# The Ribosome Regulates the GTPase of the β-subunit of the Signal Recognition Particle Receptor

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*Abstract.* Protein targeting to the membrane of the ER is regulated by three GTPases, the 54-kD subunit of the signal recognition particle (SRP) and the α- and β-subunit of the SRP receptor (SR). Here, we report on the GTPase cycle of the β-subunits of the SR (SRβ). We found that SRβ binds GTP with high affinity and interacts with ribosomes in the GTP-bound state. Subsequently, the ribosome increases the GTPase activity of SRβ and thus functions as a GTPase activating protein for SRβ. Furthermore, the interaction between SRβ and the ribosome leads to a reduction in the affinity of SRβ for guanine nucleotides. We propose that SRβ regulates the interaction of SR with the ribosome and thereby allows SRα to scan membrane-bound ribosomes for the presence of SRP. Interaction between SRP and SRα then leads to release of the signal sequence from SRP and insertion into the translocon. GTP hydrolysis then results in dissociation of SR from the ribosome, and SRP from the SR.

Key words: signal recognition particle receptor • GTPase • ribosome • translocation • endoplasmic reticulum

TARGETING of nascent secretory and membrane proteins to the membrane of the ER involves the interaction between the signal recognition particle (SRP)[1] (Walter and Blobel, 1981) and SRP receptor (SR; also known as docking protein; Gilmore et al., 1982; Meyer et al., 1982). SRP contacts the signal sequence via its 54-kD subunit, mediates an arrest of nascent chain elongation, and interacts with the SR in the ER membrane (reviewed by Walter and Johnson, 1994; Lütcke, 1995). At the ER membrane, the signal sequence is released from SRP54 and is inserted into the channel of the translocon that is formed by the subunits of the Sec61p complex (Brundage et al., 1990; High and Dobberstein, 1991; Görlich and Rapoport, 1993; Mothes et al., 1994; for review see Corsi and Schekman, 1996; Matlack et al., 1998).

The targeting of nascent proteins to the ER membrane is regulated by three GTPases, SRP54, the 70-kD α-subunit (SRα), and the 30-kD β-subunit of SR (SRβ; Bernstein et al., 1989; Connolly and Gilmore, 1989; Römisch et al., 1989). GTPases bind and hydrolyze GTP and can exist in three states. Binding of GTP induces a conformational change that turns the GTPase into an active state. Hydrolysis of GTP to GDP switches this active state off. The empty state is usually an intermediate between the exchange of GDP for GTP. GTP hydrolysis and GTP binding can be regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), respectively (Bourne et al., 1991).

Initial work showed that the SR-dependent release of the signal sequence from SRP requires GTP (Connolly and Gilmore, 1989) and that the subsequent dissociation of SRP from the SR requires GTP hydrolysis (Connolly et al., 1991). Analysis of GTP binding and hydrolysis by individual GTPases or combinations revealed that free SRP only binds guanine nucleotides weakly. In addition, binding of SRP54 to free signal peptides further reduces the affinity for nucleotides (Miller et al., 1993). If, however, a signal sequence of a nascent polypeptide chain and the ribosome is contacted by SRP54, the affinity of SRP54 for GTP is increased (Bacher et al., 1996). The GTP-primed SRP ribosome–nascent chain complex (RNC) has a high affinity for SR in the ER membrane (Bacher et al., 1996). The GTP-bound form of the SRα contributes to the stabilization of the SRP–SR complex (Rapiejko and Gilmore, 1997). Signal sequence transfer from SRP54 to the translocon only occurs when both SRP54 and SRα are in their GTP-bound form (Rapiejko and Gilmore, 1992, 1997;

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1. *Abbreviations used in this paper:* GAP, GTPase activating protein; GEFs, guanine nucleotide exchange factors; PPL, preprolactin; RNC, ribosome–nascent chain complex; SR, signal recognition particle receptor; SRα, α-subunit of the SR; SRβ, β-subunit of the SR; SRP, signal recognition particle.

Bacher et al., 1996). GTP hydrolysis by SRP54 and SRα leads to the dissociation of SRP from its receptor (Rapiejko and Gilmore, 1997). Experiments with XTP-specific mutants of the procaryotic homologues of SRP54 (P48/Ffh) and SRα (FtsY) suggest that these proteins function as GAPs for each other (Powers and Walter, 1995).

Little is known about the function of SRβ and the regulation of its GTPase cycle. The β-subunit is a type I integral membrane protein associated with the membrane by an $NH_2$-terminal transmembrane segment (Miller et al., 1995). The COOH-terminal GTPase domain is exposed to the cytoplasmic side of the membrane. It is most closely related to the ARF and Sar1p subfamily of GTPases, which function in vesicular trafficking. The SRβ interacts with the $NH_2$-terminal domain of the SRα, and thereby anchors it to the membrane. The SRα can be released from the β-subunit by carbonate extraction at pH 12.5 (Miller et al., 1995) or mild proteolysis and high salt treatment (Meyer et al., 1982). Under the latter conditions, a 60-kD soluble fragment of the SRα is released into the cytosol. Work with the yeast homologue of SRβ has shown that the interaction between the two SR subunits is important for their function. Furthermore, it was found that a soluble form of SRβ is also functional (Ogg et al., 1998).

To investigate the GTPase cycle of the SRβ, we analyzed GTP binding and hydrolysis in the presence of RNCs or SRP and liposomes containing SR, Sec61p complex, or translocating chain-associating membrane (TRAM) protein. Our results suggest that the ribosome contacts SRβ in its GTP-bound state, stimulates GTP hydrolysis of SRβ, and leads to a release of SRβ-bound GDP.

## Materials and Methods

### Materials

General chemicals were from Merck or Sigma Chemical Co. α[$^{32}$P]GTP (3,000 Ci/mmol), [$^{35}$S]methionine, and the ECL system were purchased from Nycomed Amersham, Inc.

### Purification and Reconstitution of SR, TRAM Protein, and Sec61p Complex

SR was purified by immunoaffinity chromatography (Migliaccio et al., 1992; Görlich and Rapoport, 1993). Antibodies were raised in rabbits to a peptide corresponding to residues 137–150 of human SRα, coupled to keyhole limpet haemocyanin with sulphosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulpho-SMCC; Pierce Chemical Co.). The antibodies were affinity-purified against the immobilized peptide (Sulpholink gel; Pierce Chemical Co.) and then immobilized on protein A–Sepharose (Pharmacia Biotech, Inc.) with dimethylsubermidate. The affinity column was then used to purify SR from a digitonin extract of dog pancreas rough microsomes, essentially as described by Görlich and Rapoport (1993). The yield of SR was ~1 mg from 30,000 equivalence of rough microsomes.

Sec61p complex was purified from a ribosome-associated membrane protein fraction by ion-exchange chromatography according to Görlich and Rapoport (1993). TRAM protein was purified as described (Görlich et al., 1992a). Protein purity was assessed by 10–15% SDS PAGE (Laemmli, 1970) and silver staining (Heukeshoven and Dernick, 1988).

Detergent exchange of translocon components from digitonin to deoxy-BigCHAP, followed by reconstitution into proteoliposomes was performed as described (Görlich and Rapoport, 1993).

### Purification of SRP and RNCs

SRP was purified from a high salt extract of canine rough microsomes by gel filtration (Sephadex G-150), followed by ion-exchange chromatography (DEAE–Sepharose) according to Martoglio et al. (1998). SRP was then further purified by sucrose density centrifugation (Walter and Blobel, 1983).

RNC complexes bearing preprolactin 86mer nascent chains (PPL86) were synthesized in the wheat germ lysate translation system (Bacher et al., 1996). Translation was allowed to proceed for 10 min at 25°C in the presence of unlabeled amino acids. Further initiation of synthesis was blocked by the addition of $^7$methyl guanosine-5′-monophosphate ($^7$me-GMP) to 2 mM. After a further incubation at 25°C for 10 min, the RNCs were stabilized by the addition of 2 mM cycloheximide. Translation reactions were adjusted to 500 mM KOAc and then the RNCs isolated by centrifugation through a sucrose cushion (1 M sucrose, 25 mM Hepes-KOH, pH 7.8, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM cycloheximide, and 1 mM DTT) for 1 h at 400,000 *g* at 4°C. The RNCs were then resuspended in half the original volume of the translation reaction in HMC buffer (25 mM Hepes-KOH, pH 7.8, 5 mM $Mg(OAc)_2$, 1 mM cycloheximide) with 500 mM KOAc and treated with 10 mM NEM for 10 min at 25°C before addition of 20 mM DTT. The RNCs were then reisolated by centrifugation as before.

RNCs were finally resuspended in HMDC buffer (HMC with 1 mM DTT) and 150 mM KOAc at a concentration of 56 $OD_{260}$ units/ml.

### GTP Cross-linking Assay

SR, purified and reconstituted into liposomes (SR liposomes; Görlich and Rapoport, 1993), was mixed at 25 nM in the presence or absence of purified RNCs and/or purified SRP (5.6 $OD_{260}$/ml) with 0.5 μM α[$^{32}$P]GTP (3,000 Ci/mmol) or concentrations as indicated in 50 mM Tris-OAc, pH 7.8, 150 mM KOAc, 2 mM DTT, 5 mM $Mg(OAc)_2$, 2 mM cycloheximide. After incubation for 20 min on ice and 5 min at 25°C, the 10 μl reactions were transferred onto a silanized glass plate precooled on ice-cold metal blocks and irradiated with UV light at 4,000 W/cm$^2$ in a stratalinker™ for 5 min to cross-link the radiolabeled GTP to the proteins (Miller et al., 1993; Bacher et al., 1996). The solutions were then transferred to an Eppendorf tube and proteins precipitated with an equal volume of 20% TCA in the presence of 0.15% deoxycholic acid and 10 mM GTP. The pellet was washed with 10% TCA and with 80% acetone to remove uncross-linked radiolabeled nucleotides, and analyzed by 12.5% SDS-PAGE (Laemmli, 1970), followed by PhosphorImaging. Quantification was done using the PhosphorImager (Fuji). Curves connecting data points and the apparent dissociation constants ($K_d$) were calculated using the nonlinear regression program GraphPad Prism™ (GraphPad Software Inc.).

### GTP Hydrolysis Assay

SR, purified and reconstituted into liposomes (Görlich and Rapoport, 1993), was mixed at 25–50 nM in the presence or absence of purified RNCs (8.4 $OD_{260}$/ml) and/or purified SRP (25–50 nM) with 0.5 μM α[$^{32}$P]GTP (3,000 Ci/mmol) in 50 mM Tris-OAc, pH 7.8, 150 mM KOAc, 2 mM DTT, 5 mM $Mg(OAc)_2$, and 2 mM cycloheximide at 25°C for the indicated time points.

Aliquots of the samples were spotted onto polyethyleneimine cellulose thin-layer plates; α[$^{32}$P]GDP was resolved from α[$^{32}$P]GTP using 0.75 M $KH_2PO_4$, pH 3.3, as solvent. Radioactive TLC spots were quantitated using a PhosphorImager. The percentage of GTP hydrolysis was calculated from the amount of α[$^{32}$P]GDP divided by the sum of the amounts of α[$^{32}$P]GTP and α[$^{32}$P]GDP.

### Floatation Assay

Liposomes lacking or containing 50 nM SR or trypsinized SR were incubated in 50 mM Tris-OAc, pH 7.8, 150 mM KOAc, 2 mM DTT, 5 mM $Mg(OAc)_2$, 2 mM cycloheximide with purified RNCs (2,8 $OD_{260}$/ml) containing [$^{35}$S]methionine-labeled PPL86 and 0.5 mM GMPPNP or GDP in the presence or absence of 50 nM SRP. Wheat germ cytosol (1.8 μl), which was previously depleted of ribosomes by pelleting the ribosomes at 400,000 *g* for 20 min, was subsequently added to 5 μl reactions to block unspecific binding of ribosomes to liposomes lacking SR. After 30 min incubation at 25°C, 60 μl of ice-cold sucrose buffer containing 2.2 M sucrose, 10 mM Tris-OAc, pH 7.8, 500 mM KOAc, 2 mM DTT, and 5 mM $Mg(OAC)_2$ was added, thoroughly mixed, and layered under a 100 μl 1.8 M sucrose cushion containing CR buffer (50 mM Tris-OAc, pH 7.8, 500 mM KOAc, 2 mM DTT, 5 mM $Mg(OAc)_2$, 2 mM cycloheximide, 0.5 mM guanine nucleotides) in a TLA 100 tube that was preincubated with a 10 mg/ml BSA/PBS solution. A 0.25 M sucrose cushion in CR buffer (40 μl) was laid over the 1.8 M sucrose cushion. After centrifugation at 400,000 *g* for 60 min, the amount of labeled PPL86 in the top and

bottom fractions was determined by scintillation counting. The amount of labeled PPL86 that was recovered in the top fraction in the absence of liposomes was taken as background and was subtracted.

## *Results*

### *GTP Binding to SRβ*

To test GTP binding to SR subunits, we used UV light-mediated cross-linking of α[$^{32}$P]GTP to purified SR reconstituted into liposomes (SR liposomes). This approach allows analysis of GTP binding to each SR subunit (Miller et al., 1993; Bacher et al., 1996). Fig. 1 A shows the purified SR analyzed by SDS-PAGE and silver staining. α[$^{32}$P]GTP cross-linked to SR liposomes is revealed after SDS-PAGE followed by PhosphorImaging, and shows labeling of both SRα (70 kD) and SRβ (30 kD). An unidentified protein of ~50 kD (Fig. 1 A) was also found in various amounts, as has been reported previously (Miller et al., 1995).

To determine the apparent affinity of SRα for GTP, we added increasing concentrations of unlabeled GTP to the cross-linking reactions with α[$^{32}$P]GTP. Proteins were analyzed by SDS-PAGE, the amount of label in SRα was quantified after PhosphorImaging, and was plotted against the concentration of added GTP. From these data, an apparent $K_d$ of 14 μM was calculated (Fig. 1 B). To determine the apparent affinity of SRβ for GTP, we used increasing amounts of α[$^{32}$P]GTP in the cross-linking reactions. The amount of labeled SRβ was plotted against the concentration of α[$^{32}$P]GTP (Fig. 1 C). We determined an apparent $K_d$ of 20 nM GTP for SRβ. Thus, the affinity of SRβ for GTP is ~700-fold higher than the affinity of SRα for GTP.

To test whether translocon components affect α[$^{32}$P]GTP cross-linking to the SR subunits, we used SR liposomes containing, in addition, the Sec61p complex and the TRAM protein. Fig. 2 shows the purified proteins analyzed by SDS-PAGE and silver staining, and the proteins cross-linked to α[$^{32}$P]GTP after SDS-PAGE and PhosphorImaging. As can be seen in Fig. 2, cross-linking of α[$^{32}$P]GTP to SRα and SRβ is not changed by the inclusion of SR/Sec61p/TRAM liposomes to the assay (Figs. 1 A and 2). Furthermore, we found that the $K_d$ of SRβ and

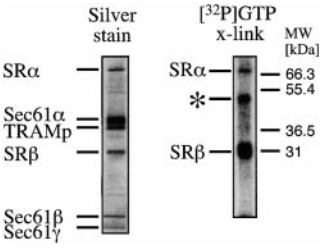


*Figure 2.* GTP binding to SR in the presence of TRAM and the Sec61p complex. Purified SR, purified TRAM protein, and purified Sec61p complex reconstituted in liposomes were analyzed by SDS-PAGE (10–15% acrylamide gel), followed by silver staining (left). Liposomes containing SR (25 nM), Sec61p complex (~200 nM), and TRAM protein (~200 nM) were incubated with 0.3 μM α[$^{32}$P]GTP. Samples were UV irradiated and subsequently analyzed by SDS-PAGE (12.5% acrylamide gel) and PhosphorImaging. An unidentified protein of ~50 kD, found in various amounts in SR preparations, was also labeled with α[$^{32}$P]GTP and is marked by an asterisk.

SRα for GTP remained unchanged in the presence of SR/Sec61p/TRAM liposomes, as compared with SR liposomes (data not shown). Thus, we conclude that neither the TRAM protein, nor the Sec61p, influence α[$^{32}$P]GTP cross-linking to the SRα or SRβ.

To test whether a component of the targeting complex affects GTP binding to SR, we added purified SRP and/or purified RNCs bearing PPL nascent chains of 86 amino acids to SR liposomes. As shown in Fig. 3 A (lanes 1 and 2), the presence of SRP did not affect α[$^{32}$P]GTP cross-linking to either SRα or SRβ. When RNCs were added, α[$^{32}$P]GTP cross-linking to SRα remained the same, however, α[$^{32}$P]GTP cross-linking to SRβ was strongly reduced (Fig. 3 A, lane 3). This indicates that the RNC interacts with SR and selectively reduces α[$^{32}$P]GTP cross-linking to SRβ. Reduction in α[$^{32}$P]GTP cross-linking to SRβ was also seen when SRP was added in addition to RNCs (Fig. 3 A, lane 4). In this case, cross-linking of α[$^{32}$P]GTP to SRP54 was found to be increased in the presence of RNC, as shown previously (Fig. 3 A, compare lanes 2 and 4; Bacher et al., 1996).

The apparent affinity of SRβ for GTP in the presence of RNCs was determined as described. We found that the ap-

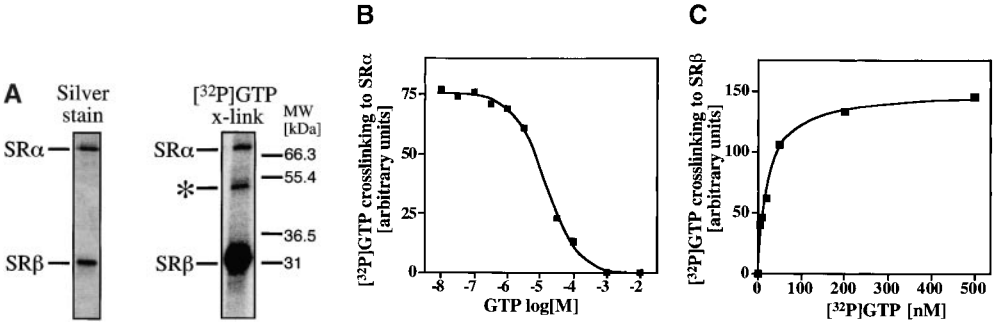


*Figure 1.* Binding of GTP to SR. A, Purified SR reconstituted in liposomes analyzed by SDS-PAGE followed by silver staining (left). α[$^{32}$P]GTP was incubated with SR liposomes (25 nM) and cross-linked to SR by UV irradiation. The sample was subsequently analyzed by SDS-PAGE and PhosphorImaging (right). An unidentified protein of ~50 kD, found in various amounts in SR preparations, was also labeled with α[$^{32}$P]GTP and is marked by an asterisk. B, Competition of α[$^{32}$P]GTP cross-linking to SR (25 nM) reconstituted into liposomes by increasing concentrations of GTP. Radiolabeled SRα was quantified using a PhosphorImager and plotted against the concentration of GTP. Curve connection data points and the apparent $K_d$ of SRα for GTP (14 μM) were calculated by a nonlinear regression program. C, Cross-linking of increasing concentrations of α[$^{32}$P]GTP to SR (25 nM) reconstituted into liposomes. Radiolabeled SRβ was quantified using a PhosphorImager and plotted against the concentration of α[$^{32}$P]GTP. The apparent $K_d$ of SRβ for GTP was 20 nM.

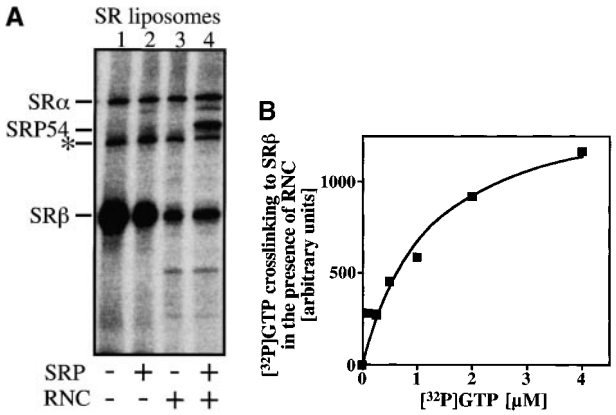


*Figure 3.* GTP binding to SR in the presence of RNC. A, Cross-linking of radiolabeled GTP to SR in the presence or absence of RNC and/or SRP. α[$^{32}$P]GTP (0.3 μM) was incubated with liposomes containing purified SR (25 nM) in the presence or absence of RNC (5.6 $OD_{260}$/ml) and/or 20 nM SRP. Samples were UV irradiated and subsequently analyzed by SDS-PAGE and PhosphorImaging. An unidentified protein of ~50 kD, found in various amounts in SR preparations, was also labeled with α[$^{32}$P]GTP and is marked by an asterisk. B, Cross-linking of increasing concentrations of α[$^{32}$P]GTP (500 Ci/mmol) to SR (25 nM) in the presence of RNC (8.4 $OD_{260}$/ml). Radiolabeled SRβ was quantified using the PhosphorImager and plotted against the concentration of α[$^{32}$P]GTP. The apparent $K_d$ of SRβ for GTP in the presence of RNC was 1 μM. GTP cross-linking assay was performed at low temperature (0°C) to reduce GTP hydrolysis. α[$^{32}$P]GDP was <1% of α[$^{32}$P]GTP in the assay, as determined by thin-layer chromatography.

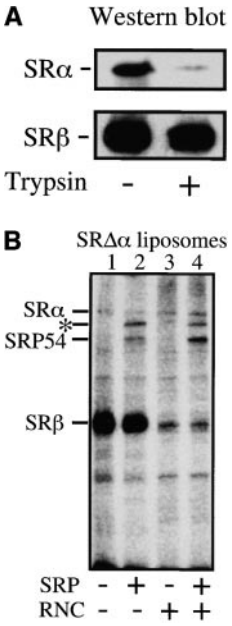


*Figure 4.* GTP binding to SRβ in the presence of RNC. A, SR liposomes were treated with 2 ng/ml of trypsin (SRΔα liposomes) and analyzed by SDS-PAGE, followed by Western blotting using antibodies raised against SRα and SRβ. B, Cross-linking of radiolabeled GTP to the SRβ in the presence of RNC. Liposomes containing 25 nM SRβ (SRΔα-liposomes) were incubated with 0.3 μM α[$^{32}$P]GTP in the presence or absence of RNC (8.4 $OD_{260}$/ml) and/or 20 nM SRP. Samples were UV irradiated and subsequently analyzed by SDS-PAGE and PhosphorImaging. An unidentified protein of ~65 kD in SRP preparations was also labeled with α[$^{32}$P]GTP and is marked by an asterisk.

parent $K_d$ was 1 μM, ~50-fold higher than that observed in the absence of RNCs (Fig. 3 B). Cross-linking of α[$^{32}$P]GDP to SRβ was also found to be reduced by the interaction with RNC (data not shown). This indicates that GTP, as well as GDP, binding to SRβ is decreased by RNC.

We next asked whether the decrease in α[$^{32}$P]GTP cross-linking to SRβ is caused by a direct interaction between RNCs and SRβ, or if it requires the presence of both SR subunits. To test the latter possibility, we removed SRα by mild trypsin digestion (Meyer and Dobberstein, 1980). Fig. 4 A shows that SRα was largely removed by treatment with 2 ng/ml trypsin and high salt, whereas SRβ resisted proteolysis and remained bound to the membranes. Liposomes containing the β-subunit of SR (SRΔα liposomes) were tested in the α[$^{32}$P]GTP cross-linking assay. Strong α[$^{32}$P]GTP cross-linking to SRβ was seen, while α[$^{32}$P]GTP cross-linking to SRα was greatly reduced (Fig. 4 B, lane 1). The presence of SRP did not affect α[$^{32}$P]GTP cross-linking to SRβ (Fig. 4 A, lane 2). When SRΔα liposomes were combined with RNCs, α[$^{32}$P]GTP cross-linking to SRβ was reduced similarly, as was seen with SR liposomes (Fig. 4 B, lanes 3). SRP had no effect on the RNC-mediated reduction of α[$^{32}$P]GTP cross-linking to SRβ (Fig. 4 B, lane 4). This indicates that RNCs directly interact with SRβ, resulting in reduced α[$^{32}$P]GTP cross-linking.

### *GTP Hydrolysis by SR and SRβ*

We next investigated the effect of the RNC–SR interaction on GTP hydrolysis. SR liposomes were incubated with RNC and/or SRP and GTP hydrolysis determined by chromatographic analysis of α[$^{32}$P]GDP generated in the assay. No significant GTP hydrolysis was observed with SR liposomes or RNC alone (Fig. 5 A). However, when SR liposomes were combined with RNCs an increase of GTP hydrolysis was observed (Fig. 5 A). This confirms that RNC interacts with SR and indicates that RNC stimulates GTP hydrolysis by SR. As shown previously, a large additional stimulation of GTP hydrolysis is observed when SRP is also added (Bacher et al., 1996). This stimulation of GTP hydrolysis by SRP was about eight times that observed in the presence of RNCs and SR alone (Fig. 5 A). This was calculated from the initial slope of the GTP hydrolysis curves shown in Fig. 5 A. SRP alone or in combination with SR or RNC showed only background level of GTP hydrolysis.

To investigate the RNC-stimulated GTP hydrolysis of SR in more detail, we used increasing concentrations of RNCs in the assay. When SR liposomes were tested alone, or in the presence of SRP, we found that the amount of GTP hydrolyzed at a given time point was saturable (Fig. 5 B). This indicates a specific interaction between RNC and SR.

To identify the subunit of the SR that hydrolyzes GTP in the presence of RNC, we used the SRΔα liposomes containing only SRβ. SRΔα liposomes alone, or combined with SRP, showed only background levels of GTP hydrolysis as observed with SR liposomes (Fig. 5 C, lanes 1, 2, 5, and 6). When SRΔα liposomes were combined with RNC, the stimulation of GTP hydrolysis was similar to that observed with SR liposomes (Fig. 5 C, lanes 3 and 7), suggesting that the RNC directly interacts with SRβ and stimulates its GTP hydrolysis. Addition of SRP and RNC to SRΔα liposomes did not significantly enhance GTP hydrolysis above the level seen with RNC alone (Fig. 5 C, lanes 3 and 4). A significant further stimulation of GTP hydrolysis is, as expected, observed with SR liposomes in the presence of RNC and SRP (Fig. 5 C, lanes 7 and 8).

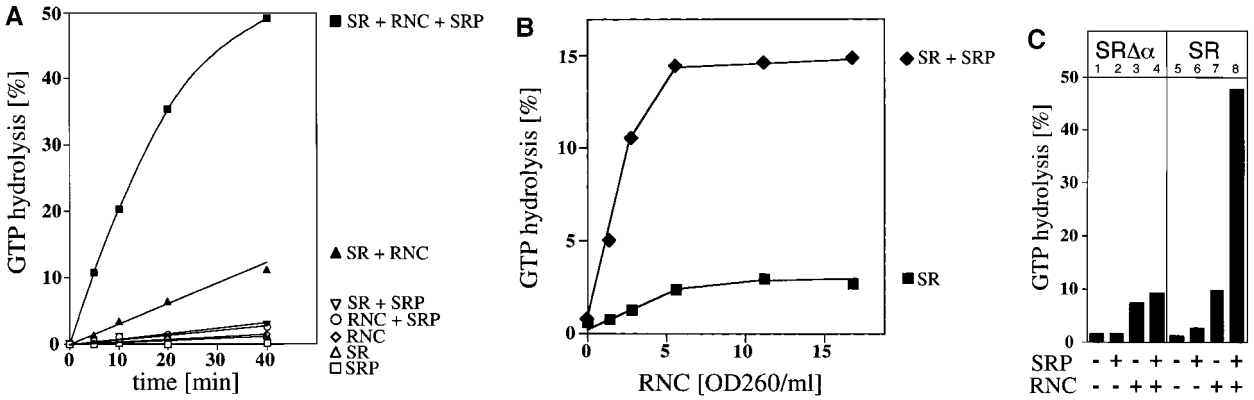


*Figure 5.* GTP hydrolysis by SR and SRβ in the presence of RNC. A, Hydrolysis of 0.5 μM α[$^{32}$P]GTP in the presence of different combinations of SR liposomes (40 nM SR), RNC (8.4 $OD_{260}$/ml), and SRP (50 nM). GTP hydrolysis was stopped by spotting aliquots at different time points onto polyethyleneimine cellulose thin-layer plates. α[$^{32}$P]GDP was resolved from α[$^{32}$P]GTP and the amount of α[$^{32}$P]GDP and α[$^{32}$P]GTP analyzed by PhosphorImaging. The amount of GTP hydrolyzed was plotted against the incubation time. B, GTP hydrolysis by SR (25 nM) in the presence of different concentrations of RNC. GTP hydrolysis was stopped after 10 min and the amount of α[$^{32}$P]GDP/GTP analyzed by PhosphorImaging. GTP hydrolysis was plotted against RNC concentrations. Background hydrolysis of GTP by RNC was subtracted. C, GTP hydrolysis by SRβ in the presence of RNC. SRΔα liposomes (40 nM, lanes 1–4) or SR liposomes (lanes 5–8) were incubated with 0.5 μM α[$^{32}$P]GTP in the presence or absence of 50 nM SRP and/or RNC (8.4 $OD_{260}$/ml). GTP hydrolysis was stopped after 40 min and the amount of α[$^{32}$P]GDP/GTP analyzed by PhosphorImaging. Background hydrolysis of GTP by RNC or RNC and SRP, respectively, was subtracted.

This also has been observed previously, and reflects the reciprocal GTPase stimulation of SRP54 and SRα (Miller et al., 1993; Bacher et al., 1996; Rapiejko and Gilmore, 1997). This indicates that RNCs stimulate the GTPase activity of SRβ, independent of the presence of SRα.

### *GTP-dependent Interaction of RNC with SR and SRΔα Liposomes*

The observation that RNC reduces α[$^{32}$P]GTP cross-linking to SRβ and stimulates GTP hydrolysis by SRβ indicates that the RNC contacts SRβ. To test this directly, we allowed interaction of RNC to SR liposomes and then floated SR liposomes with bound RNC to the top of a sucrose gradient to separate them from unbound RNCs. To test for a GTP dependence of this binding, we performed the assays in the presence of either GDP or the nonhydrolyzable GTP analogue GMPPNP. SR liposomes were incubated with purified RNCs bearing $^{35}$S-labeled PPL86. To reduce unspecific binding to the lipids, we included wheat germ cytosol from which endogenous ribosomes had been removed. SR liposomes were then floated and the amount of nascent chains ($^{35}$S-labeled PPL86) associated with the floated SR liposomes and in the pellet was determined. $^{35}$S-labeled PPL86 nascent chains were not found associated with liposomes lacking SR (Fig. 6, lane 1). In the presence of GDP, only a small amount of $^{35}$S-labeled PPL86 nascent chains were found associated with SR-liposomes (Fig. 6, lane 2). In contrast, with GMPPNP, a significantly increased amount of nascent chains was recovered with the floated SR liposomes (Fig. 6, lane 3). This suggests that RNCs bind to the SR liposomes in a GTP-dependent manner. To test the effect of SRP on RNC interaction with SR liposomes, we included SRP in the assay system. We found that, even in the presence of GDP, a further increase in RNC binding to SR liposomes compared with the absence of SRP (Fig. 6, lanes 4 and 2). But, in the presence of GMPPNP, a substantially higher amount of RNCs was found associated with SR liposomes (Fig. 6, lane 5). Taken together, this suggests two GTP-dependent interactions, one between the ribosome and SR and the other between SRP and SR.

To test whether the RNC binds to SRβ in the absence of SRα, we used SRΔα liposomes in the floatation assay. As was seen with SR liposomes, a significantly higher amount of RNC floated with SRΔα liposomes in the presence of GMPPNP, as compared with GDP (Fig. 6, lanes 6 and 7). These data suggest that the RNC directly interacts with SRβ in a GTP-dependent manner. The addition of SRP in the assay led to an increased binding of RNC to SRΔα liposomes in the presence of GDP, as was seen with SR liposomes. This might point to a GTP-independent interaction between SRP and SRβ. In the presence of GMPPNP, a further increase in binding was observed, but much less

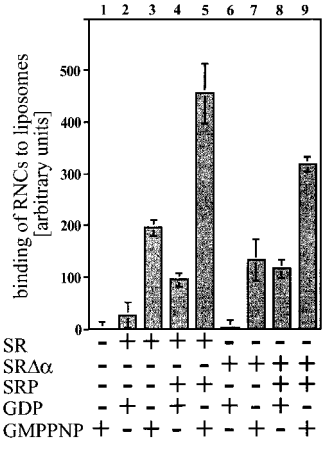


*Figure 6.* Interaction of RNC with SRβ in the presence of guanine nucleotides. Liposomes lacking SR (lane 1), SR liposomes (50 nM SR; lanes 2–5), or SRΔα liposomes (50 nM SRβ; lanes 6–9) were incubated with 2.8 $OD_{260}$/ml RNCs containing $^{35}$S-labeled PPL86 and guanine nucleotides in the presence or absence of 50 nM SRP. The liposomes were floated and recovered in the top fraction. Liposome-bound RNCs containing $^{35}$S-labeled PPL86 were then quantified by scintillation counting. Bars indicate mean values of three independent experiments with SD.

than observed with SR liposomes (Fig. 6, lanes 5 and 9). This indicates that the binding of SRP to SRα is drastically reduced, whereas the binding between SRβ and RNC is not affected (Fig. 6, lanes 2, 3, 6, and 7).

## *Discussion*

The functions of GTPases are regulated by guanine–nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), which mediate GTP binding and stimulation of GTP hydrolysis, respectively. Here, we show that the ribosome interacts with SRβ in its GTP-bound state, functions as a GAP for SRβ, and reduces the affinity of SRβ for guanine nucleotides. Previously, it has been shown that the ribosome functions as a GEF for SRP54 by increasing its affinity for GTP. Thus, the ribosome regulates the GTPases of the SRP/SR targeting system at two stages, first after signal sequence recognition by SRP54 (Bacher et al., 1996) and then at the ER membrane when it contacts SRβ.

To identify components that regulate the SRβ GTPase, we have used liposomes containing purified SR alone or together with translocon components, namely Sec61p complex, TRAM protein, and components of the targeting complex, namely RNC and SRP. Consistent with previous observations, we found that SRα alone has a very low affinity for GTP. The apparent $K_d$ of SRα for GTP was ~14 μM (Fig. 1 B). This is in good agreement to the $K_d$ of 10 μM, which has been previously reported (Miller et al., 1995). However, we found a considerably higher affinity of GTP for SRβ alone or SRβ in association with SRα ($K_d$ = 20 nM) than previously reported by Miller ($K_d$ = 1 μM; Miller et al., 1995). In the experiments shown here, SR reconstituted into liposomes was used, whereas previously, detergent solubilized SR was used. It is therefore conceivable that GTP binding to SRβ is reduced in the presence of detergents. However, in all studies, SRβ has been found to have a higher affinity for GTP than SRα.

Including components of the translocation machinery into the proteoliposomes with SR did not affect GTP binding to SRα nor to SRβ, suggesting that these components do not directly regulate the GTPases of SR. In contrast, RNCs were found to drastically reduce GTP binding to SRβ. In addition, they specifically stimulate GTP hydrolysis by SRβ. We suggest that the RNC induces a conformational change of the GTPase domain of SRβ that leads to both an increased GTP hydrolysis and a reduced guanine nucleotide binding. As free SRβ binds GTP with high affinity, interaction of SRβ with the ribosome first induces hydrolysis of bound GTP, and the resulting GDP is then bound with low affinity. The low GDP affinity might increase the dissociation of the bound GDP, creating an empty state of the GTPase domain.

SRP alone or in combination with RNC showed no effect on GTP binding and hydrolysis by SRβ, indicating that it functionally interacts only with SRα. When SRβ is associated with SRα, the presence of SRP leads to the observed burst in GTP hydrolysis via the reciprocal stimulation of GTP hydrolysis by SRP54 and SRα, which was previously shown (Powers and Walter, 1995; Rapiejko and Gilmore, 1997).

The difference in regulation of SRβ and SRP54/SRα is in agreement with the difference in the primary GTPase domain structure of these molecules. The GTPase domains of SRP54 and SRα are related and contain an insertion box that stabilizes the nucleotide-free form of the proteins, resulting in the low affinity for GTP (Freymann et al., 1997; Montoya et al., 1997). In contrast, the GTPase domain of SRβ is structurally distinct and falls into its own subfamily of GTPases (Miller et al., 1995).

Ribosomes can bind to ER membranes independently of a nascent chain or SRP (Borgese et al., 1974). This suggests that there are ribosome receptor proteins at the ER membrane. Several ribosome receptors have been identified. Based on ribosome binding assays (Borgese et al., 1974), ribosome receptors of 34 kD (Tazawa et al., 1991; Ichimura et al., 1992) and 180 kD (Savitz and Meyer, 1990, 1993) have been identified. However, both were shown not to be essential for the translocation of proteins across the ER membrane (Görlich and Rapoport, 1993). Therefore, they may play a role in modulating ribosome-binding to the ER membrane or become engaged at times when ribosomes are not active in translation or translocation (Unwin, 1979; Wanker et al., 1995). Studies on ribosome binding during ongoing translocation using SR/Sec61p liposomes have revealed that the Sec61p complex of the translocon forms the translocation channel and directly binds to the ribosome (Görlich et al., 1992b; Kalies et al., 1994; Neuhof et al., 1998). Binding of ribosomes to the Sec61p complex also has been visualized by EM and revealed contacts to the large ribosomal subunit, suggesting that the nascent chain is directly transferred from the exit site on the ribosome into the protein conducting channel of the translocon (Beckmann et al., 1997).

The interaction between ribosomes and SRβ described here is unlikely to directly contribute to binding of ribosomes to the ER membrane. For this, the high affinity binding between the Sec61p complex and the ribosome is probably sufficient (Kalies et al., 1994; Beckmann et al., 1997). Recent data with the yeast SRβ showed that a functional GTPase domain of SRβ, but not its membrane-spanning region, is required for efficient translocation (Ogg et al., 1998). This is consistent with a regulatory role of SRβ, rather than a role in binding RNC/SRP to the ER membrane.

How is the GTPase cycle of SRβ related to the function of the other two translocation GTPases, SRP54 and SRα? The first step in targeting nascent secretory and membrane proteins to the ER membrane is the interaction of SRP with the signal sequence exposed on a ribosome (Fig. 7, I). The additional interaction of SRP54 with the ribosome leads to GTP binding and an activated RNC–SRP–GTP targeting complex (Fig. 7, II; Bacher et al., 1996). Binding of the targeting complex to the ER may proceed in distinct steps and may involve, besides the core components of the Sec61p complex, many accessory factors (Hegde and Lingappa, 1997; Murphy et al., 1997; Martoglio and Dobberstein, 1998). For simplicity, we consider here only the minimal translocation machinery, the SR and the Sec61p complex of the translocon.

Because of the high affinity between ribosomes and the Sec61p complex (Kalies et al., 1994), we envisage that the first contact between the RNC–SRP–GTP with the ER membrane is the interaction between the ribosome and

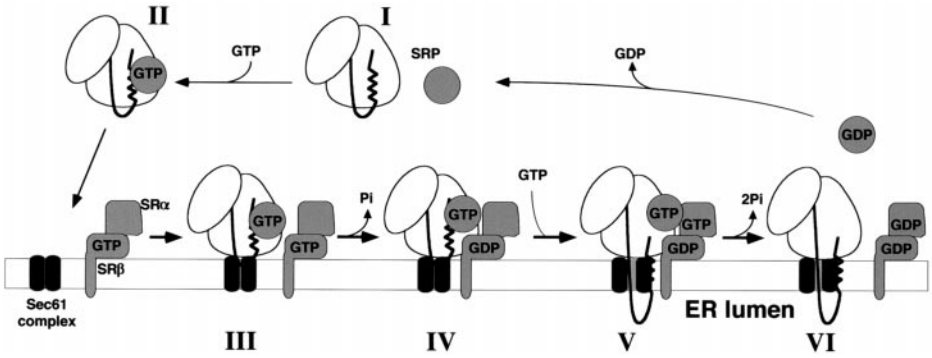


*Figure 7.* Model depicting GTP-dependent steps in SRP/SR-mediated targeting of nascent proteins to the ER membrane. We have shown here that SRβ in its GTP-bound form contacts ribosomes. This interaction stimulates GTPase activity of SRβ. SRβ is proposed to allow SRα to scan the ribosome for the presence of SRP.

the Sec61p complex (Fig. 7, III). This interaction is transient, as it can be competed by 80S ribosomes (Neuhof et al., 1998). The ribosome of a membrane-bound RNC–SRP–GTP complex may then recruit SR by interacting with SRβ–GTP (Fig. 7, IV). This would allow SRα to scan the ribosome for the presence of SRP and trigger the release of the signal sequence from SRP54 when GTP has been bound (Fig. 7, V). The dual contacts between RNC/SRP and the membrane, via an interaction between the ribosome and SRβ, and SRP and SRα may ensure that only the combination of ribosomes and SRP make a functional targeting complex.

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