The β-Subunit of the Protein-conducting Channel of the Endoplasmic Reticulum Functions as the Guanine Nucleotide Exchange Factor for the β-Subunit of the Signal Recognition Particle Receptor*

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Cotranslational protein transport to the endoplasmic reticulum is controlled by the concerted interaction of three GTPases: the SRP54 subunit of the signal recognition particle (SRP) and the α- and β-subunits of the SRP receptor (SR). SRβ is related to ADP-ribosylation factor (ARF)-type GTPases, and the recently published crystal structure of SRβ-GTP in complex with the binding domain of SRα suggested that SRβ, like all ARF-type GTPases, requires a guanine nucleotide exchange factor (GEF) for function. Searching the sequence data base, we identified significant sequence similarity between the Sec7 domain of ARF-GEFs and the cytosolic domains of the β-subunits of the two homologous heterotrimeric protein-conducting channels in yeast. Using a fluorescence nucleotide exchange assay, we show that the β-subunits of the heterotrimeric protein-conducting channels function as the GEFs for SRβ. Both the cytosolic domain of Sec61β as well as the holomeric Sec61p, when part of the isolated trimeric Sec61p complex, function as the GEF for SRβ, whereas the same Sec61β, when part of the heptameric complex that facilitates posttranslational protein transport, is inactive as the GEF for SRβ.

Of the GTPases involved in cotranslational protein transport, the signal recognition particle (SRP)β subunit SRP54 recognizes and binds to the signal sequence of the nascent chain, presented by the ribosome nascent chain complex (RNC) (1). The interaction between SRP54 and the membrane bound SRP receptor (SR) selectively targets the RNC-SRP complex to the membrane of the endoplasmic reticulum (ER). This is followed by the release of the signal sequence from SRP54 and its insertion into the protein-conducting channel (PCC) that is formed by the oligomeric assembly of the trimeric Sec61p complex (2). The nascent chain translocates into the ER lumen, while the ribosome remains attached to the PCC.

The GTPase domains of SRP54 and SRα are very similar in their structure and function. Structural studies of their prokaryotic homologues have shown that they form their own family within the GTPase superfamily (3, 4). They have low affinity for nucleotide and are both stable in their empty states (5, 6). SRP54 binds GTP when in contact with the RNC (6). The interaction between SRP54 and SRα leads to the formation of a GTP stabilized SRP-SRα complex (7). GTP hydrolysis dissociates the SRP-SRα complex (8), whereby SRP54 and SRα serve as mutual GTPase-activating proteins (GAP). This reciprocally symmetric interaction is unique among known GTPases (9).

SRβ is closely related to members of the ADP-ribosylation factor (ARF) GTPase family (10, 11). ARFs are conserved in all eukaryotes and are involved in the regulation of vesicle transport (12). SRβ is present only in eukaryotes and contains a transmembrane segment providing the membrane anchor for SRα (13). However, a truncated protein representing the cytosolic GTPase domain without the transmembrane domain is functional in protein targeting (14). The function of the GTPase domain of SRβ has long been a matter of conjecture. A recent structural and biochemical study showed that SRβ effectively binds SRα only when bound to GTP and not when bound to GDP (11), revealing that the GTP cycle of SRβ controls the association and dissociation of the heterodimeric SR.

The precise order of events that lead to the disassembly of the RNC-SRP-SR complex and the transfer of the signal sequence to the PCC are unknown. The PCC is composed of an oligomeric assembly of heterotrimeric integral membrane proteins: Sec61α, -β, and -γ. In yeast there are two homologous α-subunits (termed Sec61p and Ssh1p) and two homologous β-subunits (termed Shb1p and Shb2p). The two α- and β-subunits form distinct trimeric complexes (termed Sec61p and Ssh1p complex), each with the shared γ-subunit (termed Sss1p) (15–17). In comparison to the Sec61p complex, the Ssh1p complex interacts with signal sequences of stronger hydrophobicity (18) and appears to be involved exclusively in cotranslational protein transport. The trimeric Sec61p complex can associate with four additional subunits Sec62p, Sec63p, Sec71p, and Sec72p to form the so called heptameric complex (19), which facilitates posttranslational protein transport in yeast.

To identify a GEF for SRβ, we have searched the sequence data base for homologues of the known GEFs for ARF related GTPases in yeast. We found that the cytosolic domains of each of the β-subunits of the PCCs, Shb1p and Shb2p, showed homology to a motif that is common to ARF specific GEFs. We
have employed a fluorescence spectroscopy technique to directly monitor the nucleotide exchange of SRβ and have shown that the cytosolic domains of recombinant Shb1p and Shb2p indeed function as the GEFs for SRβ. GEF activity was retained when Shb1p was part of the trimeric Sec61p complex. In contrast, GEF activity was not expressed when Shb1p was part of the heptamer complex that functions in posttranslational translocation. We propose a model in which Shb1p and Shb2p regulate the reassociation of the SR subunits, thereby coupling targeting with translocation.

MATERIALS AND METHODS

Loading of Purified SRβ with MantGDP—The cytosolic GTPase domain of SRβ from Saccharomyces cerevisiae (residues 31–244) (11) was expressed in BL21(DE3) cells (Stratagene). SRβ31–244 was purified by covalent affinity chromatography (TALON Metal Affinity Resin, Clontech) from two liters of cell culture and eluted in 15 ml of buffer A (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5 mM β-mercaptoethanol) containing 150 mM imidazole. To remove endogenous nucleotide bound to SRβ31–244, EDTA (0.5 mM) was added to the elute, followed by an incubation at 4 °C for 30 min. The eluate was then subjected to size exclusion chromatography in the presence of 0.5 mM EDTA in buffer B (20 mM Hepes, pH 7.5, 250 mM NaCl, 1 mM DTT) (Superdex-75, Amersham). Fractions of a single Dmax peak, representing SRβ31–244, were pooled and stored at −80 °C.

A 10 µl solution of SRβ31–244 was incubated for 30 min at 4 °C with 200 µM of 3‘-(N-methylanthraniloyl)-2’-deoxyguanosine-5’-diphosphate (mantGDP) in buffer C (20 mM Hepes, pH 7.5, 100 mM KOAc, 5 mM Mg(OAc)2, 1 mM DTT, and 5 mM Mg(OAc)2). The excess of unbound mantGDP was removed by dialysis against buffer C at 4 °C for 4 h.

Purification of the Heptameric and the Sec61p Complex—The heptameric complex was immunopurified from yeast strain Sec63prA, in which the gene coding for Sec63p was tagged by in-frame integration of a DNA fragment encoding for a Factor X protease site and the immunoglobulin G (IgG)-binding domains of protein A, yielding a fusion protein of Sec63p-FactorX site-protein A (20). Cells from a 36-liter culture were lysed in lysis buffer (20 mM Hepes, pH 7.5, 400 mM sucrose, 750 mM KOAc, 2.5 mM Mg(OAc)2, 0.5 mM EDTA, and 10% glycerol) and incubated overnight at 4 °C with 1 ml IgG-Sepharose (Amersham). The cell lysate was cleared by low speed centrifugation at 6,000 g for 15 min and then centrifuged at 100,000 × g for 38 min at 4 °C to obtain a crude membrane pellet. Aliquots of the crude membrane pellet were stored at −80 °C. A 10th part of the crude membrane pellet (5 ml) was solubilized by resuspending it in 46 ml of solubilization buffer (20 mM Hepes, pH 7.5, 400 mM sucrose, 750 mM KOAc, 2.5 mM Mg(OAc)2, 0.5 mM EDTA, 3% digitonin (Calbiochem), 5 mM DTT, and 0.5 mM PMSF) and incubation at 4 °C for 30 min. The solubilized material was pelleted by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatant was diluted 1:1 with dilution buffer (20 mM Hepes, pH 7.5, 400 mM sucrose, 2.5 mM Mg(OAc)2 and incubated over night at 4 °C with 1 ml IgG-Sepharose (Cappel, Durham, NC). The resin was washed using washing buffer WLX (20 mM Hepes, pH 7.5, 100 mM KOAc, 3 mM Mg(OAc)2, 10% glycerol, 1 mM DTT) containing 0.5% digitonin and 5 mM/ml asolectin and the heptameric complex eluted by incubating with 5 µg/ml Factor X protease in 1 ml of WLX buffer for 5 h at 25 °C. To exchange detergent, pooled fractions containing the heptameric complex were incubated with Q-Sepharose (Amersham Biosciences), equilibrated and eluted with buffer WLX containing 0.3% DeoxyBigChAP (Calbiochem). The trimeric Sec61p complex was essentially purified as described previously (20). Briefly, the immunomobilized heptameric complex (see above) was incubated with buffer WLX containing 1% Triton X-100, thereby dissociating the trimeric Sec61p complex from the heptameric complex. To exchange detergent, the trimeric Sec61p complex was then bound to SP-Sepharose (Amersham Biosciences), equilibrated and cleaved WLX containing 0.3% DeoxyBigChAP. The trimeric Sec61p complex was essentially purified as described previously (20). Briefly, the immunomobilized heptameric complex (see above) was incubated with buffer WLX containing 1% Triton X-100, thereby dissociating the trimeric Sec61p complex from the heptameric complex. To exchange detergent, the trimeric Sec61p complex was then bound to SP-Sepharose (Amersham Biosciences), equilibrated and cleaved WLX containing 0.3% DeoxyBigChAP. The purified SRβ31–244 was incubated with 1 mM factor X protease in 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5 mM β-mercaptoethanol containing glutathione-Sepharose (Amersham Biosciences).

SRβ Nucleotide Exchange Assay—Steady-state fluorescence intensity was measured using a SPEX Fluorolog FL3-11 photon-counting spectrophotometer with a single grating excitation monochromator and a 450-watt xenon lamp. Samples were excited at 352 nm (1 nm bandwidth) and emission was detected at 440 nm (5-nm bandwidth). All spectral measurements were done at 25 °C in buffer J (20 mM Hepes, pH 7.5, 350 mM KOAc, 5 mM Mg(OAc)2, 10% glycerol, 1 mM EDTA, and 0.3% DeoxyBigChAP) using a 4 × 4-mm quartz cuvette. The release of mantGDP from SRβ31–244 was measured in real time as a decrease in fluorescence.

Purification of Ribosomas and Ribosome Nascent Chain Complexes—Ribosomes and ribosome nascent chain complexes were purified as described previously (2).

RESULTS

Shb1p and Shb2p Share Sequence Homology with the Sec7 Domain—GEFs for several families of GTPases have been functionally and structurally characterized. Interestingly, GEFs vary dramatically in structure. However those specific for one family of GTPases usually have the same fold. Accordingly, the GEFs for the GTPases of the ARF family share a common functional region of about 200 amino acids, the Sec7 domain (12). Taking the structural similarity between SRβ and ARF into account, a similarity between the respective GEFs is also conceivable. Searching the sequence data base, we could identify significant sequence similarity between the cytosolic domains of the two β-subunits of the PCCs in yeast and the Sec7 domain (Fig. 1). The sequence similarity involves the region of Sec7 that is crucial for GEF function, namely the F-G loop and helix H (21). Based on these findings, we tested whether Shb1p and Shb2p are able to promote the exchange of nucleotide for SRβ.

Shb1p and Shb2p Function as the GEFs for SRβ—A fluorescence spectroscopy assay was used, which allows one to monitor the nucleotide exchange on a G-protein in real-time and at high resolution. It had been shown that the fluorescent signal of mantGDP is dependent on the environment of the probe and hence on the amount of mantGDP bound by a G-protein (22). Recombinant yeast SRβ31–244 was purified from E. coli and endogenously bound nucleotide removed by size exclusion chromatography in the presence of EDTA. The removal of nucleotide was confirmed by high performance liquid chromatography analysis and determined to be complete (data not shown). SRβ31–244 was reloaded by incubation with an excess of mantGDP and unincorporated mantGDP was removed by dialysis. The reloading of SRβ31–244 with mantGDP resulted in a substantial increase of emitted fluorescence compared with mantGDP alone. The SRβ31–244-dependent increase averaged to 20%, indicating that mantGDP was successfully bound by SRβ31–244 (Fig. 2A).

The SBH1 gene in yeast codes for an 8.7-kDa protein with a COOH-terminal transmembrane domain. Its homologue Shb2p
is slightly larger with a size of 9.6 kDa. To test whether Sbh1p and Sbh2p are directly involved in the nucleotide exchange of SRβ, we purified the recombinant NH2-terminal cytosolic domains of Sbh1p (Sbh1pΔC) and Sbh2p (Sbh2pΔC) by GST affinity chromatography (Fig. 2D, as indicated). Equimolar amounts of mantGDP-SRβ31–244, GST-Sbh1pΔC, or GST-Sbh2pΔC in the presence of an excess of GDP were incubated at 25 °C. The reactions were excited at 352 nm and the emission recorded at 441 nm. When incubating mock buffer with mantGDP-SRβ31–244, no decrease of fluorescence could be detected (Fig. 2B). This result indicates that SRβ31–244 binds nucleotide tightly and does not exchange with the solution by itself. When using GST alone in the assay, no decrease in fluorescence could be detected either (data not shown). In the presence of GST-Sbh1pΔC or GST-Sbh2pΔC, a substantial decrease of fluorescence could be measured resulting from the exchange of nucleotide. The observed decrease of fluorescence averaged 12% after 15 min (Fig. 2B) and reached a plateau after a decrease of 14% (data not shown). The obtained data were best fitted as a sum of two exponential decays. When a 10-fold excess of either GST-Sbh1pΔC or GST-Sbh2pΔC over SRβ31–244 was used in the assay, the decrease of fluorescence averaged 50%, indicating that GST-Sbh1pΔC and GST-Sbh2pΔC do exchange the nucleotide of SRβ stoichiometrically rather than catalytically (data not shown).

**Sbh1p Functions as the GEF for SRβ in the Trimeric Sec61p but Not in the Heptamer Complex—Sbh1p is assembled into two different PCCs in yeast. It is not only part of the trimeric Sec61p complex, involved in cotranslational protein transport, but also part of the heptameric complex that is exclusively involved in posttranslational protein transport. To test whether the GEF activity for SRβ is dependent on the assembly state of Sbh1p we purified the trimeric Sec61p and the heptameric complex from yeast (Fig. 2D, as indicated) and tested both detergent-solubilized complexes in the nucleotide exchange assay.

We incubated equimolar amounts of mantGDP-SRβ31–244 with trimeric Sec61p complex or heptamer complex in the presence of an excess of GDP at 25 °C. When mantGDP-SRβ31–244 was incubated with purified trimeric Sec61p complex, mantGDP was efficiently exchanged with GDP, resulting in a decrease of fluorescence that averaged to 17% after 15 min (Fig. 2C) and reached a plateau after a decrease of 20% (data not shown). The obtained data were best fitted as a sum of two exponential decays. When using equimolar amounts of mantGDP-SRβ31–244 and purified heptameric complex, no decrease in fluorescence could be detected (Fig. 2C).

The ribosome had been reported to reduce the affinity of SRβ for nucleotide (23) and therefore was proposed to function as the GEF for SRβ (24). To test whether the ribosome is able to promote the exchange of nucleotide for SRβ, we used purified translating and non-translating ribosomes in the GTPase exchange assay. In our system, neither translating nor non-translating ribosomes were able to exchange the nucleotide of SRβ (data not shown).

We conclude that Sbh1p and Sbh2p are the GEFs for SRβ and are able to promote the exchange of nucleotide without the other subunits of the trimeric Sec61p complex. Furthermore, Sbh1p is able to promote the nucleotide exchange for SRβ when assembled into the trimeric Sec61p complex but not when assembled into the heptameric complex.

**DISCUSSION**

Based on biochemical and structural data, a recent study had suggested that SRβ requires a GAP and GEF to complete its GTP switch cycle (11). Without a GEF, the nucleotide exchange in most GTPases occurs very slowly under physiological conditions (12). In agreement with this, we could not detect any intrinsic nucleotide exchange activity of purified SRβ, which indicates that SRβ relies on an extrinsic GEF.

Searching the sequence data base, we have identified a sequence similarity between the β-subunits of the two homologous trimeric PCCs in yeast and the conserved Sec7 domain that is present in all ARF-GEFs. The residues in contact with ARF1 in the ARF1-Sec7 complex form a binding groove made up of two regions: the α-helix H and the loop connecting the preceding helices F and G of the all-α-helical Sec7 domain (21), forming a functional module. This module is not a folded domain, but is rather held in shape by the remainder of the Sec7 domain, which forms the surrounding architectural scaffold. Therefore it appears that the SRβ binding groove of Sbh1p/Sbh2p is presented in a different structural context. Sbh1p and Sbh2p are not related to the Sec7 domain except for the functional module. The cytosolic domains of Sbh1p and Sbh2p consist of only about 60 residues and thus represent the smallest functional GEFs to date. We note that not all residues that are important for Sec7 function are conserved in Sbh1p/Sbh2p. Therefore the guanine nucleotide exchange of SRβ will also differ from ARF GEFs in some detail.

Using a fluorescence nucleotide exchange assay, we show that recombinant proteins comprising the cytosolic domains of Sbh1p and Sbh2p indeed function as the GEFs for SRβ. Despite
the small size of their cytosolic domains, Shb1p and Shb2p are able to efficiently promote the exchange of nucleotide for SRβ. We find that Shb1p is also functional as the GEF for SRβ when assembled into the detergent-solubilized trimeric Sec61p complex isolated from yeast. A direct interaction between the trimeric Sec61p complex and SRβ is supported by the fact that a protein interaction screen has found the trimeric Sec61p complex and its homologue, the Sh1p complex to be in the proximity of SRβ (18). The calculated reaction rates at which the trimeric Sec61p complex, Shb1p, and Shb2p promoted nucleotide exchange are very similar, which indicates that both β-subunits promote the nucleotide exchange equally well, independent of their assembly state with the other subunits of the trimeric complexes. Interestingly, however, when Shb1p was assembled into the detergent-solubilized heptameric complex isolated from yeast, it did not express its GEF activity. This suggests, that the cytosolic domain of Shb1p in this complex might not be accessible to SRβ.

The β-subunit of the SR is an eukaryotic feature; the SRα homologue in bacteria directly associates with the membrane (1). Interestingly, there is no bacterial homologue for Shb1p. It is tempting to speculate that in higher organisms, the SRβ subunit evolved in concert with its GEF, not merely to provide a membrane anchor for SRα but also to provide an additional regulatory step to the translocation process; the interaction between these two proteins in the eukaryotic ER membrane, one a component of the targeting machinery (SR), the other a component of the trimeric PCCs, suggests that these proteins might link the two processes in a controllable fashion.

The association of the SR subunits is controlled by the nucleotide bound state of SRβ (11). With SRβ in its GDP bound form, the SR subunits dissociate. The β-subunits of the trimeric PCCs, acting as the GEFs for SRβ, reload SRβ with GTP and therefore control the reassociation of the SR subunits. It is conceivable that the β-subunits of the occupied PCC are inaccessible to SRβ. This would prevent the nucleotide exchange and the heterodimerization with SRα. As a result the targeting machinery could not be linked to the occupied PCC. Shb1p can be cross-linked to Spc25p, a subunit of the signal peptidase complex (25). Inaccessibility of the β-subunits of the PCCs may, therefore, not only be caused by the RNC complex binding to the PCCs, but also by the lateral recruitment within the plane of the ER membrane of other integral ER membrane proteins, such as the signal peptidase complex, the oligosaccharyltransferase or the additional subunits recruited to form the heptameric complex which is involved in posttranslational transport.

It had been reported that the ribosome decreases the affinity of SRγ for nucleotide (23). From these results it had been concluded that the ribosome might stabilize an empty state of SRγ and therefore could function as its GEF. However, using either purified non-translating or translating ribosomes in our assay had no effect on the nucleotide exchange of SRγ.

Our data show a specific function for the β-subunits of the two homologous PCCs and allows for a refined model of the cotranslational targeting process that had previously been suggested (11) (Fig. 3). In a first step, the SRP recognizes the signal sequence and binds to the RNC complex. The interaction between the ribosome and the SRP results in the binding of GTP by SRP54 (6). Shb1p assembled into the trimeric Sec61p complex functions as the GEF for SRβ, loading it with GTP. This nucleotide exchange reaction triggers the assembly of the SR subunits in the proximity of the trimeric Sec61p complex. Only with the SR in its assembled state, can the RNC-SRP complex be targeted to the ER membrane where SRP54 and SRα form a GTP stabilized complex. Next, the trimeric Sec61p complex replaces the SRP at the exit site of the ribosome, the signal sequence is transferred to the trimeric Sec61p complex and translocation starts. GTP hydrolysis of SRβ would lead to the dissociation of the SR subunits (11). In a last step, SRP54 and SRα act as mutual GAPs, thereby resolving the SRP-SRα interaction.

The precise timing and coordination of the events during the signal sequence transfer still remain poorly understood. Signal sequence transfer to the trimeric Sec61p complex requires GTP binding by SRP54, SRα and SRβ but not necessarily GTP hydrolysis (26). The events triggering the GTP hydrolysis of the three GTPases and the release of the signal sequence remain to be elucidated. Further biochemical characterization will be necessary to completely understand the GTPase cycle of SRβ.

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