTP over the cognate XTP when it forms a complex with cpSRP54. This strongly suggests that Asp283, in addition to conferring nucleotide specificity to cpFtsY, also contributes to interactions at the dimer interface. The behavior of cpFtsY(D283N) is reminiscent of an XTP-specific mutant of

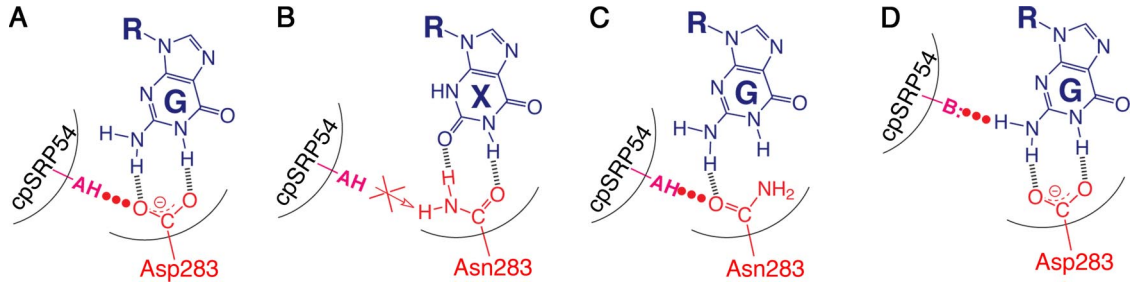

Figure 8. Model for the interactions of cpSRP54 with the side chain of cpFtsY Asp283 or with GTP. (A–C) Proposed interactions between the side chain of residue 283 with a hydrogen bond donor from cpSRP54 (-AH) for the wild-type cpSRP54•cpFtsY complex (A) and the cpSRP54•cpFtsY(D283N) complex with XTP (B) or GTP (C) bound to cpFtsY(D283N). (D) The GTP bound to cpFtsY interacts with a hydrogen bond acceptor (-B:) from cpSRP54.

the *E. coli* SRP GTPase, Ffh(D251N), which also prefers GTP over XTP when it forms a complex with FtsY (Shan and Walter, 2005). The crystal structure confirms that Asp251 makes an important interface contact with Lys390 from FtsY (Egea *et al.*, 2004; Focia *et al.*, 2004). A similar interaction could be formed by Asp283 of cpFtsY with a hydrogen bond donor (−AH) at the interface of the cpSRP54•cpFtsY complex (Figure 8A). When cpFtsY(D283N) is bound to XTP, mutation of Asp283 to Asn destroys this interface contact and compromises the interaction between the two GTPases (Figure 8B). In contrast, replacement of XTP with GTP no longer constrains Asn283 in this particular configuration; a rotation around the C^{β}–C^{γ} bond can reposition the carbonyl oxygen of Asn283 close to the hydrogen bond donor from cpSRP54, thus restoring this interface contact (Figure 8C). Alternatively, the exocyclic amino group of GTP could directly interact with a hydrogen bond acceptor from cpSRP54 (Figure 8D, -B:), therefore, replacement of GTP with XTP compromises the cpSRP54–cpFtsY(D283N) interaction. In either scenario, our results map the G-IV motif of cpFtsY and its bound nucleotide to the dimer interface between the two GTPases, and demonstrate the presence of extensive cross-talk between the two GTPase sites.

Perspective

The results here help rationalize why the chloroplast SRP targeting pathway bypasses the requirement for the SRP RNA, because the SRP and SR GTPases from chloroplast can interact efficiently with one another without the help from the RNA. The novel cpSRP43 protein, which together with cpSRP54 forms the chloroplast SRP, has often been viewed as a functional replacement for the SRP RNA. Our results show that the chloroplast GTPases have evolved to efficiently interact with one another. Although a previous study has suggested a moderate effect of cpSRP43 on the stimulated GTPase reaction between cpSRP54 and cpFtsY (Goforth *et al.*, 2004), a more thorough kinetic analysis showed that the cpSRP43 protein does not provide additional acceleration of the interaction between these two chloroplast GTPases (Supplemental Figure 2). Therefore, cpSRP43 does not replace all of the functions of the SRP RNA. This novel chloroplast protein may have evolved to mediate other important roles of the SRP RNA in the protein targeting reaction, such as recognition of the cargo protein (Schuenemann *et al.*, 1998; Tu *et al.*, 2000). Analogously, the SRP RNA may have been evolved to interact with ribosomal RNAs during cotranslational protein targeting in the classical SRP pathways (Rinke-Appel *et al.*, 2002; Halic *et al.*, 2004, 2006; Schaffitzel *et al.*, 2006).

It is fascinating to speculate on the evolutionary origin of the vast difference in the interaction kinetics between the SRP and SR GTPases from chloroplast versus those from classical SRP pathways, and why cells have evolved the SRP RNA to deal with the inefficient interaction between the SRP and SR GTPases in the classical pathway. An intriguing possibility is that the slow interaction kinetics between the SRP and SR GTPases in classical pathways provides additional opportunities for regulation and for improving fidelity. Compared with the time scale of protein targeting, the interaction kinetics between SRP and SR is still relatively slow even with the RNA present. The ribosome and/or the cargo protein, however, further accelerate the interaction between the two GTPases (Zhang, X., and Shan, S., unpublished data). The SRP RNA, bound in vicinity to the signal-sequence binding site, could mediate this additional stimulation of the SRP–SR interaction in response to cargo binding. In this way, the SRP RNA potentially provides a checkpoint to improve the fidelity of the classical SRP pathway, which needs to sort a diverse array of cellular proteins and to recognize signal sequences with different amino acid composition. In contrast, a much smaller number of proteins needs to be sorted inside the stroma of chloroplast, and the posttranslational cpSRP pathway seems to be dedicated for delivery of the LHCP family of proteins to the thylakoid membrane. More specific binding interactions between cpSRP and its cargo protein can be established to ensure the fidelity of the cpSRP pathway, and this alleviates the need to build in additional fidelity checkpoints by using the SRP RNA as a kinetic regulator.

ACKNOWLEDGMENTS

We thank Dr. Ralph Henry (University of Arkansas) for the bacterial expression vectors for cpSRP54 and cpFtsY, Dr. Judith Campbell and the rest of the Shan laboratory for comments on the manuscript, and Dr. Peter Walter for insightful discussions and intellectual support. S.S. was supported by career awards from the Burroughs Wellcome Fund and the Camile and Henry Dreyfus Foundation. P.J.A. is supported by the Bray fellowship.

REFERENCES

Bishop, A. *et al.* (2000). Unnatural Ligands for engineered proteins: new tools for chemical genetics. Annu. Rev. Biophys. Biomol. Struct. *29*, 577–606.

Buskiewicz, I., Kubarenko, A., Peske, F., Rodnina, M. V., and Wintermeyer, W. (2005). Domain rearrangement of SRP protein Ffh upon binding 4.5S RNA and the SRP receptor FtsY. RNA *11*, 947–957.

Connolly, T., Rapiejko, P. J., and Gilmore, R. (1991). Requirement of GTP hydrolysis for dissociation of the signal recognition particle from its receptor. Science *252*, 1171–1173.

Egea, P. F., Shan, S., Napetschnig, J., Savage, D. F., Walter, P., and Stroud, R. M. (2004). Substrate twinning activates the signal recognition particle and its receptor. Nature *427*, 215–221.

Focia, P. J., Shepotinovskaya, I. V., Seidler, J. A., and Freymann, D. M. (2004). Heterodimeric GTPase core of the SRP targeting complex. Science *303*, 373–377.

Franklin, K. E., and Hoffman, N. E. (1993). Characterization of a chloroplast homologue of the 54-kDa subunit of the signal recognition particle. J. Biol. Chem. *268*, 22175–22180.

Freymann, D. M., Keenan, R. J., Stroud, R. M., and Walter, P. (1997). Structure of the conserved GTPase domain of the signal recognition particle. Nature *385*, 361–364.

Freymann, D. M., Keenan, R. J., Stroud, R. M., and Walter, P. (1999). Functional changes in the structure of the SRP GTPase on binding GDP and Mg^{2+}GDP. Nat. Struct. Biol. *6*, 793–801.

Gawronski-Salerno, J., Coon, Y.J.S., Focia, P. J., and Freymann, D. M. (2006). X-ray structure of the *T. Aquaticus* Ftsy: GDP complex suggests functional roles for the C-terminal helix of the SRP GTPases. Proteins *66*, 984–995.

Gilmore, R., Blobel, G., and Walter, P. (1982a). Protein translocation across the endoplasmic reticulum: I. Detection in the microsomal membrane of a receptor for the signal recognition particle. J. Cell Biol. *95*, 463–469.

Gilmore, R., Walter, P., and Blobel, G. (1982b). Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. J. Cell Biol. *95*, 470–477.

Goforth, R. L., Peterson, E. C., Yuan, J., Moore, M. J., Kight, A. D., Lohse, M. B., Sakon, J., and Henry, R. L. (2004). Regulation of the GTPase cycle in post-translational signal recognition particle-based protein targeting involves cpSRP43. J. Biol. Chem. *279*, 43077–43084.

Gorlich, D., Prehn, S., Hartmann, E., Kalies, K. U., and Rapoport, T. A. (1992). A mammalian homolog of Sec61p and SecYp is associated with ribosomes and nascent polypeptides during translocation. Cell *71*, 489–503.

Halic, M., Becker, T., Pool, M. R., Spahn, C.M.T., Grassucci, R. A., Frank, J., and Beckmann, R. (2004). Structure of the signal recognition particle interacting with the elongation-arrested ribosome. Nature *427*, 808–814.

Halic, M., Blau, M., Becker, T., Mielke, T., Poll, M. R., Wild, K., Sinning, I., and Beckmann, R. (2006). Following the signal sequence from ribosomal tunnel exit to signal recognition particle. Nature *444*, 507–511.

Huber, D., Boyd, D., Xia, Y., Olma, M. H., Gerstein, M., and Beckwith, J. (2005). Use of thioredoxin as a reporter to identify a subset of *Escherichia coli* signal sequences that promote signal recognition particle-dependent translocation. J. Bacteriol. *187*, 2983–2991.

Hwang, Y. W., and Miller, D. L. (1987). A mutation that alters the nucleotide specificity of elongation factor Tu, a GTP regulatory protein. J. Biol. Chem. *262*, 13081–13085.

Jagath, J. R., Rodnina, M. V., and Wintermeyer, W. (2000). Conformational changes in the bacterial SRP receptor FtsY upon binding of guanine nucleotides and SRP. J. Mol. Biol. *295*, 745–753.

Jagath, J. R., Rodnina, M. V., Lentzen, G., and Wintermeyer, W. (1998). Interaction of guanine nucleotides with the signal recognition particle from *Escherichia coli*. Biochemistry *37*, 15408–15413.

Keenan, R. J., Freymann, D. M., Stroud, R. M., and Walter, P. (2001). The signal recognition particle. Annu. Rev. Biochem. *70*, 755–775.

Li, X., Henry, R., Yuan, J., Cline, K., and Hoffman, N. (1995). A chloroplast homologue of the signal recognition particle subunit SRP54 is involved in the posttranslational integration of a protein into thylakoid membranes. Proc. Natl. Acad. Sci. USA *92*, 3789–3793.

Lu, Y., Qi, H.-Y., Hyndman, J. B., Ulbrandt, N. D., Teplyakov, A., Tomasevic, N., and Bernstein, H. D. (2001). Evidence for a novel GTPase priming step in the SRP protein targeting pathway. EMBO J. *20*, 6724–6734.

Montoya, G., Svensson, C., Luirink, J., and Sinning, I. (1997). Crystal structure of the NG domain from the signal recognition particle receptor FtsY. Nature *385*, 365–368.

Moser, C., Mol, O., Goody, R. S., and Sinning, I. (1997). The signal recognition particle receptor of *Escherichia coli* (FtsY) has a nucleotide exchange factor built into the GTPase domain. Proc. Natl. Acad. Sci. USA *94*, 11339–11344.

Padmanabhan, W., and Freymann, D. M. (2001). The conformation of bound GMPPNP suggests a mechanism for gating the active site of the SRP GTPase site. Structure *9*, 859–863.

Peluso, P., Herschlag, D., Nock, S., Freymann, D. M., Johnson, A. E., and Walter, P. (2000). Role of 4.5S RNA in assembly of the bacterial signal recognition particle with its receptor. Science *288*, 1640–1643.

Peluso, P., Shan, S., Nock, S., Herschlag, D., and Walter, P. (2001). Role of SRP RNA in the GTPase cycles of Ffh and FtsY. Biochemistry *40*, 15224–15233.

Poritz, M. A., Strub, K., and Walter, P. (1988). Human SRP RNA and *E. coli*. 4.5S RNA contain a highly homologous structural domain. Cell *55*, 4–6.

Powers, T., and Walter, P. (1995). Reciprocal stimulation of GTP hydrolysis by two directly interacting GTPases. Science *269*, 1422–1424.

Rinke-Appel, J., Osswald, M., Knoblauch, K., Mueller, F., Brimacombe, R., Sergiev, P., Avdeeva, O., Bogdanov, A., and Dontsova, O. (2002). Crosslinking of 4.5S RNA to the *Escherichia coli* ribosome in the presence or absence of the protein Ffh. RNA *8*, 612–625.

Schaffitzel, C., Oswald, M., Berger, I., Ishikawa, T., Abrahams, J. P., Koerten, H. K., Koning, R. I., and Ban, N. (2006). Structure of the *E. coli* signal recognition particle bound to a translating ribosome. Nature *444*, 503–506.

Schuenemann, D., Gupta, S., Persello-Cartieaux, F., Klimyuk, V. I., Jones, J.D.G., Nussaume, L., and Hoffman, N. E. (1998). A novel signal recognition particle targets light-harvesting proteins to the thylakoid membranes. Proc. Natl. Acad. Sci. USA *95*, 10312–10316.

Shan, S., and Walter, P. (2003). Induced Nucleotide Specificity in a GTPase. Proc. Natl. Acad. Sci. USA *100*, 4480–4485.

Shan, S., and Walter, P. (2005). Molecular crosstalk between the nucleotide specificity determinant of the SRP GTPase and the SRP receptor. Biochemistry *44*, 6214–6222.

Shan, S., Stroud, R., and Walter, P. (2004). Mechanism of association and reciprocal activation of two GTPases. Plos Biol. *2*, e320.

Shepotinovskaya, I. V., and Freymann, D. M. (2001). Conformational change of the N-domain on formation of the complex between the GTPase domains of *Thermus aquaticus* Ffh and FtsY. Biochem. Biophys. Acta *1597*, 107–114.

Simon, S. M., and Blobel, G. (1991). A protein-conducting channel in the endoplasmic reticulum. Cell *65*, 371–380.

Spanggord, R. J., Siu, F., Ke, A., and Doudna, J. A. (2005). RNA-mediated interaction between the peptide-binding and GTPase domains of the signal recognition particle. Nat. Struct. Mol. Biol. *12*, 1116–1122.

Struck, C.R.J., Toschka, H. Y., Specht, T., and Erdmann, V. A. (1988). Common structural features between eukaryotic 7SL RNAs, eubacterial 4.5S RNA and scRNA and archaebacterial 7S RNA. Nucleic Acids Res. *16*, 7740–7746.

Tu, C.-J., Schuenemann, D., and Hoffman, N. E. (1999). Chloroplast FtsY, Chloroplast Signal Recognition Particle, and GTP are required to reconstitute the soluble phase of light-harvesting chlorophyll protein transport into thylakoid membranes. J. Biol. Chem. *274*, 27219–27224.

Tu, C. J., Peterson, E. C., Henry, R., and Hoffman, N. E. (2000). The L18 domain of light-harvesting chlorophyll proteins binds to chloroplast signal recognition particle 43. J. Biol. Chem. *275*, 13187–13190.

Walter, P., and Blobel, G. (1982). Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature *299*, 691–698.

Walter, P., and Blobel, G. (1983). Disassembly and reconstitution of signal recognition particle. Cell *34*, 525–533.

Walter, P., and Johnson, A. E. (1994). Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. Annu. Rev. Cell Biol. *10*, 87–119.

Walter, P., Ibrahimi, I., and Blobel, G. (1981). Translocation of proteins across the endoplasmic reticulum I. Signal Recognition Protein (SRP) binds to *in vitro* assembled polysomes synthesizing secretory protein. J. Cell Biol. *91*, 545–550.

Weijland, A., Parlato, G., and Parmeggiani, A. (1994). Elongation factor TuD138N, a mutant with modified substrate specificity, as a tool to study energy consumption in protein biosynthesis. Biochemistry *33*, 10711–10717.

Wilson, C., Connolly, T., Morrison, T., and Gilmore, R. (1988). Integration of membrane proteins into the endoplasmic reticulum requires GTP. J. Cell Biol. *107*, 69–77.

Yuan, J., Kight, A., Goforth, R. L., Moore, M., Peterson, E. C., Sakon, J., and Henry, R. (2002). ATP stimulates signal recognition particle (SRP)/FtsY-supported protein integration in chloroplasts. J. Biol. Chem. *277*, 32400–32404.

Zhong, X.-M., Chen-Hwang, M.-C., Hwang, Y. W. (1995). Switching nucleotide specificity of Ha-Ras p21 by a single amino acid substitution at aspartate 119. J. Biol. Chem. *270*, 10002–10007.