The h-region of twin-arginine signal peptides supports productive binding of bacterial Tat precursor proteins to the TatBC receptor complex

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The twin-arginine translocation (Tat) pathway transports folded proteins across bacterial membranes. Tat precursor proteins possess a conserved twin-arginine (RR) motif in their signal peptides that is involved in their binding to the Tat translocase, but some facets of this interaction remain unclear. Here, we investigated the role of the hydrophobic (h-) region of the Escherichia coli trimethylamine N-oxide reductase (TorA) signal peptide in TatBC receptor binding in vivo and in vitro. We show that besides the RR motif, a minimal, functional h-region in the signal peptide is required for Tat-dependent export in Escherichia coli. Furthermore, we identified mutations in the h-region that synergistically suppressed the export defect of a TorA[KQ]-30aa-MalE Tat reporter protein in which the RR motif was replaced with a lysine-glutamine pair. Strikingly, all suppressor mutations increased the hydrophobicity of the h-region. By systematically replacing a neutral residue in the h-region with various amino acids, we detected a positive correlation between the hydrophobicity of the h-region and the translocation efficiency of the resulting reporter variants. In vitro crossing-linking of residues located in the periplasmically-oriented part of the TatBC receptor to TorA[KQ]-30aa-MalE reporter variants harboring a more hydrophobic h-region in their signal peptides confirmed that unlike in TorA[KQ]-30aa-MalE with an unaltered h-region, the mutated reporters moved deep into the TatBC-binding cavity. Our results clearly indicate that, besides the Tat motif, the h-region of the Tat signal peptides is another important binding determinant that significantly contributes to the productive interaction of Tat precursor proteins with the TatBC receptor complex.

The twin-arginine translocation (Tat)2 system operates in parallel with the well studied general secretion (Sec) pathway (reviewed in Ref. 1) and translocates fully folded, often cofactor-containing or even oligomeric proteins across the cytoplasmic membrane of bacteria and archaea (reviewed in Refs. 2–8). It is closely related to the thylakoid APh pathway of plant chloroplasts (reviewed in Ref. 9). Tat-dependent precursor proteins are targeted to the Tat translocone by N-terminal signal peptides, consisting of a positively charged amino-terminal n-region that harbors the highly conserved Tat consensus motif S/T-R-R-X-F-L-K, a central hydrophobic core (h-region), and a polar carboxyl-terminal c-region containing the signal peptide recognition site (10). Various site-directed mutagenesis studies have shown that the two adjacent twin-arginine residues (RR) within the Tat consensus motif, from which the name of the Tat pathway is derived, are important determinants for the productive recognition and binding of Tat signal peptides by the Tat translocone. Depending on the sensitivity of the reporter system used in the respective studies, even the conservative substitution of the twin-arginines by a lysine pair either significantly reduced or completely abolished transport in most cases (11–17), whereas single mutations of one of the two consecutive arginines were largely tolerated and only reduced the export efficiency (11, 15, 18).

In Escherichia coli four genes (tatA, tatB, tatC, and tatE) encode the membrane-integral components of the Tat translocone that assemble on demand to form an active translocone. Previous studies revealed that the TatB and TatC components are assembled into higher-order 1:1 complexes in which at least four TatB monomers form a dome-like core structure that is surrounded by an outer ring of TatC molecules, thereby creating a deeply membrane-embedded binding cavity for the Tat substrate (17, 19). The involvement of the TatBC complex in binding N-terminal signal peptides of Tat-dependent substrates has been confirmed by various experimental procedures, such as the inhibition of substrate binding by antibodies against the thylakoidal orthologues of TatB and TatC, Hcf106 and cpTatC (20), co-immunoprecipitation or co-purification of Tat precursors and the Hcf106-cpTatC and TatBC complex, respectively (20, 21), the identification of suppressor mutations in TatB and TatC that restore the export of a transport-incom-
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potent Tat precursor (22, 23), as well as the identification of cross-links between Tat signal peptides and the TatBC complex (13, 17, 24–26). Substrate binding to TatBC subsequently triggers the proton motive force (PMF)-dependent recruitment of TatA oligomers (27), which are assumed to allow the passage of the folded protein through the membrane probably either by forming a size-fitting protein-conducting channel (28, 29) or, due to their short transmembrane domains, by destabilizing/weakening the membrane (30, 31). There is experimental evidence that Tat precursors might be first inserted into the lipid bilayer preceding their recognition by the TatBC receptor (32–34).

Several studies revealed that TatC is primarily responsible for the recognition and binding of the Tat consensus motif present in Tat signal peptides. Using site-directed cross-linking analysis, the cytosolic N terminus and the first cytosolic loop of TatC were shown to contact the signal peptide close to the RR motif in the n-region upon membrane binding. Vice versa, Tat precursors carrying photocross-linkers next to the RR motif were found in close proximity to TatC (13, 15, 17, 24, 35, 36). The cross-links between TatC and the Tat signal peptide were strictly dependent on an intact RR motif and were not obtained when the arginine residues had been replaced by a lysine pair (13, 17, 36). Importantly, the interactions between TatC and the precursor did not require the presence of TatB, indicating a hierarchy in the targeting process (13). According to this model and the findings of recent cross-linking studies, TatC acts as the primary substrate receptor that first recognizes the Tat signal peptide via its RR motif. Subsequently, the signal peptide insertion activity of TatC triggers a deep, hairpin loop-like insertion of the signal peptide into an enclosed binding cavity jointly formed by TatB and TatC (37), a process that was shown to strictly depend on the presence of an intact RR motif (16). In this so-called stage of advanced binding, the signal peptide is primarily located in close proximity to TatB, which in addition to the Tat consensus motif, also contacts the hydrophobic core (h-region) of the signal peptide as well as the surface-exposed regions of the folded mature domain, suggesting a cage-like structure of the TatB monomers surrounding the folded Tat substrate (13, 25).

As noted above, the RR motif is indisputably a major binding determinant of Tat precursor proteins to the Tat translocase. Here, we examined the contribution of the hydrophobic core of Tat signal peptides in TatBC receptor binding and analyzed the importance of the hydrophobicity of the h-region of the TMAO (trimethylamine N-oxide) reductase (TorA) signal peptide for in vivo Tat transport. We found that, besides an intact RR motif, a minimal uninterrupted h-region in the signal peptide is required for Tat-dependent export in E. coli. Furthermore, we identified mutations in the hydrophobic core that compensate for the loss of the RR residues and significantly restored the export of an otherwise transport-defective TorA[KQ1]-30aa-MalE reporter protein by increasing the hydrophobicity of the h-region. A positive correlation between the hydrophobicity of the hydrophobic core and the translocation efficiency of the respective reporter variants could be observed, indicating that the newly introduced hydrophobic residues might increase the binding affinity of the Tat precursor proteins to the hydrophobic substrate-binding cavity formed by TatB and TatC. Importantly, in vitro cross-linking results strongly suggest that the mutations in the h-region exert their effect on the advanced-stage binding of the TorA signal peptide to the TatBC receptor complex. Based on our combined results, we propose that the h-region of Tat signal peptides significantly contributes to the overall binding affinity and thus, besides the Tat consensus motif, is a second major binding determinant of Tat precursors to the TatBC receptor complex.

Results

A minimal uninterrupted hydrophobic core in the TorA signal peptide is required for the Tat-dependent export of a TorA-MalE precursor protein

Previously, a sensitive reporter system based on the Tat-specific fusion protein TorA-MalE was established that allows an easy in situ detection of Tat-dependent protein translocation on indicative media (22, 23). The plasmid-encoded TorA-MalE reporter protein consists of the signal peptide of the Tat substrate trimethylamine N-oxide reductase (TorA) fused to the mature protein part of the normally Sec-dependent maltose-binding protein (MalE). Because the presence of MalE in the periplasm is strictly required for maltose uptake (38), the Tat-specific export of TorA-MalE into the periplasm is directly linked with the ability of cells to utilize maltose and thus to grow on maltose minimal medium (MMM) as well as to form red colonies on MacConkey maltose (MCM) agar plates (22, 23, 39, 40).

Studies on the thylakoidal ΔpH-dependent pathway, which is closely related to the Tat system in bacteria, revealed that both the RR motif and the hydrophobic region of the signal peptide are required for efficient transport in plant chloroplasts (20, 41, 42). To determine whether an intact hydrophobic region is also a prerequisite for effective Tat-dependent export in bacteria, we replaced a hydrophobic valine residue (position 23) in the center of the h-region by a negatively-charged aspartate, resulting in an interruption of the hydrophobic core (Fig. 1A). In contrast to the positive control expressing the unaltered TorA-MalE precursor protein, the export of the mutated TorA[V23D]-MalE variant was completely abolished because the respective strain GSJ101 (a malE-negative derivative of the tat deletion strain DADE) co-expressing the Tat translocase did not grow on MMM and formed pale colonies on MCM (Fig. 1B). The export defect of TorA[V23D]-MalE was also directly demonstrated by cell fractionation experiments using EDTA-lysozyme spheroplasting to yield a combined cytosolic/membrane (C/M) fraction and a periplasmic fraction (P), which were subsequently separated by SDS-PAGE followed by Western blotting analysis using MalE-specific antibodies. Several MalE-derived polypeptides, which are present in the combined fraction of C/M of the positive control, represent the unprocessed precursor protein and its cytosolic degradation products, whereas mature-sized MalE that has been translocated across the cytoplasmic membrane can be detected in the periplasmic fraction (P) (Fig. 1B, lanes 3 and 4) (40). In full agreement with the in situ phenotypes described above, the unprocessed precursor accumulates in the cytosol and no mature MalE can be detected in the periplasmic
fraction of GSJ101 co-expressing the Tat translocase and the mutated TorA[V23D]-MalE reporter (Fig. 1B, lanes 1 and 2), showing that the negatively-charged aspartate in the hydrophobic core is not tolerated by the bacterial Tat system and renders the precursor inactive for translocation.

We previously demonstrated that a set of mutations in the TatBC receptor can suppress the export defect of another transport-incompetent TorA-MalE variant, TorA[KQ]-MalE, in which the crucial RR residues had been replaced with a lysine-glutamine pair, most likely by sufficiently increasing the overall binding affinity of the signal peptide to the TatBC-binding site and thus allowing export (22, 23). To investigate whether suppressing mutations in the Tat translocase can in principle also restore transport of the export-defective TorA[V23D]-MalE reporter, the E. coli strain GSJ101 expressing this mutated TorA-MalE variant was transformed with a library of mutagenized, plasmid-borne genes, encompassing TorA-MalE/TorA[V23D]-MalE precursor in the C/M fraction; asterisk, TorA-MalE/TorA[V23D]-MalE-derived degradation products in the C/M fraction. Positions of molecular weight markers are indicated on the left margin. The phenotypes of the respective strains on MMM (−, no growth; +++, fast growth) and C/M (pale, red) agar plates are shown in the boxes at the bottom of the figure.

for growth on MMM, were not associated with the tat genes, rather they were found to affect the hydrophobic region of the normally transport-incompetent precursor protein (Fig. 2A). Four different types of mutated reporter variants could be identified that were strictly Tat-specific, as judged by the inability to grow with maltose as the sole carbon and energy source in the absence of a functional Tat-translocase (Fig. 2B). In these reporter variants, the negatively-charged aspartate was either replaced with a neutral glycine or a hydrophobic tyrosine residue (TorA[V23G]-MalE; TorA[V23Y]-MalE), or several amino acids including the aspartate residue were deleted in the center of the hydrophobic core (TorA[ΔT22-V23]-MalE; TorA[ΔG19-G25]-MalE) (Fig. 2A). The export of the mutated TorA-MalE reporter variants was analyzed 1) indirectly by plate assays and 2) directly by determining the amount of MalE in the periplasm after membrane translocation. As shown in Fig. 2B, GSJ101 co-expressing the tat genes and TorA[V23G]-MalE, TorA[V23Y]-MalE, and TorA[ΔT22-V23]-MalE, respec-
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Traditionally, showed efficient growth on MMM and the formation of red colonies on MCM. In contrast, slow growth on MMM and the formation of light red colonies on MCM was observed with GSJ101 (pTorA[ΔG19-G25]-MalE, pHSG-TatABCE) harboring a severely shortened hydrophobic region in the signal peptide. Furthermore, the amounts of mature MalE in the periplasmic fractions of GSJ101 (pHSG-TatABCE) expressing the reporter variants TorA[V23G]-MalE and TorA[ΔT22-V23]-MalE, respectively, were found to be quite similar or somewhat lower compared with the positive control GSJ101 (pTorA-MalE, pHSG-TatABCE) (Fig. 2B, compare lane 1 with lanes 3 and 5). Strikingly, the replacement of Asp-23 with a hydrophobic tyrosine residue seemed to result in an even more efficient export compared with the unaltered reporter, indicating that an increased hydrophobicity of the h-region might enhance substrate-receptor interactions during productive binding (Fig. 2B, compare lanes 1 and 4). Furthermore, a positive correlation between the length of an intact hydrophobic core and the translocation efficiency of the respective reporter variants could be noticed. In full agreement with the results obtained from the plate assays, a deletion of two amino acid residues in the h-region of the reporter variant TorA[ΔT22-V23]-MalE allowed for a significantly better export compared with a deletion of seven residues, which caused a substantial truncation of the h-region (Fig. 2B, compare lanes 5 and 6). These combined results clearly show that a minimal uninterrupted hydrophobic region of the TorA signal peptide is required for Tat-dependent export in E. coli.

Mutations in the h-region of the TorA signal peptide can suppress the export defect of a TorA[KQ]-30aa-MalE precursor protein

In the previous paragraphs, it was shown that, besides an intact RR motif in the TorA signal peptide, a minimal functional hydrophobic core must additionally be present to allow export by the E. coli Tat translocase. We therefore hypothesize that a certain hydrophobicity of the signal peptide is necessary to provide a critical amount of interactions within the hydrophobic TatBC-binding cavity needed for productive binding and thus, for effective export. In the following, we aimed to determine whether the h-region of the TorA signal peptide contributes to the overall binding affinity and thus is directly involved in the binding process to the TatBC receptor complex. Analogous to the previous isolation of suppressing mutations in TatBC, which restored export of the otherwise transport-incompetent TorA[KQ]-MalE reporter protein (22, 23), we now performed a genetic screen for intragenic mutations specifically located within the hydrophobic core of the TorA signal peptide. Here, the export-defective reporter protein TorA[KQ]-30aa-MalE was used as the starting point for the following mutagenesis studies that, in addition to the TorA signal peptide, also contains the first 30-amino acid residues of the mature part of TorA fused to the mature protein region of MalE.

Using pTorA[KQ]-30aa-MalE as template, the entire TorA signal peptide was mutagenized by error prone PCR. The resulting error prone PCR fragments were cloned into the low-copy vector pBR1MCS-2 carrying the torA[KQ]-30aa-MalE fusion gene, thereby replacing the otherwise unaltered TorA[KQ] signal by a pool of randomly mutagenized signal peptides. The constructed library of mutated pTorA[KQ]-30aa-MalE plasmids was subsequently used for transformation of GSJ101 (pHSG-TatABCE) and the selection for intragenic suppressors of the export defect of TorA[KQ]-30aa-MalE on MMM agar plates. After up to 5 days of incubation, the formation of single colonies was observed. Ninety-five colonies were randomly chosen, tested for reproducible growth on MMM, and further analyzed with respect to the DNA sequence of the mutated torA[KQ]-30aa-MalE fusion genes.

DNA sequencing revealed different types of mutated TorA[KQ]-30aa-MalE precursors carrying single or multiple amino acid alterations in the TorA signal peptide. A reversion of the KQ mutation in the Tat consensus motif to RR was found in 14 mutants, however, in case of six suppressors (designated iKQ-X for intragenic KQ suppressor with X being the respective amino acid alteration), the mutations were solely located within the hydrophobic region or at the boundary between the n- and h-regions (Fig. 3A). Interestingly, all of these single mutations were substitutions of the respective amino acids by more hydrophobic residues, suggesting that suppression of the export defect occurs by means of an increase of the overall hydrophobicity and thus of the quality of the h-region.

First, we tested whether the isolated TorA[KQ]-30aa-MalE variants were still strictly exported via the Tat pathway by analyzing their in situ phenotypes on indicative media in the absence of a functional Tat translocase in the AmalEΔtatABCE deletion mutant GSJ101. In all cases, no growth on MMM and the formation of pale colonies on MCM agar plates was observed, clearly excluding the possibility that export of the TorA[KQ]-30aa-MalE suppressor mutant reporters had occurred via the Sec pathway (Fig. 3B).

Next, the mutated TorA[KQ]-30aa-MalE reporter variants were further analyzed with respect to the suppressing activities of the corresponding mutations either by analyzing the export of MalE indirectly by MMM and MCM plate assays or directly by determining the amount of MalE in the periplasmic fractions after cell fractionation (Fig. 3B, supplemental Fig. S1A). The relative export efficiency (reflected by the amount of matured MalE present in the periplasm) of strain GSJ101 (pTorA-30aa-MalE; pHSG-TatABCE), to which all further relative translocation efficiencies described in the present work will be related, was set to 100% (Fig. 3C). As shown in Fig. 3B and supplemental Fig. S1A, strains expressing the various mutated reporter proteins showed diverse in situ phenotypes in the plate assays because they formed differently colored colonies on MCM agar plates and exhibited different growth rates with maltose as the sole carbon and energy source. The mutations T22I, G25V, and G25W, located quite in the center of the hydrophobic region showed the highest suppressing activities, resulting in the formation of red or, in case of G25W, light red colonies on MCM and the fastest growth on MMM of the corresponding strains. In comparison, the substitutions A16V and T22A promoted clearly weaker suppression phenotypes on the indicator plates (i.e., slow growth on MMM and pale colonies on MCM). However, in all cases, significant growth on MMM was observed in contrast to the negative control GSJ101 (pHSG-TatABCE) expressing the export-defective TorA[KQ]-30aa-
MaLE reporter with an unaltered h-region. The latter finding indicates that the amino acid alterations, present in the h-region, are in fact the reason for the suppression of the transport defect. Furthermore, the export behavior conferred by the different substitutions in the h-region suggested that a functional relationship exists between hydrophobicity of the TorA signal peptide and the export efficiency of the respective reporter proteins. A replacement of Thr-22 or Gly-25 with a highly hydrophobic isoleucine or valine residue, respectively, evidently resulted in much higher export of the corresponding reporter variants compared with substitutions by less hydrophobic amino acids, i.e. alanine or tryptophan, as indicated by faster growth on MMM and the formation of red colonies on MCM (Fig. 3B, lanes 4–7). As shown in Fig. 3B, various MaLE-derived polypeptides corresponding to the unprocessed precursor and its cytosolic degradation products can be detected in the C/M fractions of GSJ101 (pHSG-TatABCE) expressing the unaltered TorA-30aa-MaLE reporter (positive control; lane 1), the export-defective TorA[KQ]-30aa-MaLE (negative control; lane 2), or the various TorA[KQ]-30aa-MaLE reporter variants harboring the h-region-located suppressor mutations (lanes 3–8). Significant amounts of mature-sized MaLE were only detected in the P fractions of GSJ101 co-expressing the Tat translocase together with the positive control and the suppressor mutants iKQ-T22I, iKQ-G25V, and iKQ-G25W, respectively, suggesting that the exported amounts of MaLE are in the other cases too low to be detectable at the protein level. Quantification of the chemiluminescence signals further confirmed that the export efficiencies were generally quite low compared with the positive control (100%), with values between 0.9% observed for the weakest isolated suppressor mutant iKQ-T22I and 13.5% for iKQ-G25V (Fig. 3C). Nevertheless, the combined data strongly suggest that mutations in the h-region can compensate to a certain degree for the loss of the crucial RR residues and restore the export of the TorA[KQ]-30aa-MaLE reporter by increasing the hydrophobicity of the TorA signal peptide and thus likely enhancing the substrate-receptor interactions within the hydrophobic TatBC-binding cavity. Hence, the present results clearly support the hypothesis that the hydrophobic core might be directly involved in the productive binding of the TorA signal peptide to the TatBC receptor complex.

**Combinations of mutations in the h-region of the TorA signal peptide synergistically suppress the export defect of a TorA[KQ]-30aa-MaLE precursor protein**

As shown before, mutations in the h-region of the TorA signal peptide can suppress the export defect of a TorA[KQ]-30aa-MaLE precursor protein by increasing the hydrophobicity of the signal peptide. Next, we addressed the question whether combinations of those single h-region-located mutations would further enhance the export of a TorA[KQ]-30aa-MaLE precursor. For the construction of five various double mutants, the corresponding single mutations were introduced into the pTorA[KQ]-30aa-MaLE reporter with an unaltered h-region. The latter finding indicates that the amino acid alterations, present in the h-region, are in fact the reason for the suppression of the transport defect. Furthermore, the export behavior conferred by the different substitutions in the h-region suggested that a functional relationship exists between hydrophobicity of the TorA signal peptide and the export efficiency of the respective reporter proteins. A replacement of Thr-22 or Gly-25 with a highly hydrophobic isoleucine or valine residue, respectively, evidently resulted in much higher export of the corresponding reporter variants compared with substitutions by less hydrophobic amino acids, i.e. alanine or tryptophan, as indicated by faster growth on MMM and the formation of red colonies on MCM (Fig. 3B, lanes 4–7). As shown in Fig. 3B, various MaLE-derived polypeptides corresponding to the unprocessed precursor and its cytosolic degradation products can be detected in the C/M fractions of GSJ101 (pHSG-TatABCE) expressing the unaltered TorA-30aa-MaLE reporter (positive control; lane 1), the export-defective TorA[KQ]-30aa-MaLE (negative control; lane 2), or the various TorA[KQ]-30aa-MaLE reporter variants harboring the h-region-located suppressor mutations (lanes 3–8). Significant amounts of mature-sized MaLE were only detected in the P fractions of GSJ101 co-expressing the Tat translocase together with the positive control and the suppressor mutants iKQ-T22I, iKQ-G25V, and iKQ-G25W, respectively, suggesting that the exported amounts of MaLE are in the other cases too low to be detectable at the protein level. Quantification of the chemiluminescence signals further confirmed that the export efficiencies were generally quite low compared with the positive control (100%), with values between 0.9% observed for the weakest isolated suppressor mutant iKQ-T22I and 13.5% for iKQ-G25V (Fig. 3C). Nevertheless, the combined data strongly suggest that mutations in the h-region can compensate to a certain degree for the loss of the crucial RR residues and restore the export of the TorA[KQ]-30aa-MaLE reporter by increasing the hydrophobicity of the TorA signal peptide and thus likely enhancing the substrate-receptor interactions within the hydrophobic TatBC-binding cavity. Hence, the present results clearly support the hypothesis that the hydrophobic core might be directly involved in the productive binding of the TorA signal peptide to the TatBC receptor complex.

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A[KQ]-30aa-MalE vector by site-directed mutagenesis and the resulting pTorA[KQ]-30aa-MalE plasmid variants were subsequently transformed into GSJ101 expressing the tatABCE genes. In suppressor double mutants iKQ-G25V/G28W and iKQ-G25W/G28W, the single mutations G25V and G25W, which already showed relatively strong or moderate suppressing activities in the in situ plate assays (i.e., efficient growth on MMM; red and light red colonies on MCM, respectively; Fig. 10870 J. Biol. Chem. (2017) 292(26) 10865–10882

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A. TorA (wt) ---SRRFLAQLGGLTVAGMLGPSL---
TorA[KQ] ---SKQRFLAQGLTVAGMLGPSL---
TorA[KQ, G25V, G28W] ---SKQRFLAQGLTVAMLWPSL---
TorA[KQ, G25W, G28W] ---SKQRFLAQGLTVAWMLWPSL---
TorA[KQ, A16V, G25W] ---SKQRFLVQGGLTVAGMLGPSL---
TorA[KQ, A16V, G28W] ---SKQRFLVGLTVAGMLWPSL---
TorA[KQ, T22A, G28W] ---SKQRFLAQGLAVAGMLWPSL---

B. TorA wt KQ KQ G25V KQ G25W KQ G25V G28W KQ G25W G28W
P
C/M

C. TorA wt KQ KQ T22A KQ G25W KQ G28W KQ T22A G25W G28W

D. TorA wt KQ KQ A16V KQ G25W KQ G28W KQ A16V G25W G28W KQ A16V T22A G25W G28W

E. Relative export efficiency [%]

TorA: wt KQ A16V T22A G25V G28W
Role of h-region of Tat signal peptides

4B; supplemental Fig. S1B) were combined with one of the weakest isolated suppressing mutation, G28W, located in the C-terminal half of the h-region. Both double mutant reporter proteins promoted a stronger suppression phenotype in the plate assays reflected by faster growth on MMM and the formation of red colonies on MCM of the corresponding strains in comparison to the suppressing activities conferred by the respective single mutations. Importantly, the export of these reporter variants was strictly Tat-specific and not due to an (at least partial) export via the Sec pathway, because no export was detectable in the in situ plate assays in the absence of a functional Tat translocase (Fig. 4B). In full agreement with the in situ phenotypes, a striking increase of MalE export into the periplasm conferred by the combined mutations was also observed at the protein level. As shown in Fig. 4, B and E, the respective single mutations were found to synergistically contribute to the suppression of the TorA[KQ]-30aa-MalE export defect because the export efficiencies observed for the double mutants iKQ-G25V/G28W and iKQ-G25V/G28W (28.2 and 19.2%, respectively) were significantly higher than the sum of the translocation efficiencies observed for the corresponding single mutants iKQ-G25V (13.5%), iKQ-G25V (3.7%), and iKQ-G28W (1.4%). The synergy between two suppressing mutations in the h-region is especially evident when the mutations G25W and G28W, which by themselves did not possess sufficient suppressing activities to allow for the formation of red colonies on MCM, were combined, thereby giving rise to a strong suppressor double mutant that showed highly efficient export into the periplasm.

All mutations being combined so far mapped to the C-terminal half of the h-region. To test whether the observed synergistic effects of double mutations on the translocation efficiency of a TorA[KQ]-30aa-MalE precursor protein depend on their localization in the hydrophobic core, mutations distributed all over the h-region were combined giving rise to the double mutant reporter variants iKQ-A16V/G25W, iKQ-A16V/G28W, and iKQ-T22A/G28W. As described in the previous paragraphs, the presence of single mutations A16V, T22A, G25W, and G28W, respectively, in the h-region was sufficient to allow low, but nevertheless, significant export of a TorA[KQ]-30aa-MalE precursor protein reflected by the ability of the corresponding cells to grow with maltose as the sole carbon source (Figs. 3B and 4, C and D). Although only very low levels of TorA[KQ]-30aa-MalE export were promoted by those single suppressing mutations, resulting in relative export efficiencies of 0.9 to 3.7%, a substantial increase in the relative translocation efficiency was found for GSJ101 co-expressing the Tat translocase and the corresponding double mutant reporter proteins iKQ-A16V/G25W (14.7%), iKQ-A16V/G28W (25.2%), or iKQ-T22A/G28W (12.6%) (Fig. 4E). The amounts of the exported mutated TorA[KQ]-30aa-MalE variants in the periplasmic fractions of the respective strains totally reflected their phenotypic behavior observed on indicative agar plates (i.e., highly efficient growth on MMM; deep red colonies on MCM) (Fig. 4, C and D, supplemental Fig. S1, C and D). Also for these TorA[KQ]-30aa-MalE variants, Sec-dependent translocation could be excluded (Fig. 4, C and D). Taken together, the results clearly demonstrate that the isolated suppressing mutations in the h-region, when combined, synergistically act together in promoting export of the otherwise transport-incompetent TorA[KQ]-30aa-MalE precursor protein irrespective of their particular position within the hydrophobic core of the TorA signal peptide. The latter finding strongly suggests that the entire h-region is directly involved in the binding process of Tat signal peptides to the TatBC receptor complex.

The hydrophobicity of the h-region of the TorA signal peptide correlates with the export efficiency of the corresponding TorA[KQ]-30aa-MalE reporter variants

As noticed above, hydrophobicity of the h-region seemed to be correlated to the export efficiency of the respective TorA[KQ]-30aa-MalE reporter variants, because an increase in hydrophobicity resulted in increased export into the periplasm (Figs. 3, B and C, and 4, B–E). To investigate the correlation between hydrophobicity of the h-region and the translocation efficiency of the corresponding Tat precursor in more detail, further site-directed mutagenesis studies were performed. As shown in Fig. 5A, the amino acid glycine at position 25 in the center of the h-region was substituted by hydrophobic (methionine, cysteine, or alanine) or neutral/hydrophilic (threonine, serine, or asparagine) residues, respectively. Because many hydrophobicity scales for amino acids have been developed to date with significant variations in the hydrophobicity values, we chose a range of scales that were derived by different methods and under various conditions for the following comparative analyses (43–48) (Table 1). However, some common trends in the distribution of the chosen amino acid residues can be observed within these scales. Whereas non-polar amino acids,

Figure 4. Combinations of mutations in the h-region of the TorA signal peptide synergistically suppress the export-defect of a TorA[KQ]-30aa-MalE precursor protein.

A, amino acid sequences of TorA-30aa-MalE reporter variants encompassing the Tat consensus motif (dashed line) and the entire h-region of the TorA signal peptide. Positions of the particular mutations are underlined. B–D, subcellular localization of TorA-30aa-MalE-derived polyptides. Cells were fractionated into a P fraction and a combined C/M fraction by EDTA-lysozyme spheroplasting. The samples of the fractions corresponding to an identical localization in the hydrophobic core, mutations distributed all over the h-region were combined giving rise to the double mutant reporter variants iKQ-A16V/G25W, iKQ-A16V/G28W, and iKQ-T22A/G28W. As described in the previous paragraphs, the presence of single mutations A16V, T22A, G25W, and G28W, respectively, in the h-region was sufficient to allow low, but nevertheless, significant export of a TorA[KQ]-30aa-MalE precursor protein reflected by the ability of the corresponding cells to grow with maltose as the sole carbon and energy source (Figs. 3B and 4, C and D). Although only very low levels of TorA[KQ]-30aa-MalE export were promoted by
Table 1
Hydrophobicity scales for amino acid residues

| Amino acid | Monera et al. (43) | Kyte and Doolittle (44) | Hopp-Woods (45) | Argos (46) | Eisenberg and Weiss (47) | Engelman et al. (48) |
|------------|-------------------|------------------------|-----------------|------------|-------------------------|---------------------|
| Met        | 74                | 1.9                    | −1.3            | 2.96       | 0.26                    | 3.4                 |
| Val        | 76                | 4.2                    | −1.5            | 1.14       | 0.54                    | 2.6                 |
| Cys        | 49                | 2.5                    | −1.0            | 1.23       | 0.04                    | 2.0                 |
| Ala        | 41                | 1.8                    | −0.5            | 1.56       | 0.25                    | 1.6                 |
| Gly        | 0                 | −0.4                   | 0               | 0.62       | 0.16                    | 1.0                 |
| Thr        | 13                | −0.7                   | −0.4            | 0.91       | −0.18                   | 1.2                 |
| Ser        | −5                | −0.8                   | 0.3             | 0.81       | −0.26                   | 0.6                 |
| Asn        | −28               | −3.5                   | 0.2             | 0.27       | −0.64                   | −4.8                |

Table 5. Mutations in the h-region of the TorA signal peptide suppress the export defect of a TorA[KQ]-30aa-MalE precursor protein by increasing the overall hydrophobicity of the signal peptide. A, amino acid sequences of TorA-30aa-MalE reporter variants encompassing the Tat consensus motif (dashed line) and the entire h-region of the TorA signal peptide. Positions of the particular mutations are underlined. B and C, subcellular localization of TorA-30aa-MalE-derived polypeptides. Cells were fractionated into a P fraction and a combined C/M fraction by EDTA-lysozyme spheroplasting. The samples of the fractions corresponding to an identical amount of cells were subjected to SDS-PAGE and immunoblotting using anti-MalE antibodies. The positive control was E. coli GSJ101 containing plasmids pTorA-30aa-MalE and pHSG-TatABCE (lane 1). The other samples correspond to GSJ101 co-expressing the tatABCE genes and the export-defective TorA[KQ]-30aa-MalE reporter (lane 2). B, amino acid sequences of TorA-30aa-MalE reporter variants encompassing the Tat consensus motif (dashed line) and the entire h-region of the TorA signal peptide. Positions of the particular mutations are underlined. B, subcellular localization of TorA-30aa-MalE-derived polypeptides. Cells were fractionated into a P fraction and a combined C/M fraction by EDTA-lysozyme spheroplasting. The samples of the fractions corresponding to an identical amount of cells were subjected to SDS-PAGE and immunoblotting using anti-MalE antibodies. The positive control was E. coli GSJ101 containing plasmids pTorA-30aa-MalE and pHSG-TatABCE (lane 1). The other samples correspond to GSJ101 co-expressing the tatABCE genes and the export-defective TorA[KQ]-30aa-MalE reporter (lane 2). D, relative export efficiencies of the analyzed TorA-30aa-MalE reporter variants in strains expressing the Tat translocase. The amount of exported MalE protein in the P fraction of strains GSJ101 co-expressing the tatABCE genes and TorA-30aa-MalE or one of the mutated TorA[KQ]-30aa-MalE reporter variants was determined in at least three different independent experiments via quantification of the chemiluminescence signals. The signals were recorded by a CCD camera and subsequently analyzed by the program AIDA 4.50 (Raytest). The average values are indicated by horizontal marker lines; standard deviations by error bars. The relative export efficiency of the positive control GSJ101 (pTorA-30aa-MalE, pHSG-TatABCE) was set to 100%.
i.e. glycine, alanine, valine, and methionine, generally exhibit the highest hydrophobicity values, the polar amino acids threonine, serine, and asparagine can be considered as rather non-hydrophobic and hydrophilic residues. Despite the polarity of its thiol group, cysteine was found to stabilize hydrophobic interactions in micelles (49) and associate with hydrophobic regions of proteins, and thus is grouped among the hydrophobic amino acids in most hydrophobicity scales (50).

Based on the results obtained from the mutagenesis studies above, we expected that a replacement of Gly-25 with the more hydrophobic residues Met, Cys, or Ala would restore the export of a TorA[KQ]-30aa-MalE precursor protein to various degrees, dependent on the hydrophobic character of the respective amino acid that has been introduced into the hydrophobic core. As shown in Fig. 5B, all TorA[KQ]-30aa-MalE reporter protein variants, in which the native glycine residue has been replaced with more hydrophobic amino acids, were significantly translocated into the periplasm in a Tat-dependent manner, as indicated by growth on MMM and the formation of light red or red colonies on MCM of the respective strains harboring a functional Tat translocase. The observed export efficiencies paralleled the hydrophobicity of the introduced amino acids as shown 1) by the various in situ phenotypes on MCM (i.e. differences in red staining) and 2) by the quantification of the amounts of MalE-derived polypeptides in the periplasm after cell fractionation. As described above, the mutation G25V in the h-region was already classified as a relatively strong suppressor of the export defect of the TorA[KQ]-30aa-MalE precursor protein, conferring an export efficiency of 13.8% (Fig. 5, B, lane 3, and D). Surprisingly, the replacement of glycine with methionine resulted in an even higher MalE export (19.8%) (Fig. 5, B, compare lanes 3 and 4, and D). According to most popular hydrophobicity scales, methionine is assumed to be generally less hydrophobic than valine, however, the scale of Argos et al. (46) or Engelman et al. (48) places methionine above valine toward the hydrophobic end of the spectrum. In full agreement with the great majority of hydrophobicity scales, substitution of Gly-25 by cysteine or alanine, which compared with a valine substitution leads to a smaller increase of the hydrophobicity of the TorA[KQ] signal peptide, only promoted lower translocation efficiencies of 11.2 and 6.4%, respectively, and the formation of light red colonies of the corresponding cells on MCM (Fig. 5, B, lanes 5 and 6, and D).

Furthermore, we replaced Gly-25 with less hydrophobic, rather neutral/hydrophilic amino acid residues (i.e. threonine, serine, and asparagine) (Fig. 5C). Consistent with the corresponding hydrophobicity values, the amino acid alterations G25S and G25N were not expected to suppress the export defect of the TorA[KQ]-30aa-MalE reporter, whereas the effect of a replacement of Gly-25 with threonine on the export is rather unclear due to significant differences in the relative degree of hydrophobicity among the scales (43–48). However, in the case of all substitutions of glycine by less hydrophobic residues as well as by threonine, no export of the corresponding TorA[KQ]-30aa-MalE reporter variants was detected, as shown by the inability of the respective strains to grow on MMM in contrast to GS101 co-expressing the Tat translocase and the intragenic suppressor mutant iKQ-G25V described above (Fig. 5C). Besides, no significant differences in the translocation efficiency of these mutated reporter variants compared with the negative control (i.e. TorA[KQ]-30aa-MalE with an unaltered h-region) could be observed (Fig. 5D), confirming that a suppression of the export defect in fact requires the introduction of a more hydrophobic amino acid residue into the h-region. The latter finding, showing that a replacement of Gly-25 with any amino acid did not per se permit export of TorA[KQ]-30aa-MalE, further excludes that the observed effects are solely due to the removal of the α-helix-breaking glycine residue and that the resulting conformational changes of the signal peptide (i.e. extension of the α-helical structure), rather than the hydrophobicity might facilitate membrane insertion and thus, affect translocation, as it was previously shown for Sec signal peptides (51).

Our combined results clearly show that a correlation exists between the overall hydrophobicity of the h-region of the TorA signal peptide and the resulting translocation efficiency of the respective TorA[KQ]-30aa-MalE variants. Furthermore, our finding that MalE export in the absence of an intact RR motif, specifically required an increase in overall hydrophobicity of the h-region, provides further evidence for the participation of the hydrophobic core in the productive binding of Tat signal peptides to the TatBC receptor complex. This conclusion is also supported by analysis of the various h-regions by using the ΔG predictor program (52, 53), demonstrating that all mutant reporter proteins that show export possess a decreased ΔG value for their h-region compared with the unaltered TorA[KQ]-30aa-MalE reporter protein (supplemental Table S2).

Contacts between the h-region-coupled suppressor mutants and TatB/TatC confirm deep insertion of the reporter variants into the TatBC-binding cavity

So far, the suppressing activities of the h-region-located mutations were analyzed by in vivo transport assays. Because the export of the normally transport-incompetent TorA[KQ]-30aa-MalE precursor protein was significantly restored, we proposed that the mutations exert their effect by increasing the overall binding affinity of the respective reporter variants to the hydrophobic TatBC-binding site, thereby compensating for the lack of the binding contacts between the missing RR residues and TatBC. To directly analyze whether these suppressing mutations promote an advanced stage of signal peptide binding deep within the TatBC-binding cavity, site-specific in vitro cross-linking experiments were performed. The photo-activatable cross-linker p-benzoyl-phenylalanine (Bpa) was introduced at the N-terminal position Ile-4 of TatB or at residue Val-202 located in the transmembrane helix 5 (TM5) of TatC, as both residues were previously found to contact deep inserted RR precursors in the advanced binding stage (17). Additionally, Bpa was placed at position Leu-9 in the cytoplasmically localized N terminus of TatC, which was shown to constitute part of the primary recognition and binding site for the Tat consensus motif (13, 22). Inside-out inner-membrane vesicles (INV) were prepared from E. coli strains expressing the individual Bpa-containing variants of TatB and TatC, respectively, and were incubated with the in vitro synthesized and radioactively labeled TorA-30aa-MalE-derived reporter protein variants.
Cross-linking was induced by irradiation with ultraviolet light. Subsequently, radiolabeled cross-linking products were separated by SDS-PAGE and visualized by phosphorimaging. As shown in Fig. 6A, UV-light induced two prominent higher molecular cross-linking products when the Bpa variant Ile-4 of TatB was incubated with the transport-competent TorA[RR]-30aa-MalE (wt). Those adducts correspond by size to one or two TatB molecules binding to the precursor protein. These TatB adducts disappear by using the transport-incompetent TorA[KQ]-30aa-MalE precursor protein (KQ). As expected from the results of our preceding in vivo studies, 1:1 TorA-30aa-MalE-TatB complexes were primarily obtained with reporter variants exhibiting the highest export efficiencies in vivo. The most prominent contacts could be observed with the suppressor mutant iKQ-T22I. For a further in-depth characterization, we therefore chose the mutant reporter protein iKQ-T22I. To verify the embedment of this reporter variant in a binding cavity that is jointly formed by both Tat components TatB and TatC, we performed a cross-linking experiment using the Bpa variant of TatC (TatC(V202Bpa)). In full agreement with a deep insertion, TatC carrying Bpa at position Val-202 located in the TM5 in the trans-sided part of the lipid bilayer cross-linked to iKQ-T22I (Fig. 6B). No such cross-link was obtained with the transport-incompetent TorA[KQ]-30aa-MalE reporter harboring an unaltered h-region. In contrast, no TatC-iKQ-T22I adduct was visible when the cross-linker had been placed at residue Leu-9 in the cytoplasmic N terminus of TatC (supplemental Fig. S2).

The results described above clearly demonstrate that the TatABC translocase of the INV allows advanced-stage binding of the mutated TorA[KQ]-30aa-MalE variants, even if they carry an inactive KQ motif that prevents productive primary recognition of the signal peptide by TatC. Thus, we strongly suggest that mutations in the h-region exert their effect by directly affecting the binding step of the TorA signal peptide to the TatBC receptor complex upon insertion into the TatBC-binding cavity and prior to the PMF-dependent translocation of Tat substrates across the membrane. This was further elucidated by the use of two inhibitors of the PMF, CCCP and DCCD. CCCP dissipates the PMF by acting as protonophore, whereas DCCD interferes with the ATP-dependent generation of the proton gradient by the vesicles via ATPase inhibition. Recently we found that DCCD additionally prevents the insertion of Tat signal peptides into the TatBC-receptor complex.3

Figure 6. Suppressors of the TorA[KQ]-30aa-MalE export defect harboring mutations in the h-region of the TorA signal peptide are inserted deep into the TatBC-binding cavity. TorA-30aa-MalE reporter variants containing wild-type (wt) or mutated TorA* signal peptides harboring mutations in the RR motif and/or the h-region of the TorA signal peptide indicated above the lines were synthesized and radioactively labeled by in vitro transcription/translation in the presence of E. coli INV, which contained TatABC. Bpa was incorporated either (A, C) at the Ile-4 position in the N terminus of TatB or (B) at the Val-202 position in TM5 of TatC. In samples labeled with a +, cross-linking was initiated by irradiation with ultraviolet light or mock treated (−). Radiolabeled cross-linking products were separated by SDS-PAGE and visualized by phosphorimaging. Indicated are the positions of molecular size standard proteins, the various TorA*-30aa-MalE precursor proteins (p) and the cross-linked TatB-TorA*-30aa-MalE (black star) or TatC-TorA*-30aa-MalE (black square) complexes. C, effect of CCCP and DCCD on cross-linking between residue Ile-4 in TatB and TorA[KQ, T22I]-30aa-MalE (iKQ-T22I).

3 A.-S. Blümmel et al., unpublished data.
Cross-linking of iKQ-T22I to residue Ile-4 in the trans-exposed N terminus of TatB was not affected by CCCP confirming that its formation did not require the PMF. Strikingly, however, no cross-linking products were obtained in the presence of DCCD. The latter finding provides direct evidence that the observed contacts between iKQ-T22I and TatB strictly depend on an insertion of the TorA signal peptide into the TatBC-binding cavity and thus, reflect a post-targeting, however, pre-translational binding event.

**Mutations in the h-region of the TorA signal peptide and the TatBC receptor complex synergistically suppress the export defect of a TorA[KQ]-30aa-MalE precursor protein**

Finally, we analyzed whether the export of the h-region-coupled suppressor mutants can be further boosted by a previously identified suppressor mutation located in the TatBC receptor complex (22, 23). To test this, we combined the L9F mutation in the cytoplasmically localized N terminus of TatC with h-region-located single mutations A16V and T22I, respectively, as well as the double mutation G25V/G28W. Export of the mutated TorA[KQ]-30aa-MalE variants was then analyzed on indicator plates and directly by cell fractionation experiments (Fig. 7). As already described by Kreutznerbeck et al. (22, 23), the L9F mutation in TatC was sufficient to partially restore the export of the otherwise translocation-incompetent TorA[KQ]-30aa-MalE precursor proteins into the periplasm (9.6%), allowing significant growth on MMM and the formation of pink colonies on MCM of the respective strain (Fig. 7, A and B, lanes 4, and C). The presence of the additional mutations in the h-region of the TorA[KQ] signal peptide, however, had a remarkable effect on the export of the TorA[KQ]-30aa-MalE precursor protein by the mutant TatABC[L9F]E translocase. Although comparatively quite low levels of TorA[KQ]-30aa-MalE export by the wild-type Tat translocase were promoted by the mutations A16V, T22I, and G25V/G28W, resulting in relative translocation efficiencies of 1.4, 11.7, and 26.6%, respectively, a combination of these mutations with the L9F mutation in TatC resulted in a substantial increase in the export of the corresponding mutant reporter proteins iKQ-A16V (35.6%), iKQ-T22I (65.7%), and iKQ-G25V/G28W (82.0%), respectively (Fig. 7, A, compare lanes 6 and 8 with lanes 5 and 7; B, compare lane 6 with lane 5; and C). This was also reflected by faster growth on MMM and the rapid formation of red colonies on MCM of the respective strains (Fig. 7, A and B, compare phenotypes after 15 and 24 h of incubation). These observed effects were found to be synergistic because the export efficiencies of the h-region-coupled suppressor mutants by the mutant TatABC[L9F]E translocase were strikingly higher than the sum of the translocation efficiencies conferred by the TatC mutation L9F (9.6%) and the h-region-located mutations A16V (1.4%), T22I (11.7%), and G25V/G28W (26.6%), respectively. These results show that the previously identified suppressor mutation L9F in the TatBC receptor complex and the h-region-located mutations synergistically act together in restoring export of the otherwise translocation-defective TorA[KQ]-30aa-MalE precursor protein and we propose that both types of mutations improve the binding of the mutated TorA[KQ]-30aa-MalE precursor proteins to the TatBC receptor complex.

**Discussion**

In the present study, we have analyzed the role of the hydrophobic region of Tat signal peptides in *E. coli* TatBC receptor binding using *in vivo* and *in vitro* strategies. First, we addressed a rather general question of whether Tat-specific export in *E. coli* requires an intact hydrophobic core in the amino-terminal signal peptides of Tat precursor proteins. In full agreement with similar studies on the thylakoidal APh-dependent pathway (20, 41, 42), an introduction of a negatively-charged aspartate residue into the center of the hydrophobic core of the TorA signal peptide was not tolerated and rendered the TorA-MalE precursor protein completely defective for transport via the *E. coli* Tat translocase. Replacement of the aspartate by neutral or hydrophobic amino acids, or deletions within the hydrophobic core that removed the aspartate residue and a different number of adjacent amino acids restored export. However, translocation efficiency of the TorA-MalE precursor protein was drastically reduced when the hydrophobic region of the TorA signal peptide was severely truncated. A conceivable inference from these results therefore might be that a minimal length of an uninterrupted h-region in the signal peptide is required to establish a productive interaction of Tat precursor proteins with the hydrophobic TatBC-binding cavity.

To examine whether the h-region of the TorA signal peptide is directly involved in the binding process to the TatBC receptor complex, we aimed to identify mutations specifically located within the hydrophobic core that restore Tat-dependent export of a TorA[KQ]-30aa-MalE precursor protein, in which the crucial RR residues had been replaced by a lysine-glutamine pair. Previously, we identified such mutations in the TatBC receptor complex that significantly and, when combined, even synergistically suppressed the export defect of a similar TorA[KQ]-MalE precursor protein (22, 23). Assuming that in this case binding of the respective precursor to the unaltered TatBC receptor complex was most likely too weak to establish a productive interaction resulting in effective transport, we proposed that the suppressing mutations in the Tat translocase either directly or indirectly alter the signal peptide binding pocket in such a way that missing or weakened binding contacts between the QQ residues at the positions of the two arginine residues in the Tat consensus motif and the binding cavity are compensated by newly introduced or strengthened interactions between the binding pocket and one or more regions located elsewhere in the signal peptide (see “Discussion” section in Refs. 22 and 23). If so, then it seems that a critical threshold for the overall binding affinity must be exceeded to allow productive binding and consequently, effective translocation of Tat precursor proteins.

In this work, we identified six different mutations located in the h-region of the TorA signal peptide that significantly restored the export of a TorA[KQ]-30aa-MalE precursor protein. Because all isolated mutations substituted neutral or weakly hydrophobic amino acids by more hydrophobic residues, it is reasonable to suppose that the suppression of this export defect occurs by means of an increase of the overall hydrophobicity of the h-region. In support of this assumption, we showed that 1) combinations of two single suppressing
mutations, leading to a substantial increase of the hydrophobicity of the TorA signal peptide, act synergistically in promoting export of the TorA[KQ]-30aa-MalE precursor protein, 2) solely substitutions of a neutral glycine residue in the h-region of the export-defective TorA[KQ] signal peptide by more hydrophobic residues restored export, and 3) that a correlation exists between the respective hydrophobicity of the h-region of the TorA signal peptide and the translocation efficiency of the corresponding precursor protein. In full agreement with our previous proposal (22, 23), our present results strongly suggest that the newly introduced hydrophobic residues enhance the hydrophobic interactions within the intramembrane TatBC-binding groove and thus, sufficiently increase the overall binding affinity required for productive binding. This suggestion is in line

Figure 7. Combinations of mutations in the h-region of the TorA signal peptide and the mutation L9F in TatC synergistically suppress the export defect of a TorA[KQ]-30aa-MalE precursor protein. A and B, subcellular localization of TorA-30aa-MalE-derived polypeptides. Cells were fractionated into a P fraction and a combined C/M fraction by EDTA-lysozyme spheroplasting. The samples of the fractions corresponding to an identical amount of cells were subjected to SDS-PAGE and immunoblotting using anti-MalE antibodies. The positive controls were E. coli GSJ101 containing plasmids pTorA-30aa-MalE and pHSG-TatABCE (lane 1) or pHSG-TatABC[L9F]E (lane 2). The other samples correspond to GSJ101 co-expressing the wild-type tatABC or mutated tatABC[L9F]E genes and the export-defective TorA[KQ]-30aa-MalE reporter (lanes 3 and 4, respectively) or one of the TorA[KQ]-30aa-MalE reporter variants containing single mutations or double mutations in the h-region of the TorA signal peptide as indicated above the lanes. wt, wild-type; p, wt or mutated TorA-30aa-MalE precursor in the C/M fraction; m, mature form of MalE in the P fraction; asterisk, wt or mutated TorA-30aa-MalE-derived degradation products in the C/M fraction. Positions of molecular mass markers are indicated on the left margin. The phenotypes of the respective strains on MMM (–, no growth; +, slow growth; ++, moderate growth; ++++, fast growth) and MCM (pale, light red, pink, red) agar plates after 15 and 24 h of incubation are shown in the boxes at the bottom of the figure. C, relative export efficiencies of the analyzed TorA-30aa-MalE reporter variants in strains expressing the wild-type or mutant TatABC[L9F]E translocase. The amount of exported MalE protein in the P fraction of strains GSJ101 co-expressing the wild-type tatABC or mutated tatABC[L9F]E genes and TorA-30aa-MalE or one of the mutated TorA[KQ]-30aa-MalE reporter variants was determined in at least three different independent experiments via quantification of the chemiluminescence signals. The signals were recorded by a CCD camera and subsequently analyzed by the program AIDA 4.50 (Raytest). The average values are indicated by horizontal marker lines; standard deviations by error bars. The relative export efficiency of the positive control GSJ101 (pTorA-30aa-MalE, pHSG-TatABCE) was set to 100%.

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with experimental evidence that demonstrates that the entire signal peptide is inserted into the enclosed TatBC-binding cavity in a hairpin loop-like orientation (16, 37), with the h-region being positioned in close proximity to the transmembrane domain of TatB lining up with the TM5 of TatC (17). Thus, the hydrophobic core provides a large potential interaction site for the hydrophobic residues encompassing the TatBC signal peptide-binding site.

To give a direct proof that the h-region-located mutations exert their effects by affecting binding to the TatBC receptor complex, in vitro cross-linking studies were performed. In fact, in case of mutants that exhibited high export efficiencies in vivo, cross-linking products corresponding to 1:1 complexes between the reporter variants and TatB appeared upon UV irradiation of the INVs when the photocross-linker Bpa had been placed at position Ile-4 in the periplasmically-oriented N terminus of TatB (see Fig. 6A). Because this residue was previously found to contact deep inserted RR precursors in the stage of advanced binding (17), these results strongly suggest that the signal peptides of the mutated TorA[KQ]-30aa-MalE reporter variants harboring suppressing mutations in the h-region must have been threaded deep into the TatBC-binding cavity, even in the absence of an intact RR motif. No such contacts were obtained with the transport-incompetent TorA[KQ]-30aa-MalE precursor protein harboring mutations solely in the RR motif.

Surprisingly, by far the most prominent contacts were observed with the suppressor mutant iKQ-T22I, which exhibits only moderate export efficiency in vivo, whereas a lower amount of cross-linking products was obtained with the strongest in vivo suppressor iKQ-G25V/G28W. A likely explanation for this discrepancy was that the substitution of two α-helix-breaking glycine residues resulted in far more drastic conformational changes of the signal peptide compared with the replacement of threonine by isoleucine. Consequently, particular residues in TatB or TatC might be no longer located close enough to be efficiently cross-linked to the signal peptide. One could speculate that the transmembrane helices of TatB and TatC might be even pushed apart by the double mutant iKQ-G25V/G28W resulting in substantial conformational alterations of the TatBC-binding cavity. Considering all tested reporter variants, the spatial configuration of iKQ-T22I most likely resembles the conformation of the native TorA signal peptides that is perfectly adapted to the TatBC-binding site, as indicated by the prominent cross-linking products obtained with the unaltered TorA-MalE precursor protein.

In total agreement with the deep insertion of signal peptides into an enclosed binding cavity formed by both TatB and TatC (16, 37), iKQ-T22I also cross-linked to TatC when Bpa was introduced into the distal part of TM5 (Val-202) (see Fig. 6B), however, no contacts were obtained between iKQ-T22I and the cytoplasmic N terminus of TatC, which constitutes part of the recognition and binding site for the Tat consensus motif (see supplemental Fig. S2). The latter finding strongly suggests that the specific primary recognition of the Tat consensus motif by TatC, which was shown to exclusively depend on an intact RR motif (13, 16), is apparently not a prerequisite for insertion of the signal peptide into the TatBC binding pocket. This finding is seemingly at odds with the binding model of Alami et al. (13) who proposed a clear hierarchy in the targeting steps. Accordingly, the Tat signal peptide is first recognized by TatC via the RR motif before the RR precursor protein is transferred to TatB and bound in a cavity jointly formed by both Tat components. Our results would be consistent with the model above, if the remaining unaltered residues of the Tat consensus motif were sufficient to ensure recognition by TatC and the subsequent deep insertion of the signal peptide harboring a KQ at the RR position into the intramembrane binding groove. Although the less strictly conserved residues of the Tat consensus motif were shown to likely contribute to the specific binding to TatC (11, 54), the lack of contacts between the suppressor mutant iKQ-T22I and the signal peptide recognition site of TatC strongly suggests that primary recognition of the Tat consensus motif by TatC rather did not occur in the case of the TorA[KQ] signal peptide.

Recently, we demonstrated that DCCD not only inhibits the ATP synthase and thus, the generation of a proton gradient, but also prevents insertion of Tat precursors into the TatBC receptor complex.3 By adding CCCD and DCCD to Tat[−]INVs, we showed that the TatB-iKQ-T22I adducts disappeared upon DCCD treatment only (see Fig. 6C). Thus, we conclude that the observed contacts between iKQ-T22I and TatB strictly depend on an insertion of the TorA signal peptide into the TatBC-binding cavity and so, reflect a post-targeting, however, pre-translational binding event.

Irrespective of the effects the particular amino acid alterations might have on the conformation of the TorA signal peptide, we clearly demonstrated that the additional mutations in the h-region affect the binding of the signal peptide to the TatBC receptor complex most likely at a stage where the signal peptide has been transferred into an advanced-stage precursor-binding site that reaches out as far as the periplasmically oriented end of the transmembrane helix of TatB aligned to TM5 of TatC. Because significant export of the respective reporter variants was observed in vivo, mutations in the h-region evidently restored a productive interaction between the otherwise export-defective TorA[KQ]-30aa-MalE precursor protein and the TatBC receptor complex.

In support of this conclusion, we performed a final experiment and analyzed the export of the h-region-coupled suppressor mutants by one of the mutant Tat translocases identified in previous studies, TatABC[L9F]E, which allowed significant export of a normally transport-incompetent TorA[KQ]-MalE precursor protein (21, 22). Remarkably, we found that the h-region-located mutations and the L9F mutation in the cytoplasmically localized N terminus of TatC synergistically act together in the suppression of the export defect of TorA[KQ]-30aa-MalE, providing further evidence that all these mutations affect interactions between the substrate and the TatBC receptor complex at the stage of deep binding, resulting in an optimized adaptation of the mutated TorA[KQ] signal peptide to the TatBC-binding cavity (see Fig. 7). Recently it was shown that suppressor mutations located in the N-terminal part of TatB can restore the export of otherwise transport-defective Tat precursor proteins by mimicking the substrate-driven conformational changes required for TatA recruitment in the
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absence of substrate binding (19, 55). As a consequence, binding of signal peptides with even low affinity to the TatBC receptor complex was found to be sufficient to trigger the formation of an active TatABC translocase (51). The L9F mutation in TatC, however, is located in the cytoplasmic domains of the receptor complex, which constitute part of the recognition and binding site for the Tat consensus motif and thus, most likely acts in a different manner. Because no contacts between the suppressor mutant iKQ-T22I and position Leu-9 of TatC could be detected (see supplemental Fig. S2), which would be indicative of a direct interaction with the substrate, we propose that the L9F mutation acts rather indirectly by causing conformational changes in the TatBC receptor complex that result in strengthened substrate-receptor interactions elsewhere within the TatBC-binding cavity.

The combined results of this study prompted us to suggest the following model for the targeting and binding of the TorA[KQ]-30aa-MalE reporter variants to the Tat translocase, which shares main similarities with the two-stage binding mechanism proposed in previous studies (15, 56). First, the Tat precursor proteins approach the TatBC receptor complex either directly from the cytosol or via a membrane-lipid associated form (32–34). Subsequently, the Tat signal peptide is recognized and superficially bound by the cytoplasmically localized domains of the TatBC receptor complex. Different from the model of Alami et al. (13), we suggest that the first targeting step does not strictly depend on the RR residues and involves the recognition of a more extended area of Tat signal peptides also encompassing other residues than the arginine pair of the twin-arginine motif. Along this line, less strictly conserved residues of the Tat consensus motif were shown to most likely contribute to the specificity of initial recognition of the signal peptide by the TatBC receptor complex (11, 23, 54). In addition, Alami et al. (13) reported residual contacts between the h-region of the pre-SufI signal peptide and TatB, also in case when the RR residues were replaced by KK. Furthermore, McDevitt et al. (21) demonstrated that pre-SufI[KK] can be co-purified with the TatBC receptor complex, strongly suggesting that association with TatBC does not strictly require the twin-arginine residues. In accordance with the two-stage binding model proposed by Panahandeh et al. (15), we would predict that the initial interaction of the Tat precursor protein with the cytosolic domains of the TatBC receptor complex subsequently causes conformational changes of the TatBC receptor that lead to an insertion of the signal peptide deep into the membrane. Substrate-driven conformational changes of the Tat translocase have recently also been shown to be responsible for the displacement of TatB from the common TatA/TatB-binding site on TatC and the recruitment of TatA to the substrate-loaded TatBC receptor complex, thereby activating the Tat translocase for the actual translocation event (19, 55). An advanced stage binding of the TorA[KQ] signal peptide was indeed confirmed by the identification of cross-links between the h-region-coupled suppressor mutant precursor proteins and residues located in the periplasmically-oriented part of the TatBC receptor complex (see Fig. 6, A and B). In this deep insertion mode of the signal peptide, the reported mutations in the h-region most likely permit productive binding of the otherwise export-defective TorA[KQ]-30aa-MalE reporter variants by increasing the hydrophobic interactions between the signal peptide and residues encompassing the hydrophobic signal peptide-binding site on the TatBC receptor complex, which finally exceed the critical threshold for the overall binding affinity and restore export. In support of this conclusion, site-specific cross-linking studies confirmed contacts between several residues of the h-domain of the pre-SufI or TorA signal peptide and TatB (13, 17), which were strictly dependent on the presence of TatC and did not disappear upon dissipation of the PMF, strongly suggesting that these interactions most likely reflect such an advanced stage binding event prior to the PMF-dependent recruitment of TatA and the actual translocation event. Nevertheless, it is very likely that an efficient primary recognition of an unaltered Tat consensus motif by TatC positively affects translocation efficiency by promoting high-affinity binding, which was shown to persist even during the whole translocation process (24) and that cannot be achieved by the sole increase of the overall hydrophobicity of the signal peptide. Taken together, our results clearly indicate that, besides the twin-arginine motif, the h-region of Tat signal peptides is a second major binding determinant that plays a pivotal role in the binding process of Tat precursor proteins to the TatBC receptor complex.

Experimental procedures

Bacterial strains, plasmids, and culture conditions

*E. coli* strains and plasmids used in this study are listed in Table 2. Cells were grown at 37 °C in Luria Bertani medium (57), minimal medium (58) supplemented with 0.4% (w/v) maltose (MMM), or MacConkey agar base medium (Difco) supplemented with 1% (w/v) maltose (MCM). If required, isopropyl β-D-thiogalactopyranoside was used at a 0.1 mM concentration. Antibiotics were supplemented at following concentrations: kanamycin, 50 mg/liter; chloramphenicol, 25 mg/liter.

DNA manipulations

All DNA manipulations followed standard procedures (59). Oligonucleotides used in this study are listed in supplemental Table S1. The correctness of all newly constructed plasmids was verified by DNA sequencing. Plasmid pTorA-30aa-MalE was constructed by crossover PCR. Two DNA fragments were amplified using primer pairs TorA-30aa_fw1 and TorA-30aa_rev2 or TorA-30aa_fw3 and TorA-30aa_rev4, respectively, and the chromosomal torA gene of *E. coli* or pTorA-MalE plasmid as templates, respectively. Subsequently, both fragments were fused and amplified using TorA-30aa_fw1 and TorA-30aa_rev4 as primers. The resulting PCR fragment was digested with Hpal and EcoRI and ligated into Hpal/EcoRI-digested linearized pTorA-MalE plasmid. For the construction of pTorA[KQ]-30aa-MalE, plasmid pTorA[KQ]-MalE was digested with BamHI and Hpal. The resulting fragment containing the KQ mutation was ligated into BamHI/Hpal-digested linearized pTorA-30aa-MalE vector. The replacement of valine by aspartate (V23D) in the h-region of the TorA signal peptide, resulting in plasmid pTorA[V23D]-MalE, was done using the QuikChange II XL Site-directed Mutagenesis Kit (Agilent) with pTorA-MalE as template and
primers hV23Dfor and hV23Drev, according to the manufacturer’s instructions.

Likewise, the combinations of h-region-located mutations A16V and G25W or A16V and G28W, respectively, were constructed using the same procedure with pTorA[KQ,G25W]-30aa-MalE and pTorA[KQ,G25W]-30aa-MalE, respectively, as templates and primers KQ_A16V-fw and KQ_A16V_rev, resulting in plasmids pTorA[KQ,A16V,G25W]-30aa-MalE and pTorA[KQ,A16V,G28W]-30aa-MalE. Plasmid pTorA[KQ,T22A,G28W]-30aa-MalE was constructed by introducing amino acid substitution T22A into pTorA[KQ,G28W]-30aa-MalE using the QuikChange® II XL Site-directed Mutagenesis Kit (Agilent) and the primers KQ_T22A-fw and KQ_T22A_rev. For the construction of pTorA[KQ,G25W,G28W]-30aa-MalE and pTorA[KQ,G25W,G28W], respectively, a DNA fragment was amplified using the forward primer intramut_torA30aaMalE_fwd and the reverse primer KQ_G25W+G28W_rev and KQ_G25W+G28W_rev, respectively, with pTorA[KQ]-30aa-MalE as template. The resulting PCR fragment was digested with BamHI and Hpal and ligated into BamHI/Hpal-digested linearized pTorA[KQ]-30aa-MalE vector. Plasmids pTorA[KQ,G25N]-30aa-MalE with ψ being Ala, Cys, Met, Asn, Thr, and Ser, respectively, were constructed as described above using the QuikChange® II XL Site-directed Mutagenesis Kit (Agilent) with pTorA[KQ]-30aa-MalE as template and primers KQ_30aa-fw and KQ_30aa_rev, respectively. For the construction of the plasmids pET22b-TorA-30aa-MalE and pET22b-TorA[KQ]-30aa-MalE, a DNA fragment encompassing the entire torA-30aa-malE or torA[KQ]-30aa-malE genes was amplified by the forward primer pET22b_NdeI_TorAx_fwd and the reverse primer pET22b_XhoI_TorAx_rev using the template pTorA-30aa-MalE or pTorA[KQ]-30aa-MalE, respectively.

### Table 2

**Bacterial strains and plasmids used in this study**

| E. coli strains | Relevant properties | Source |
|-----------------|--------------------|--------|
| DH5α | supE44, lacI1Δ169 (80lacZΔM15) hisD17 recA1 endA1 hsdR17 gyrA relA thi | 62 |
| GS100 | MC4100 X P.M1129-2X ΔmalE444 zyb 729-Tn10 | 39 |
| GS101 | DADE X P.M1129-2ΔmalE444 zyb 729-Tn10 | 39 |

| Plasmids | | |
|-----------------|--------------------|--------|
| pHSG575 | pSC101 replicon, lacZα− ; CmR | 63 |
| pHSG-TatABCE | pHSG575 derivative; carrying the tatABCE genes of *E. coli* | 40 |
| pHSG-TatABCE[L9F]E | pHSG-TatABCE harboring L9F mutation in TatC | 22 |
| pTorA-MalE | pBBR1MCS-2 carrying the torA-malE fusion gene, KanR | 39 |
| pTorA-A30aa-MalE | pBBR1MCS-2 carrying the torA-30aa-malE fusion gene with linker region consisting of 30 aa of mature TorA, KanR | This study |

* KanR, kanamycin resistance; CmR, chloramphenicol resistance; AmpR, ampicillin resistance.
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and G28W, respectively, were constructed by the same procedure as described above using pET22b_Ndel_TorAx_fw and pET22b_Xhol_TorAx_rev as primers and the corresponding pTorA[KQ, φ]-30aa-MalE plasmid variants as templates. For the construction of pET22b-TorA[KQ,G25M]-30aa-MalE, the respective codon alteration was introduced using the QuickChange® II XL Site-directed Mutagenesis Kit (Agilent) with pET22b-TorA[KQ]-30aa-MalE as template and the primers KQ_G25M_fw and KQ_G25M_rev. Plasmids expressing Bpa variants of TatB and TatC have been described (17).

**Isolation of V23D suppressor mutants**

For the isolation of mutant Tat translocases that allow the export of TorA[V23D]-MalE, a library of pHS-G-TatABC plasmids constructed in previous studies (22, 23) carrying randomly mutagenized *E. coli* tatABC genes was used to transform GSJ101 (pTorA[V23D]-MalE) by a standard heat-shock method. The transformed cells were plated on solid minimal medium containing 0.4% (w/v) maltose (MMM) and incubated at 37 °C for up to 5 days. Some of the single mutant colonies that appeared on the selection plates were randomly picked and re-streaked on the same medium, and those isolates that showed reproducible growth were chosen for further characterization. From these isolates, plasmids pHSG-TatABC and pTorA[V23D]-MalE were isolated and separated. Subsequently, the pHSG-TatABC plasmids were re-transformed into GSJ101 (pTorA[V23D]-MalE) to verify that mutations responsible for the restored export of TorA[V23D]-MalE are in fact located in the *tatABC* genes. The resulting transformatns were re-tested for growth on MMM. In all cases no growth could be observed, indicating that mutations suppressing the export defect of TorA[V23D]-MalE must have rather occurred spontaneously in the reporter plasmid pTorA[V23D]-MalE. The corresponding pTorA[V23D]-MalE plasmids were then used for DNA sequence analysis and further functional characterization.

**Isolation of h-region-coupled suppressor mutants**

The entire signal peptide of the TorA[KQ]-30aa-MalE reporter protein was mutagenized via error prone PCR using the GeneMorph® II Random Mutagenesis Kit (Agilent) according to the manufacturer’s instructions. Plasmid pTorA[KQ]-30aa-MalE was used as template and intramut_torAs30aMalE_fw and intramut_torAs30aMalE_rev as primers, which contained BamHI and Kpn2I restriction sites, respectively. The error prone PCR conditions were adjusted such that 1–9 mutations were introduced per kilobase of template DNA. After completion of the PCR, the amplified fragments encompassing the entire TorA signal peptide, 30 amino acids of the mature TorA and 48 amino acids of the mature MalE protein, were cut with BamHI and Kpn2I and ligated into BamHI/Kpn2I-digested linearized pTorA[KQ]-30aa-MalE. After transformation of the ligation products into *E. coli* DH5α, about 5000 clones were obtained from which a pool of mutagenized pTorA[KQ]-30aa-MalE plasmids was isolated. Small aliquots of this mutant library pool were transformed into GSJ101 (pHS-G-TatABC) by using a standard heat-shock method. The transformed cells were plated on solid minimal medium containing 0.4% (w/v) maltose and incubated at 37 °C for up to 5 days. Some of the single mutant colonies that appeared on the selection plates were randomly picked and re-streaked on the same medium, and those isolates that showed reproducible growth were chosen for further characterization. From these isolates, plasmids pHSG-TatABC and mutagenized pTorA[KQ]-30aa-MalE were isolated, separated, and pTorA[KQ]-30aa-MalE was subsequently used for DNA sequence analysis and further functional characterizations.

**Cell fractionation studies**

Fractionation of cells into a fraction containing the C/M and a P fraction was done using an EDTA-lysozyme spheroplasting method as described by Kreutzbeck et al. (22). Samples of the cell fractions corresponding to an equal amount of cells were subjected to SDS-PAGE and Western blotting using MalE-specific antibodies (22). As a control for the quality of the fractionation experiments, the subcellular localization of the cytoplasmic enzyme transaldolase B (TalB) or the cytoplasmic membrane-associated protein SecA was analyzed in parallel using TalB- and SecA-specific antibodies (22), respectively. Western blotting using anti-MalE, anti-TalB, or anti-SecA antibodies was performed by using the ECL Western blotting detection kit (GE Healthcare) according to the manufacturer’s instructions. The chemiluminescent protein bands were recorded and quantified using the Fujifilm LAS-3000 Mini CCD camera and image analyzing system together with the software AIDA 4.50 (Raytest).

**Membrane vesicles**

Inside-out INV were prepared as described (60) from *E. coli* strains BL21(DE3)* (Novagen) transformed with plasmid pEVOL-pBpF and derivatives of plasmid p8737. TatABC-INV containing Bpa variants of TatB and TatC were prepared as described (17).

**In vitro reactions**

Mutagenized variants of the precursor protein TorA-30aaMalE were synthesized by *in vitro* transcription/translation using derivatives of plasmid pET22b(+) listed in Table 2. Cell extract used for the *in vitro* synthesis was prepared (60) from *E. coli* strain Top10 (Invitrogen). Coupled transcription/translation reactions were performed in 50-μl aliquots as described by Moser et al. (60). INV were added 15 min after starting the synthesis reaction and incubated for 10 min at 37 °C. Assaying Bpa-dependent cross-linking by irradiating samples with UV-light and addition of CCCP and DCCD have been described (17, 61). SDS-PAGE using 10% gels was performed as described (60).

**Author contributions**—A. U., J. F., R. F., and M. M. conceived and coordinated the study. A. U., J. F., R. F., and M. M. designed the experiments. A. U. performed the experiments shown in Figs. 3–5, Fig. 7, and supplemental Fig. S1, and wrote the manuscript. J. F. performed the experiments shown in Figs. 6 and supplemental Fig. S2. A. S. B. helped with the experiments of J. F. F. L. performed the experiment shown in Fig. 2 and A. K. H. performed the experiment shown in Fig. 1. A. U., J. F., A. S. B., R. F., and M. M. analyzed the experiments, and revised and approved the final version of the manuscript.
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