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Mitochondrial Protein Import: Recognition of Internal Import Signals of BCS1 by the TOM Complex

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BCS1, a component of the inner membrane of mitochondria, belongs to the group of proteins with internal, noncleavable import signals. Import and intramitochondrial sorting of BCS1 are encoded in the N-terminal 126 amino acid residues. Three sequence elements were identified in this region, namely, the transmembrane domain (amino acid residues 51 to 68), a presequence type helix (residues 69 to 83), and an import auxiliary region (residues 84 to 126). The transmembrane domain is not required for stable binding to the TOM complex. The Tom receptors (Tom70, Tom22 and Tom20), as determined by peptide scan analysis, interact with the presequence-like helix, yet the highest binding was to the third sequence element. We propose that the initial recognition of BCS1 precursor at the surface of the organelle mainly depends on the auxiliary region and does not require the transmembrane domain. This essential region represents a novel type of signal with targeting and sorting functions. It is recognized by all three known mitochondrial import receptors, demonstrating their capacity to decode various targeting signals. We suggest that the BCS1 precursor crosses the TOM complex as a loop structure and that once the precursor emerges from the TOM complex, all three structural elements are essential for the intramitochondrial sorting to the inner membrane.

Targeting and translocation of most nucleus-encoded mitochondrial proteins depend on N-terminal extensions referred to as mitochondrial targeting sequences or presequences (37, 41). A presequence typically consists of about 15 to 40 amino acid residues and is enriched in positively charged residues. The ability of most presequence peptides to form an amphipathic α-helix is thought to be important for their recognition by the translocation machineries in the mitochondria (52). Biochemical studies of the past few years have demonstrated a series of interactions of the presequence during entry into mitochondria (5, 32, 40, 48). At the TOM complex, these interactions are first established with surface receptors (mainly Tom20 and Tom22), resulting in the formation of a salt-sensitive intermediate (*cis* site) (7, 25, 28, 34, 44). The presequence moves on to the other side of the outer membrane (*trans* site), where it is mainly in contact with Tom40, the main component of the translocation channel (2, 22, 35, 42). The presequences are then recognized again by the TIM23 complex, which thus facilitates further movement of the precursor into or across the inner membrane (6).

All precursor proteins of the mitochondrial outer membrane, most proteins of the intermembrane space, and some proteins of the inner membrane are devoid of a typical N-terminal presequence. For a small number of this last class of precursor proteins, the targeting signal has been identified. Some outer membrane proteins, such as the Tom20 and Tom70 subunits of the TOM complex, contain an N-terminal targeting sequence which functions also as membrane anchor segment (signal anchor) (27, 36). Others, such as Tom6 and Tom22, contain a membrane anchor segment towards their C termini (tail anchor) which, together with a segment in the cytosolic domain, comprises the information for targeting and assembly (9, 16, 18, 45). Proteins residing in the intermembrane space, like cytochrome *c* heme lyase and cytochrome c_1 heme lyase, also contain internal targeting sequences which were recently identified and found to be in the third quarter of the proteins (17). They represent a unique type of targeting signals which are clearly different from those of amphipathic amino-terminal matrix-targeting signals.

The BCS1 protein was first identified in *Saccharomyces cerevisiae* as the product of a gene required for the expression of functional Rieske iron-sulfur protein, and later it was found to function as a chaperone for the assembly of the cytochrome bc_1 complex (10, 38). The protein is anchored to the inner membrane by a single transmembrane domain (residues 51 to 68). A short N-terminal tail is exposed to the intermembrane space, while the bulk of the protein is in the matrix (N_{out}-C_{in} topology). The membrane anchor is followed by a positively charged segment (residues 69 to 83) which, like presequences, has the potential to form an amphipathic α-helix. This internal amphipathic helix was suggested to function as part of the targeting information and, together with the transmembrane segment, to facilitate the import and the intramitochondrial sorting of the BCS1 precursor (20). The precursor was suggested to form a tight loop structure during translocation across the inner membrane. Placing a folded dihydrofolate reductase (DHFR) moiety at the C terminus of BCS1 did not hamper the import and sorting of the rest of the protein (20). Thus, import in a C- to N-terminal fashion is unlikely.

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The mechanisms by which the BCS1 precursor crosses the outer membrane and how the internal import signal mediates recognition and translocation across the outer membrane are unclear.

The contributions of the various structural elements within the BCS1 precursor to the translocation process were studied. Based on our results, we suggest that the BCS1 precursor crosses the TOM complex as a loop structure. Whereas in presequence-containing proteins the presequence alone is sufficient for correct recognition and import, the BCS1 precursor contains several sequence elements that cooperate to facilitate productive import and intramitochondrial sorting.

MATERIALS AND METHODS

Yeast and *Neurospora crassa* strains and growth. Growth and handling of *N. crassa* wild-type strain 74A were as described previously (14). The yeast *BCS1* null strain and its isogenic strain W303 were as described previously (38). A yeast *tom70* null strain was obtained from Research Genetics (Huntsville, Ala.).

Construction of BCS1 mutants. pGEM4-BCS1(1-126)ΔTM-DHFR and pGEM4-BCS1(66-86)-DHFR were constructed by PCR amplification of the DNA sequences by using pGEM4-BCS1ΔTM and pGEM4-BCS1, respectively, as the template (20). Both PCR products were digested and subcloned into a pGEM4 vector containing a DHFR-encoding sequence. pGEM4-BCS1(84-126)-DHFR was constructed by PCR amplification of the DNA sequence by using as the template pGEM4-BCS1(1-126)-DHFR. DHFR-BCS1(1-250)-DHFR was constructed by PCR amplification of DHFR-BCS1(1-250) by using DHFR-BCS1 as the template (20). The PCR product was digested and subcloned into a pGEM4 vector containing a DHFR-encoding sequence. For constructing pGEM4-BCS1-Cytc$_1$, a DNA sequence encoding amino acid residues 273 to 287 of cytochrome c_1 was amplified by a PCR method. The PCR product was digested and inserted into pGEM4-BCS1ΔTM. pGEM4-BCS1-Su9 was constructed by PCR amplification of the DNA sequence encoding residues 1 to 69 of BCS1. The PCR product was digested and inserted upstream of a DNA sequence encoding pSu9(1-48)-DHFR. Next, the sequence encoding the DHFR domain was released, and a PCR product encoding residues 84 to 458 of BCS1 was ligated into the above pGEM4 vector. pGEM4-BCS1-CoxIV was constructed by PCR amplification of the DNA sequence encoding residues 84 to 458 of BCS1 and insertion of it into a pGEM4 vector. Next, the sequence encoding the presequence part of CoxIV was amplified by PCR and inserted into the above vector. Finally, a PCR product encoding residues 1 to 68 of BCS1 was ligated upstream of the CoxIV-BCS1(84-458) coding sequence. For expression in yeast cells, the sequence encoding the wild-type protein or its mutated versions was inserted into the yeast expression vector pYX142. All constructs were sequenced to ensure their correct composition.

Biochemical procedures. Isolation of mitochondria and outer membrane vesicles (OMV) from *N. crassa* was performed as described previously (33). Yeast mitochondria were isolated from strain D273-10B according to published methods (13) and resuspended in SEM buffer (250 mM sucrose, 1 mM ethylenediaminetetraacetic acid [EDTA], 10 mM morpholinepropanesulfonic acid [MOPS]-KOH [pH 7.2]) to a final concentration of 10 mg/ml and stored at −80°C. To disrupt the mitochondrial outer membrane, mitochondria were resuspended in 20 mM HEPES-HCl (pH 7.5) and incubated on ice for 30 min. Purification of *N. crassa* TOM core complex was according to a published procedure (3). Purification of the cytosolic domain of Tom70 for the pull-down assay was as described by Young et al. (54).

Import of preproteins into isolated mitochondria or binding to OMV. Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Amersham) after in vitro transcription by SP6 polymerase from pGEM4 vectors containing the gene of interest. Binding experiments with OMV were performed in buffer A (0.25 mg of bovine serum albumin [BSA]/ml, 20 mM KCl, 2.5 mM $MgCl_2$, 10 mM MOPS-KOH [pH 7.2]) in the presence of 1 mM NADPH and 1 μM methotrexate (MTX/NADPH) when indicated. After binding, the OMV were washed with EM buffer (1 mM EDTA, 10 mM MOPS-KOH [pH 7.2]) containing the indicated concentrations of KCl. Import into *N. crassa* mitochondria was performed by incubation of radiolabeled preproteins in F5 import buffer (0.5% [wt/vol] BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM NADH, 10 mM MOPS-KOH [pH 7.2]) at the indicated temperature. Protein import in yeast mitochondria was performed in SI buffer (3% BSA [wt/vol], 0.5 M sorbitol, 50 mM HEPES-KOH, 80 mM KCl, 10 mM MgAc, 2 mM KH_2PO_4, 2.5 mM EDTA, 2.5 mM $MnCl_2$, 2 mM ATP, 2 mM NADH [pH 7.2]). Protease treatment of mitochondria or OMV was performed by incubation with proteinase K (PK) or trypsin for 15 min on ice, followed by the addition for 5 min of 1 mM phenylmethylsulfonyl fluoride (PMSF) or 20× trypsin inhibitor, respectively. Import was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography, and phosphorimaging (Fuji BAS 1500).

In some experiments, a recombinant chimeric precursor was used. The chimeric precursor consisted of the N-terminal 69 amino acid residues of the presequence for *N. crassa* ATPase subunit 9 fused to the coding sequence of mouse DHFR (pSu9-DHFR). Purification was according to published procedure (51). DHFR and reduced carboxymethylated lactalbumin (RCLMA) were purchased from Sigma.

For coimmunoprecipitation, samples after import of radiolabeled precursors were dissolved in lysis buffer (3% BSA, 0.75% β-dodecyl maltoside or digitonin, 250 mM sucrose, 1 mM EDTA, 150 mM KCl, 10 mM MOPS-KOH [pH 7.2]). After a clarifying spin (20 min at 20,000 × *g*), the supernatants were incubated with antibodies that were precoupled to protein A-Sepharose beads.

Screening of peptide libraries with soluble domains of Tom receptors. The cytosolic domains of Tom receptor proteins were purified according to published procedures (7). Cellulose-bound peptide libraries were prepared by automated spot synthesis (21, 29). Peptides of 13 amino acid residues with an overlap of 10 residues covered the sequence from residues 1 to 126 of BCS1. The membranes were incubated with 150 nM soluble cytosolic domain of Tom20, Tom22, or Tom70 in binding buffer as described previously (8). After a washing, the bound protein was transferred to a polyvinylidene difluoride (PVDF) membrane (46), followed by detection with antibodies against the corresponding Tom component. Binding data were analyzed by scanning laser densitometry and quantified by utilizing the TINA program. The mean of results of three independent experiments for each peptide spot was used.

RESULTS

Recognition of BCS1 precursor by the TOM complex. A hybrid protein composed of the first 126 amino acids of BCS1 fused to mouse DHFR [BCS1(1-126)-DHFR] was synthesized in vitro and incubated with OMV isolated from *N. crassa* mitochondria. This fusion protein was shown previously to be correctly sorted to the inner membrane and imported into mitochondria with a rate and efficiency similar to those for the authentic BCS1 protein (20). The binding properties of this precursor were compared to those of a matrix-destined precursor, pSu9-DHFR, a chimeric preprotein consisting of the presequence of subunit 9 of the mitochondrial F_o-ATPase fused to DHFR. Whereas the binding of the matrix-destined precursor, pSu9-DHFR, at low temperature was completely salt sensitive, a significant fraction of BCS1(1-126)-DHFR remained bound to OMV after a treatment with 200 mM salt (Fig. 1A) (42). Therefore, hydrophobic interactions seem to play a major role in mediating binding of BCS1(1-126)-DHFR under these conditions. About half of the salt-resistant BCS1(1-126)-DHFR molecules contained a folded DHFR domain (Fig. 1A). Hence, these hydrophobic interactions are probably mediated by the BCS1 moiety of the fusion protein and not by the folded DHFR domain.

Are the interactions of BCS1 with the outer membrane mediated by the TOM complex? The precursors of BCS1wt and BCS1(1-126)-DHFR were incubated with OMV, and the TOM complex was isolated by immunoprecipitation and analyzed for bound preprotein. A substantial fraction of both precursors were coimmunoprecipitated with the TOM complex after preprotein binding at 25°C and wash with high-salt buffer (Fig. 1B). Smaller amounts of precursors were stably associated with the TOM complex when the binding was analyzed at 0°C (Fig. 1B). The relatively stable binding of BCS1

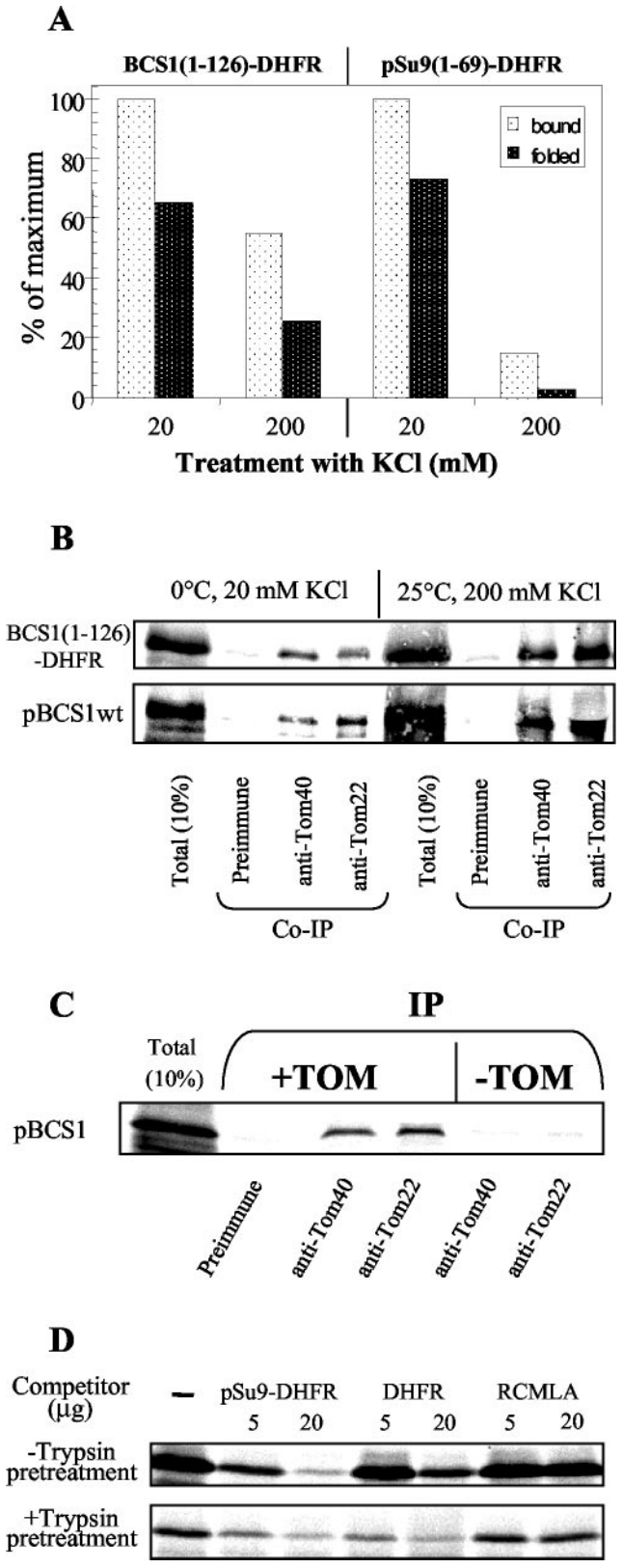

FIG. 1. Interaction of the targeting signal of BSC1 with the TOM complex. (A) BCS1(1-126)-DHFR and pSu9(1-69)-DHFR were incubated with *N. crassa* OMV for 20 min at 0°C in the presence of MTX/NADPH. OMV were then treated with buffer containing either 20 or 200 mM KCl. The OMV were reisolated and resuspended in buffer, and the suspensions were halved. One half was treated with trypsin at 0°C (folded material), while the second half was kept at 0°C (bound). Proteins were then analyzed by SDS-PAGE and phosphorimaging. The amount of protein bound at 20 mM salt was set to 100%. (B) Preprotein bound to OMV can be coimmunoprecipitated with components of the TOM complex. Radiolabeled BCS1 and BCS1(1-126)-DHFR were incubated with OMV at 0°C in the presence of MTX/NADPH or at 25°C in the absence of MTX/NADPH. The reaction mixtures were adjusted to 20 or 200 mM KCl at 0°C, and OMV were reisolated and resuspended in SEM buffer. Immunoprecipitation was performed with antibodies raised against Tom22 or Tom40 or with preimmune serum. To control for binding, an aliquot was removed before the coimmunoprecipitation and precipitated with trichloroacetic acid (TCA) (Total). (C) Precursor of BCS1 interacts with purified TOM complex. Radiolabeled precursor of BCS1 was incubated for 20 min at 25°C with purified TOM core complex. Immunoprecipitation was performed with antibodies or preimmune serum, as described for panel B. To exclude unspecific interactions, immunoprecipitation was also performed in the absence of the TOM complex (−TOM). (D) A matrix-destined precursor can outcompete the precursor of BCS1(1-126)-DHFR. Radiolabeled precursor of BCS1(1-126)-DHFR was incubated for 20 min at 25°C with either mitochondria alone (−) or with mitochondria preincubated with the indicated amounts of proteins for 2 min on ice. The mitochondria were either intact (upper panel) or pretreated with trypsin before incubation with proteins (lower panel). At the end of the import reactions, mitochondria were treated with proteinase K, washed, reisolated, and analyzed by SDS-PAGE.

constructs to the TOM complex at 0°C differs from the loose binding of the matrix-destined precursor, pSu9-DHFR, under similar conditions (see also reference 42).

We further asked whether the TOM complex alone is sufficient for the observed association. Radiolabeled BCS1 precursor could be immunoprecipitated with antibodies against Tom components after it had been incubated with purified TOM core complex (Fig. 1C). Apparently, neither lipids in bilayer form nor other proteins in the outer membrane are required for the recognition of the BCS1 precursor by the TOM complex.

To find out whether BCS1 precursor uses the general import pathway, we tested the capacity of a protein destined to the matrix, pSu9-DHFR, to outcompete the import of the BCS1 precursor. This approach was used before to demonstrate that outer membrane proteins use the TOM complex for their insertion into the outer membrane (16, 30, 43). The addition of excess amounts of pSu9-DHFR during import of radiolabeled BCS1(1-126)-DHFR to yeast mitochondria resulted in a strong reduction of import of the radiolabeled precursor (Fig. 1D, upper panel). In contrast, addition of unrelated proteins like DHFR alone (without presequence) or reduced carboxymethylated lactalbumin had only a very minor effect on the import of BCS1(1-126)-DHFR. The slight inhibitory effect of DHFR alone may result from the cryptic mitochondrial targeting signal within this protein (26) or from hydrophobic interactions of the TOM complex with unfolded DHFR. Hence, BCS1 and precursors that use the general import pathway share at least one common step in their translocation pathway.

Is the inhibitory effect solely due to competition for binding sites on the receptors of the outer membrane, or is it also due to competition for the import pore? Mitochondria were pretreated with trypsin to remove the surface receptors and incubated with radiolabeled BCS1(1-126)-DHFR. Under these

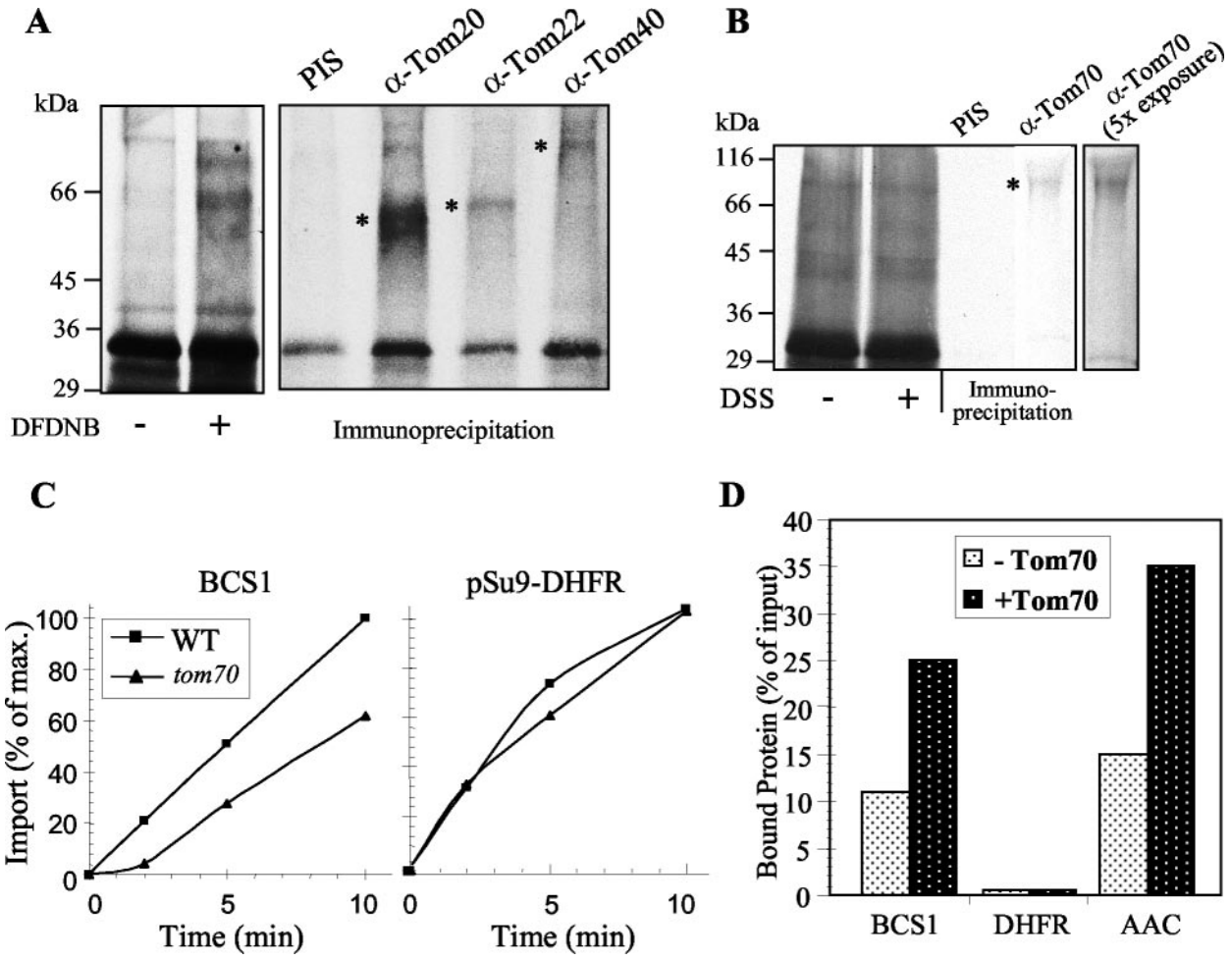

FIG. 2. Precursor of BCS1 interacts with receptor components of the TOM complex. (A) BCS1 is in the vicinity of Tom20, Tom22, and Tom40 on its insertion pathway. Radiolabeled BCS1(1-126)-DHFR precursor was incubated with isolated OMV for 30 min at 0°C. OMV were reisolated and resuspended in SEM buffer. One aliquot was left on ice (−DFDNB), while the chemical cross-linker DFDNB was added to the others for 40 min on ice. Aliquots were subjected to immunoprecipitation with antibodies against Tom20, Tom22, or Tom40 or with preimmune serum (PIS). Asterisks, adducts consisting of BCS1 cross-linked to Tom proteins. (B) BCS1 is in the vicinity of Tom70 on its insertion pathway. Radiolabeled BCS1(1-126)-DHFR precursor was incubated with isolated OMV for 2 min at 0°C. The sample was split; one aliquot was left on ice (−DSS), and the chemical cross-linker DSS was added to the other for 40 min on ice. Aliquots were subjected to immunoprecipitation with antibodies against Tom70 or with PIS. Asterisk, adduct consisting of BCS1 cross-linked to Tom70. A longer exposure of the immunoprecipitation with Tom70 is presented for clarity. (C) The *tom70* null mutation affects import of BCS1. Radiolabeled precursors of BCS1 and pSu9-DHFR were incubated at 15°C for the indicated time periods with mitochondria from either *tom70* null mutant (*tom70*) or its wild type parent (WT). At the end of the import, proteinase K was added, and mitochondria were reisolated and analyzed by SDS-PAGE. The protease-protected bands of BCS1 and mature Su9-DHFR were quantified. (D) Binding of mitochondrial preproteins to purified Tom70 cytosolic domain. The purified cytosolic domain of Tom70 was bound to an Ni-NTA column. Then radiolabeled preproteins were incubated with the bound protein for 30 min at 4°C. After a washing step, bound proteins were eluted with sample buffer and analyzed by SDS-PAGE. The total amount of each preprotein added was set to 100%.

conditions, precursors that depend on receptors for import have been shown to enter mitochondria at a lower rate due to bypass import, which occurs by their direct interaction with the general import pore (39). This was also true for BCS1(1-126)-DHFR (compare the first lanes in the upper and lower panels of Fig. 1D). In the presence of excess unlabeled pSu9-DHFR, the level of BCS1(1-126)-DHFR import was strongly reduced (Fig. 1D, lower panel). Thus, the import of BCS1 is dependent on import receptors and on components of the translocation pore.

Tom receptors are involved in the recognition of the BCS1 precursor. To obtain more information on the interaction of the BCS1 precursor with specific Tom components, we performed chemical cross-linking. Radiolabeled BCS1(1-126)-DHFR precursor was accumulated as an import intermediate in OMV, and the homobifunctional cross-linking reagent 1,5-difluoro-2,4-dinitrobenzene (DFDNB) was added. This resulted in cross-linking of BCS1 to Tom20, Tom22, and Tom40 (Fig. 2A). The Tom20-containing adduct was prominent probably because under the conditions of the binding assay (0°C), association of BCS1 with the receptors rather than with pore components is favored.

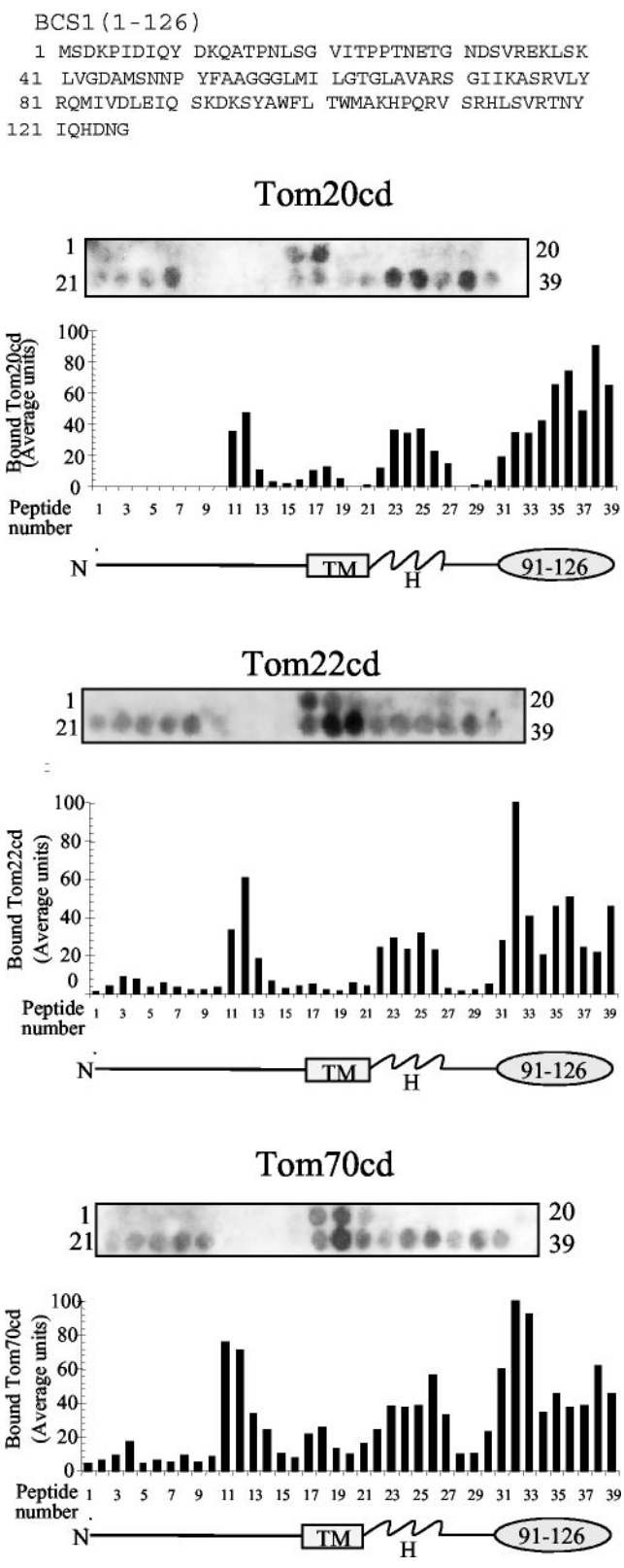

FIG. 3. Screening of a peptide library with soluble receptor domains. Cytosolic domains of the indicated Tom components (150 nM) were incubated with a peptide library on a cellulose membrane covering amino acid residues 1 to 126 of BCS1 (length of peptides, 13 residues; overlap, 10 residues). The bound proteins were blotted to PVDF membranes and decorated with the corresponding antibody. The labeling indicates the numbers of the peptides in the beginning and the end of each row. Binding was quantified by scanning densitometry from three independent experiments. The various domains of BCS1 are displayed below the corresponding peptides. TM, transmembrane domain; H, putative presequence-like helix.

Tom70 is known to promote binding and import of precursors of inner membrane proteins with internal import signals (24, 49, 53). We therefore asked whether Tom70 is involved also in the import of BCS1 precursor protein. A chemical cross-linking experiment was performed with the reagent disuccinimidyl suberate (DSS). A cross-linking adduct of BCS1 (1-126)-DHFR with Tom70 was identified (Fig. 2B). To demonstrate a functional role of Tom70 in the import of BCS1, radiolabeled BCS1 (or pSu9-DHFR as control) was imported into mitochondria isolated from either the wild-type strain or from a strain lacking Tom70. Whereas the control precursor, pSu9-DHFR, was imported into Δ*tom70* and wild-type mitochondria with similar efficiencies, the import of BCS1 into Δ*tom70* mitochondria was reduced to about one-half of the level at which BCS1 was imported into wild-type mitochondria (Fig. 2C). This reduction is similar to that reported for a well-known substrate of Tom70, AAC (23). We next investigated the capacity of Tom70 to bind the precursor of BCS1. We overexpressed in *Escherichia coli* a His-tagged version of the cytosolic domain of Tom70 (54). In a pull-down assay with Ni-nitrilotriacetic acid (Ni-NTA) beads, this recombinant protein was observed to bind specifically to radiolabeled BCS1. As controls, Tom70 interacted with the known substrate, AAC, while only background levels of the cytosolic protein, DHFR, were bound (Fig. 2D). Both BCS1 and AAC precursors contain hydrophobic stretches and hence have some unspecific binding to Ni-NTA beads. The involvement of Tom70 in the recognition of the BCS1 precursor is supported by experiments with a construct consisting of amino acid residues 66 to 86 of BCS1 fused to DHFR. This construct was efficiently cross-linked to, and coimmunoprecipitated with, Tom70 (Fig. 5C and D). Taken together, these results demonstrate that on its import pathway, BCS1 is recognized by the import receptor Tom70.

The import signal of BCS1. The region of amino acid residues 1 to 126 of BCS1 contains two putative structural elements, namely, a hydrophobic stretch (amino acids 51 to 68) that most likely forms the single transmembrane segment of the protein and an amphiphilic α-helix (amino acids 69 to 83) that resembles a presequence. Previous work has shown that both elements are essential for the proper import and sorting of the protein (20).

To determine the elements in BCS1 which bind to the receptor components of the TOM complex, peptide scans were performed. The peptide library consisted of 13-mers overlapping by 10 residues and covering residues 1 to 126. The peptides were attached via their C-terminal residues to a cellulose membrane (29). The soluble domains of the Tom receptor subunits Tom20, Tom22, and Tom70 were purified as described previously (7) and incubated with the membrane. Bound proteins were transferred to a PVDF membrane, which was then immunodecorated with antibodies against the various Tom subunits.

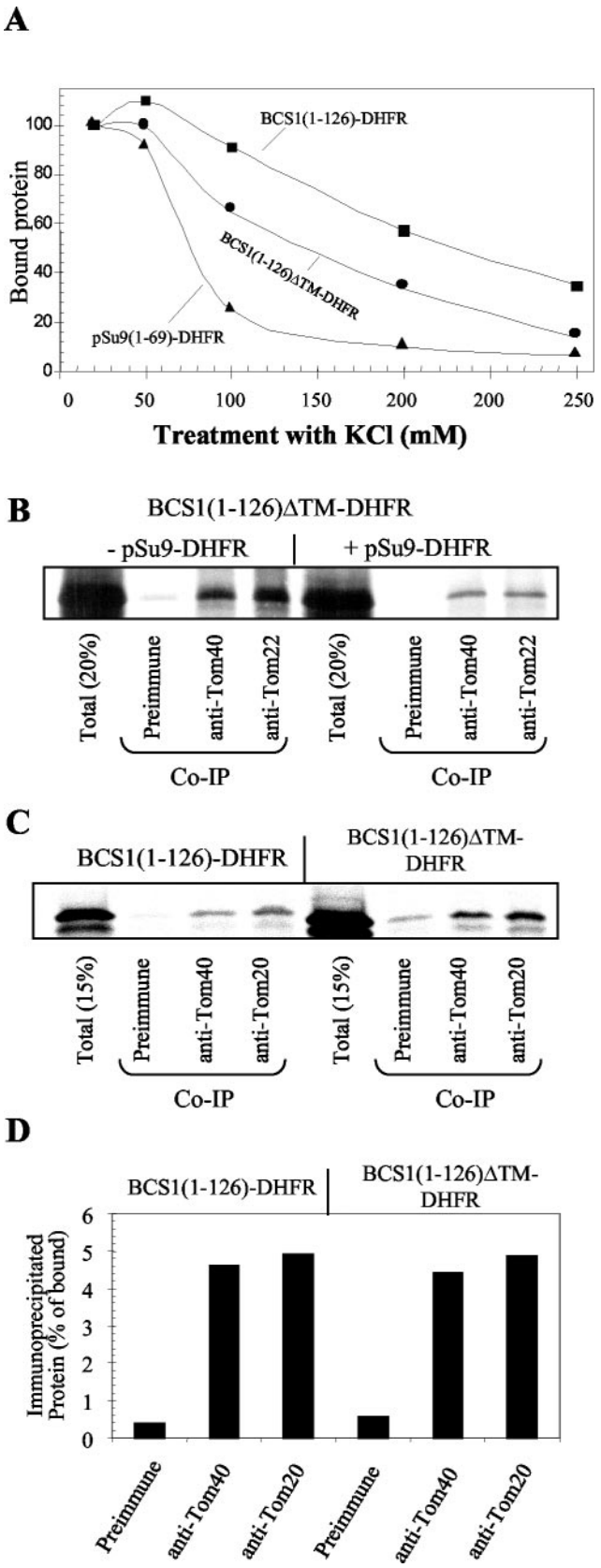

FIG. 4. BCS1 can bind to the TOM complex in the absence of the transmembrane domain. (A) The indicated radiolabeled precursors were incubated with OMV for 20 min at 0°C. Samples were then adjusted to the indicated KCl concentrations. OMV were reisolated, dissolved in sample buffer, and analyzed for bound precursor proteins by SDS-PAGE and phosphorimaging. The amount of preprotein bound at 20 mM salt was set as 100%. (B) pBCS1(1-126)ΔTM-DHFR was incubated with OMV for 20 min at 25°C in the presence or absence of excess amounts of pSu9(1-69)-DHFR. The reaction mixtures were adjusted to 200 mM KCl at 0°C, and OMV were reisolated and resuspended in SEM buffer. Immunoprecipitation was performed with antibodies raised against Tom22 or Tom40 or with preimmune serum. To control for binding, an aliquot was removed before the coimmunoprecipitation and precipitated with TCA (Total). (C) pBCS1(1-126)-DHFR and pBCS1(1-126)ΔTM-DHFR were incubated with uncoupled mitochondria for 20 min at 25°C. The mitochondria were washed with a buffer containing 50 mM KCl, reisolated, and solubilized in 0.75% digitonin buffer. Further treatment and coimmunoprecipitation were as described in the legend to Fig. 1B. (D) The bands corresponding to immunoprecipitated proteins from three experiments as described in the legend for panel C were quantified. The average values are presented as percentages of the bound material.

All three receptors bound very weakly or not at all to peptides covering the first 30 amino acid residues and to the region of the transmembrane domain (Fig. 3). Relatively strong interaction was observed with residues 31 to 46. Both peptides contain three positively charged residues in positions 35, 37, and 40 that could be involved in this binding. Moderate to high binding of all three receptors to peptides covering the presequence-like helix at residues 69 to 83 (peptides 23 to 25) was observed (Fig. 3). Binding was strongest at the region of amino acid residues 91 to 126. While Tom20 displayed highest affinity to a stretch between amino acids 103 to 126 which contains four positively charged residues, Tom22 had a clear preference for residues 94 to 106, which comprise lysine residues at both termini (Fig. 3). These results suggest that several segments of BCS1, but not the transmembrane domain, can interact with the three Tom receptors. It appears that the segment comprising residues 91 to 126 that follows the presequence-like segment plays an important role in the recognition of BCS1 by the Tom complex.

Interaction of the transmembrane and presequence-like domains of the BCS1 precursor with the TOM complex. The overall import of BCS1 into the mitochondrial inner membrane requires the transmembrane domain of the protein. On the other hand, a BCS1 construct lacking the transmembrane segment was observed to bind to the surface of mitochondria in vitro, where it was completely exposed to added protease (20). To test whether the transmembrane segment has a major role in the suggested hydrophobic interaction of BCS1 precursor with the TOM complex, we investigated the binding to OMV of a hybrid precursor protein lacking this domain, BCS1(1-126)ΔTM-DHFR. The stability of binding was reduced in the presence of higher salt concentrations (Fig. 4A). However, even under high-salt conditions, the binding of BCS1(1-126)ΔTM-DHFR with the TOM complex was more stable than that of the matrix-destined precursor, pSu9-DHFR (Fig. 4A). Thus, in addition to the transmembrane domain, further elements of BCS1 contribute to its strong interaction with the TOM complex.

The construct lacking the transmembrane domain was incubated with OMV in the presence or absence of competing excess amounts of pSu9-DHFR (Fig. 4B). Binding and precip-

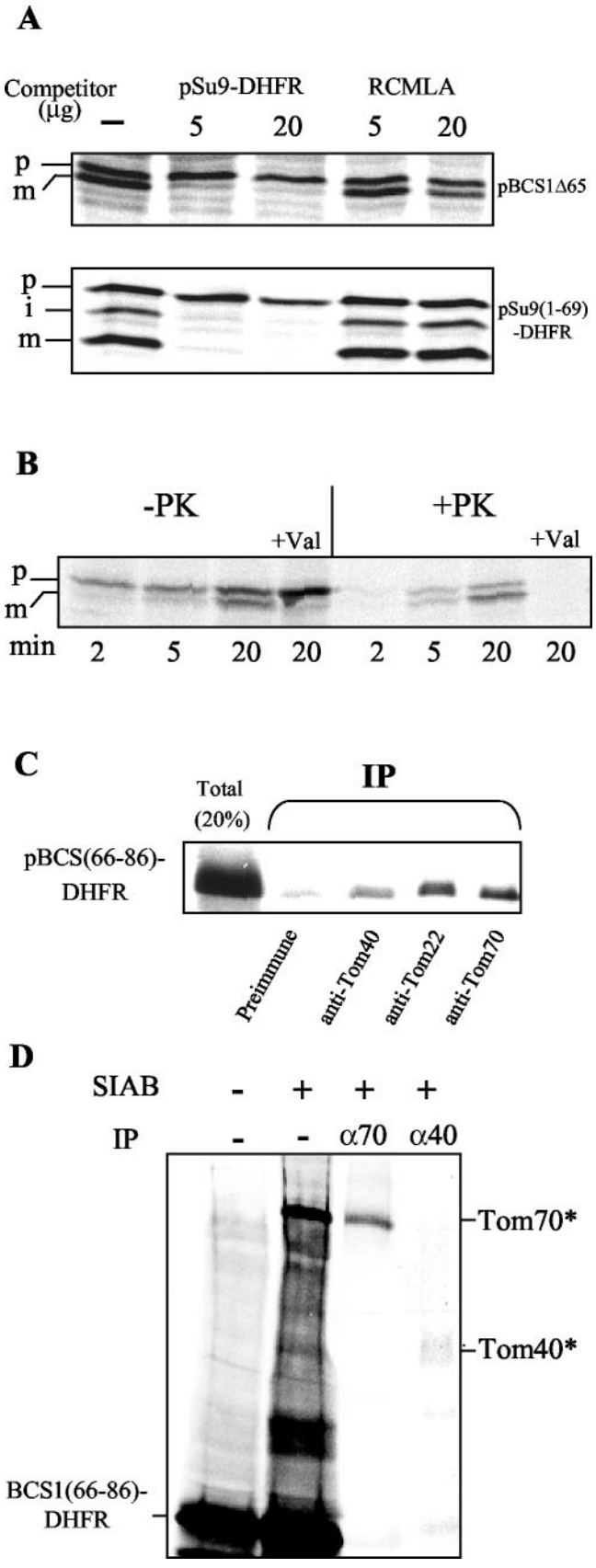

FIG. 5. The sequence comprising residues 66 to 86 of BCS1 behaves like a typical mitochondrial presequence and interacts with Tom components. (A) Radiolabeled pBCS1Δ1-65 and, as a control, pSu9 (1-69)-DHFR were incubated for 20 min at 15°C in SI buffer with either mitochondria (−) or mitochondria preincubated with the indicated amounts of competitor proteins for 2 min on ice. At the end of the import reactions, mitochondria were washed, reisolated, and analyzed by SDS-PAGE. p, precursor; i, intermediate-size form; m, mature form. (B) Residues 66 to 86 can direct a cytosolic protein into the mitochondrial matrix. Radiolabeled BCS1(66-86)-DHFR was incubated at 25°C for the indicated time periods with mitochondria in SI buffer in the absence or presence of valinomycin (1 μM). Mitochondria were reisolated, resuspended in SEM, and divided into two halves. One half was left on ice (−PK) while the other was treated with proteinase K (+PK). The import reactions were analyzed by SDS-PAGE. p, precursor form; m, mature form. (C) Residues 66 to 86 can promote interaction with the TOM complex. Radiolabeled pBCS1 (66-86)-DHFR was incubated with OMV for 20 min at 25°C. The OMV were washed with a buffer containing 100 mM KCl, reisolated, and solubilized in 0.75% β-dodecyl maltoside-containing buffer. Further treatment and immunoprecipitation (IP) were as described in the legend to Fig. 1B. (D) Radiolabeled BCS1(66-86)-DHFR precursor was incubated in the presence of MTX/NADPH with isolated OMV for 2 min at 0°C, followed by incubation for 5 min at 25°C. The chemical cross-linker *N*-succinimidyl[4-iodoacetyl]aminobenzoate (SIAB) was then added for a further 40 min at 10°C. Cross-linked samples were immunoprecipitated with antibodies against Tom70 or Tom40. Asterisks, adducts consisting of BCS1 cross-linked to Tom proteins.

itation by antibodies against Tom components were reduced when the competing precursor was present (Fig. 4B). Apparently, BCS1 precursor recognizes a binding site(s) on the general import pathway. Next, we performed comparative coimmunoprecipitation in which both BCS1(1-126)-DHFR and BCS1(1-126)ΔTM-DHFR were incubated with de-energized mitochondria and the capacity of antibodies against either Tom20 or Tom40 to precipitate the import intermediates was tested. The levels of coprecipitation were very similar for both precursors (Fig. 4C). Hence, the transmembrane domain is not required for the stable interaction with the TOM complex.

The other structural element in the import signal of BCS1 is an amphipathic helix that is similar to N-terminal mitochondrial targeting sequences and is rich in positively charged residues. We asked whether this import signal follows the general import pathway. The general import pathway was saturated with excess amounts of matrix-destined precursor, and import of radiolabeled pBCS1Δ1-65 and of pSu9(1-69)-DHFR as control was analyzed. Import of both precursor proteins was competed by the mitochondrial preprotein (Fig. 5A). Hence, pBCS1Δ1-65 is interacting with the TOM complex in a manner similar to that of matrix-destined precursors. To test whether the presequence-like segment has the capacity to target a cytosolic protein to the mitochondria, we constructed a chimeric precursor protein, BCS1(66-86)-DHFR. This precursor contains the presequence-like segment (amino acid residues 66 to 86 of BCS1) fused to the N terminus of DHFR. Upon incubation with energized mitochondria, a processed form of the preprotein was generated in a ΔΨ-dependent manner. The processed species was protected from degradation by proteinase K (Fig. 5B). Thus, the chimeric precursor was imported into the mitochondrial matrix, where it was processed by MPP. The presequence-like segment contains a potential MPP cleavage site after methionine 83. However, cleavage does not occur in the presence of an internal targeting signal (like in the native

precursor). It was efficiently processed when the N terminus of BCS1 is deleted (as in pBCS1Δ65) (20). We conclude that residues 66 to 86 of BCS1 behave like a mitochondrial targeting sequence.

We next studied the interactions of this presequence-like segment with the TOM complex. After incubation of BCS1 (66-86)-DHFR with OMV, antibodies against Tom components could precipitate the import intermediates (Fig. 5C). Furthermore, when chemical cross-linking was performed under these conditions, cross-linking adducts were formed between BCS1(66-86)-DHFR and both Tom70 and Tom40 (Fig. 5D). Thus, the interactions of this segment with the mitochondrial outer membrane are mediated by the TOM complex.

The role of the auxiliary import sequence of BCS1. An unexpected result of the peptide scan was the strong binding of all three receptors to the region of amino acid residues 91 to 126. Does this binding reflect physiologically meaningful binding to the TOM complex? We tested whether a construct lacking both the transmembrane domain and the presequence-like helix will still be able to bind the TOM complex. This BCS1 variant, BCS1Δ1-82, was found to bind OMV with a similar efficiency as that of a wild-type construct (Fig. 6A). This binding was reduced upon tryptic removal of the exposed cytosolic domains of the receptor proteins (Fig. 6A). A cytosolic protein, DHFR, displayed only background levels of binding under these conditions (not shown). To further study the capacity of residues 84 to 126 to bind the TOM complex, a fusion protein consisting of this region and of the cytosolic protein DHFR [BCS1(84-126)-DHFR] was incubated with OMV. Binding was observed which could be reduced by more than 40% upon competition with excess amounts of matrix-targeted precursor (Fig. 6B). The binding of pSu9-DHFR is presented for comparison.

The ability of amino acid residues 84 to 126 to be recognized by the TOM complex was further verified by coimmunoprecipitation. After binding of the fusion protein BCS1(84-126)-DHFR with OMV, incubation with antibodies against both Tom20 and Tom40 led to precipitation of the radiolabeled protein (Fig. 6C). This suggests a tight interaction of the fusion protein with the TOM complex. When OMV were pretreated with trypsin to remove the cytosolic domains of the import receptors and then incubated with BCS1(84-126)-DHFR, a reduced level of binding was observed. Nevertheless, also under these conditions, the precursor was attached to the pore-forming component Tom40 (data not shown). Thus, this domain interacts not only with the receptors but also with the core components of the TOM complex. To test the contribution to binding of residues 87 to 126 in the context of the full sequence, the binding of BCS1(1-86)-DHFR to OMV was compared to that of BCS1(1-126)-DHFR. The longer construct had a threefold higher binding capacity, demonstrating the auxiliary effect of residues 87 to 126 (Fig. 6D).

We conclude that the strong binding of Tom components to residues 91 to 126 of BCS1 reflects physiological affinity. In agreement with this observation, a construct lacking the first 82 amino acid residues of BCS1 was still able to bind to the mitochondrial outer membrane (20). In addition, amino acid residues 84 to 126 were found to be necessary for sorting to the inner membrane (20). Thus, amino acid residues 84 to 126 play an important role in the import of BCS1; this region is involved both in binding to the TOM complex and in correct sorting to the inner membrane.

BCS1 precursor crosses the TOM complex in a loop structure. Proteins of the carrier family and Tim23 pass through the TOM complex in a loop structure (12, 47, 53). We asked whether BCS1, also a protein with internal import signal, crosses the TOM complex in a similar manner. We constructed a protein where the DHFR moiety was fused to both termini of BCS1 and performed in vitro import experiments. The radiolabeled protein [DHFR-BCS1(1-250)-DHFR] was incubated with energized mitochondria in the presence of methotrexate, which stabilizes the folded conformation of DHFR and prevents its translocation across the outer membrane. When increasing amounts of trypsin were added under these conditions, specific proteolytic fragments of 14 to 16 kDa were formed (Fig. 7A). No such fragments were generated when BCS1 was treated with trypsin in the absence of mitochondria. In this case, only the folded DHFR domain was protected from degradation (Fig. 7A). Thus, the fragments are import specific. The protected fragments remained attached to mitochondria when the organelles were sedimented after trypsin treatment. In contrast, the folded DHFR was released to the supernatant (Fig. 7B). We suggest that the protected fragments reflect an intermediate where the internal import signal is imported into the mitochondria while both termini are still at the surface of the outer membrane.

To determine whether the import intermediate of DHFR-BCS1(1-250)-DHFR was attached to the TOM complex, we used blue native gel electrophoresis (BNGE). After import into isolated energized mitochondria and analysis by BNGE, a fraction of the precursor was found in a high-molecular-weight complex (Fig. 7C). The complex containing the accumulated radiolabeled precursor was larger than the TOM complex, apparently due to the added mass of the precursor. The observation that only part of the precursor runs together with the TOM complex is not surprising considering the fact that even components of the TOM complex, like Tom20 and Tom70, dissociate from the complex upon BNGE (15). We next verified that the precursor molecules which are found in a high-molecular-weight complex are indeed attached to the TOM complex rather than to other complexes in the inner membrane. The imported precursor, like the cytosolic domain of Tom22, was completely digested when proteinase K was added at the end of the import reaction (Fig. 7C). Hence, DHFR-BCS1(1-250)-DHFR accumulates at the outer membrane, most probably at the TOM complex.

DISCUSSION

What are the signals in a precursor with internal targeting and sorting information, and how are these signals decoded by the mitochondrial TOM complex? The inner membrane protein BCS1, used here as a model protein, was among the first mitochondrial proteins for which the internal import signal was analyzed (20). To date, only few internal import signals have been identified (4, 16–18, 27). Amino acid residues 1 to 126 of BCS1 were shown to contain all of the required information for targeting and sorting of the protein (20). Four sequence elements can be identified in this region: (i) the N-terminal residues 1 to 50, (ii) a putative transmembrane domain at

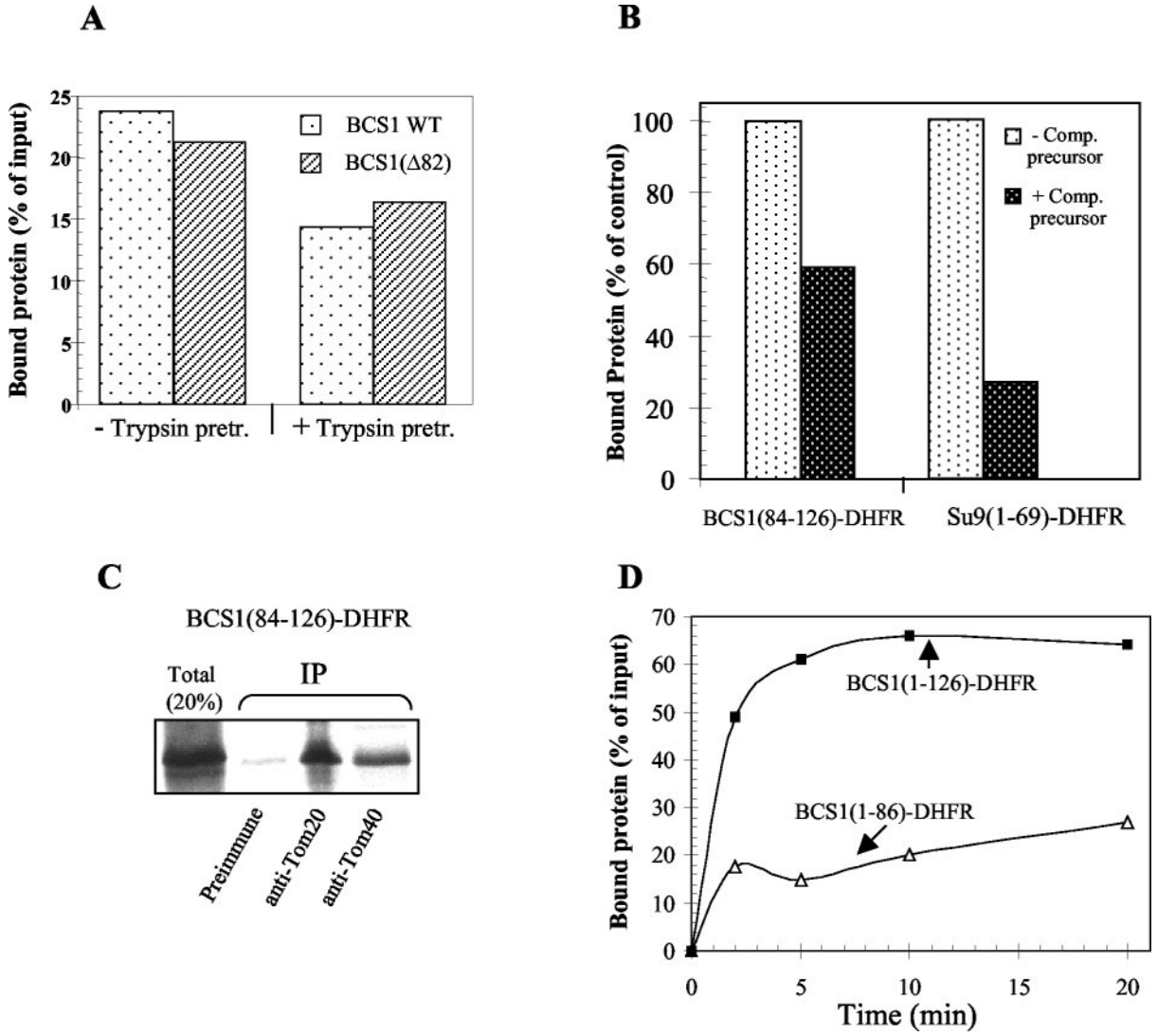

FIG. 6. The sequence comprising residues 84 to 126 of BCS1 can promote binding to the TOM complex. (A) Precursor lacking the first 82 amino acid residues of BCS1 can bind to OMV in a receptor-dependent manner. Radiolabeled BCS1 and BCS1(Δ1-82) were incubated for 20 min at 25°C with either intact OMV or OMV pretreated with trypsin. OMV were then washed with buffer containing 200 mM KCl, reisolated, and analyzed by SDS-PAGE and phosphorimaging. The average of results of three experiments is presented. (B) Residues 84 to 126 of BCS1 promote binding to the TOM complex. Radiolabeled precursors of BCS1(84-126)-DHFR or pSu9(1-69)-DHFR for comparison were incubated for 20 min at 0°C with OMV in the absence (−Comp. precursor) or presence (+Comp. precursor) of excess amounts of pSu9(1-69)-DHFR. Further treatment was as described in the legend for panel A. The amount of protein bound to untreated OMV was set to 100%. (C) BCS1(84-126)-DHFR bound to OMV can be coimmunoprecipitated with components of the TOM complex. Radiolabeled precursor was incubated for 20 min at 25°C with OMV. The OMV were then treated with buffer containing 100 mM KCl. OMV were reisolated, pelleted, and resuspended in SEM buffer. Immunoprecipitation (IP) was performed with antibodies raised against Tom20 or Tom40 or with preimmune serum. To control for binding, an aliquot was removed before coimmunoprecipitation and precipitated with TCA (Total). (D) Residues 87 to 126 of BCS1 increase binding to OMV. BCS1(1-86)-DHFR and BCS1(1-126)-DHFR were incubated with OMV in the presence of MTX/NADPH at 15°C for the indicated time periods. OMV were then washed with buffer containing 20 mM KCl, reisolated, dissolved in sample buffer, and analyzed by SDS-PAGE and phosphorimaging. For each protein, the amount of radiolabeled precursor added to the reaction was set to 100%.

amino acids 51 to 68, (iii) a presequence type helix (residues 69 to 83), and (iv) an import-auxiliary region at residues 84 to 126.

The N-terminal region does not play a role in targeting and sorting of the protein. It can be removed without affecting the import efficiency (20). Furthermore, to date, it has been found only in yeast BCS1, suggesting that it does not play a crucial role in the import or function of the protein.

The transmembrane domain is an essential element of the internal import and sorting information of the BCS1 precursor and is highly conserved among various organisms. Nevertheless, the cytosolic domains of the Tom receptors do not bind to peptides corresponding to this segment. Removing the transmembrane segment or replacing it by a transmembrane segment of another inner membrane protein impaired sorting to the inner membrane but not targeting to the outer membrane and association with the TOM complex (reference 20 and our unpublished observation). Apparently, the transmembrane domain of BCS1 does not have a role in targeting the precursor

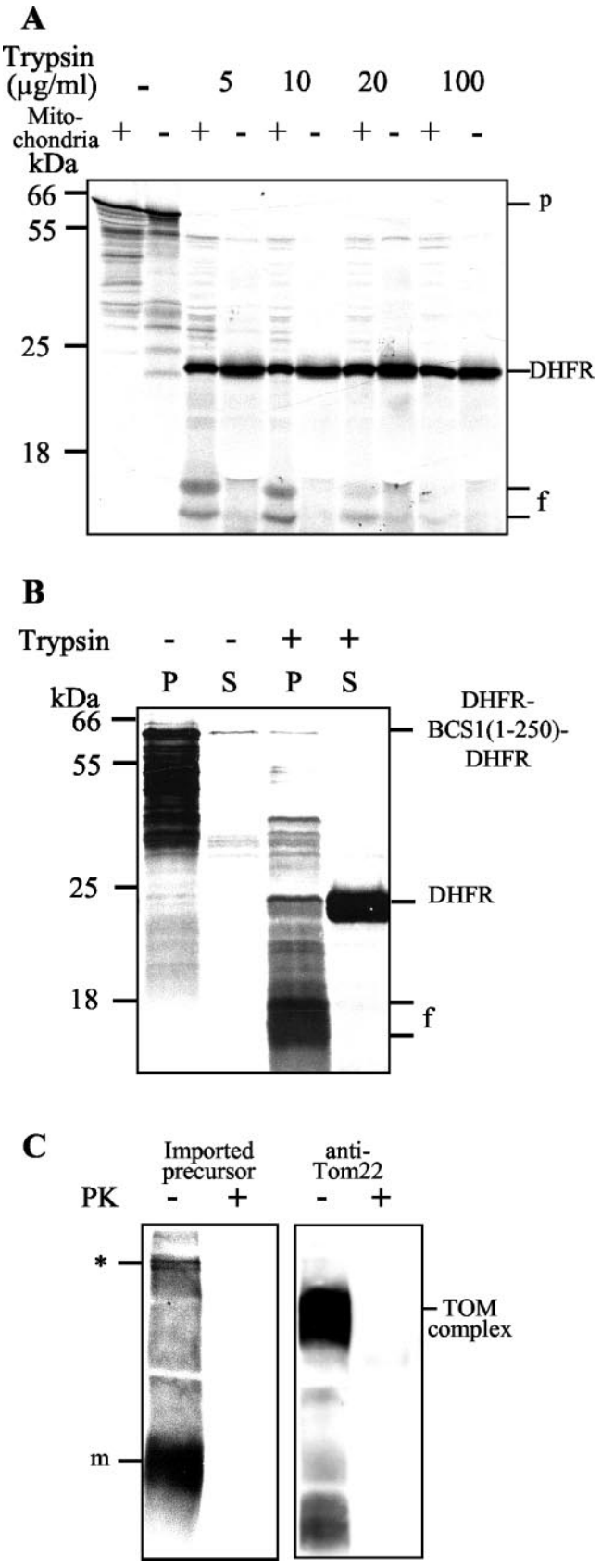

FIG. 7. BCS1 passes the TOM complex in a loop structure. (A) Radiolabeled DHFR-BCS1(1-250)-DHFR was incubated in a MTX/NADPH-containing buffer with mitochondria for 20 min at 25°C. The mitochondria were washed in a buffer containing 20 mM salt, resuspended in SEM buffer, and treated with the indicated concentration of trypsin for 15 min on ice. After inhibition of the protease by PMSF, samples were precipitated with TCA and analyzed by SDS-PAGE. The bands corresponding to the DHFR domain, the precursor protein (p), and the proteolytic fragments (f) are indicated. (B) Radiolabeled DHFR-BCS1(1-250)-DHFR was incubated with mitochondria as described in the legend for panel A. After treatment with trypsin (5 μg/ml), the mitochondria were spun down. Pellets were dissolved directly in sample buffer, while the supernatants were first precipitated with TCA and then dissolved in sample buffer. All samples were analyzed by SDS-PAGE. The bands corresponding to the DHFR domain and the proteolytic fragments (f) are indicated. (C) Radiolabeled DHFR-BCS1(1-250)-DHFR was incubated in a MTX/NADPH-containing SI buffer with mitochondria for 20 min at 25°C. The samples were halved; one aliquot was treated with proteinase K (20 μg/ml), and the other was left untreated. The mitochondria were sedimented, dissolved in buffer containing 0.4% digitonin, and analyzed by BNGE. The left panel shows the autoradiography, while the right panel represents immunodecoration of the same membrane with antibody against the cytosolic domain of Tom22. The radiolabeled precursor migrating with the TOM complex is indicated with an asterisk.

to the mitochondrial surface or in the interaction with the TOM complex. Rather, it is involved in the sorting and insertion into the mitochondrial inner membrane.

The various components in the mitochondrial import machinery were reported to have different affinities towards transmembrane segments. The cytosolic domains of Tom20 and Tom22 bind very weakly or not at all to peptides representing the transmembrane segments from another inner membrane protein with internal import information, the phosphate carrier (8). Tom70 did not bind the transmembrane domain of BCS1, while it binds part of the charged and part of the uncharged peptides of the phosphate carrier. Hence, a hydrophobic character of a segment is not sufficient to ensure its recognition by Tom70. The small Tim components (Tim9-Tim10 complex) display a completely different behavior; they have a clear preference for binding the transmembrane segments of the ADP/ATP carrier (11). Future studies on the affinity of import components towards other internal import signals will help to draw a more detailed picture of the recognition of hydrophobic stretches within mitochondrial precursors.

The presequence-like helix flanking the C terminus of the transmembrane domain bound to all three Tom receptors. Despite its similarity to matrix-targeting signals, a replacement of this segment by authentic presequences did not result in correct sorting of the mutated precursor both in vivo and in vitro (data not shown). Hence, this helix is probably involved in specific intra- or intermolecular interactions.

The strongest binding of the TOM complex was to peptides corresponding to residues 92 to 109. The potential of this segment, similar to mitochondrial presequences, to form an α-helix with positive charges on one face may explain the strong binding of import receptors to this conserved segment. While this region previously was suggested to have only a marginal effect on import (20), we show here that, in fact, this region of BCS1 precursor plays an essential role in the translocation of BCS1 across the outer membrane. A precursor lacking this region was not properly inserted into the inner membrane (20). Moreover, this region can specifically direct

the BCS1 precursor from the cytosol to the mitochondrial outer membrane. The importance of this element is underlined by its evolutionary conservation from yeast to *Drosophila* and humans (data not shown).

The interaction of the TOM complex with BCS1 translocation intermediates is different from its interactions with precursors carrying mitochondrial presequences. A recent study demonstrates that Tom20 binds the hydrophobic face of the amphiphilic helix of the presequence (1). Nevertheless, under conditions in which binding to surface receptors is prevalent, presequence-containing precursors interact with the TOM complex in a mainly electrostatic manner. BCS1 precursor was observed to interact with the TOM complex in a more hydrophobic character. Despite these different modes of binding, a recombinant preprotein can outcompete the import of BCS1. Thus, BCS1 uses the same import pore for passage across the outer membrane as preproteins destined to the matrix.

We suggest that the BCS1 precursor interacts with the TOM complex initially via residues 69 to 126 and that parts of the precursor then are moving as a loop structure into and through the TOM complex. The pore of the TOM complex is estimated to have a diameter of ca. 25 Å (22, 31, 50) and hence could accommodate such a loop structure. Moreover, the precursors of members of the carrier family and of Tim23 were also suggested to be inserted in a loopwise fashion into the TOM complex (12, 19, 53). This is in contrast to preproteins with cleavable presequences, which appear to enter the TOM complex in a linear fashion with the N terminus first. Thus, we may speculate that crossing of the TOM complex in a loop structure is a general characteristic of inner membrane proteins with internal import signal. A possible role of the auxiliary region could be to shield the transmembrane domain in order to prevent it from unproductive interactions with parts of the translocation pore. As the BCS1 precursor emerges from the translocation pore of the TOM complex, it is taken over by the TIM23 complex (20).

In conclusion, the import and intramitochondrial sorting of BCS1 require three distinct regions of the protein, namely, the transmembrane segment, the presequence-like helix, and an auxiliary region. The latter two elements are able to interact simultaneously with or sequentially to several Tom components, whereas the initial recognition does not require the transmembrane domain. The auxiliary region represents a novel type of signal with targeting and sorting functions. It is decoded by all three known mitochondrial import receptors. Such multiple interactions could increase efficiency and mediate quality control. Once the precursor emerges from the TOM complex, all three structural elements are essential for the intramitochondrial sorting to the inner membrane.

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