Charge-driven dynamics of nascent-chain movement through the SecYEG translocon

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On average, every fifth residue in secretory proteins carries either a positive or a negative charge. In a bacterium such as *Escherichia coli*, charged residues are exposed to an electric field as they transit through the inner membrane, and this should generate a fluctuating electric force on a translocating nascent chain. Here, we have used translational arrest peptides as *in vivo* force sensors to measure this electric force during cotranslational chain translocation through the SecYEG translocon. We find that charged residues experience a biphasic electric force as they move across the membrane, including an early component with a maximum when they are 47–49 residues away from the ribosomal P site, followed by a more slowly varying component. The early component is generated by the transmembrane electric potential, whereas the second may reflect interactions between charged residues and the periplasmic membrane surface.

In both prokaryotic and eukaryotic cells, proteins are translocated across lipid membranes with the aid of protein-conducting channels (translocons). In their natural context, translocons such as the bacterial SecYEG complex do not conduct ions, so as not to compromise biologically important ion gradients1, but they nevertheless act as nonselective ion channels in the sense that they conduct charged residues in the proteins being transported. In membranes that support a membrane potential, strong electric forces should be expected to act on charged residues during chain translocation, reflecting the local electric potential in the translocon channel. We reasoned that by measuring such forces at defined positions within the translocon channel we might be able to chart the electric field experienced by the nascent chain (much as electrophysiological studies have revealed a detailed picture of the electric environment in classical ion channels) and thereby be able to clarify how membrane electrostatics contributes to the dynamics of chain translocation.

**RESULTS**

We have recently shown that translational arrest peptides (APs) can be used as transplantable *in vivo* force sensors to measure forces acting on nascent polypeptide chains during cotranslational processes such as membrane-protein biogenesis2–4. APs are short stretches of polypeptide that can induce ribosomal stalling when translated5. The family of bacterial SecM APs is particularly interesting in this regard because the degree of stalling induced by these APs depends on the tension in the nascent polypeptide chain at the precise point when the most C-terminal codon of the AP is in the ribosomal A site: the higher the tension, the lower the degree of stalling3,6,7.

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To measure forces acting on charged residues during cotranslational chain translocation through the *E. coli* SecYEG translocon, we used an engineered version of the inner-membrane protein LepB as a reporter (Fig. 1a). The construct has two transmembrane segments near the N terminus (TM1 and TM2) that serve to target the ribosome–nascent chain complex to SecYEG and a large C-terminal periplasmic domain that contains a stretch of n charged or uncharged test residues (X) placed L residues upstream of a SecM AP (sequences of all constructs in Supplementary Fig. 1 and Supplementary Data Set 1). The SecM AP is followed by a 23-residue C-terminal tail to ensure that arrested and full-length LepB chains can be easily separated by SDS-PAGE.

The basic experiment is simple: a plasmid encoding a particular version of the LepB construct (characterized by the number of test residues n, the identity of the test residues X, the number of linker residues L between the nX stretch and the last residue of the AP, and the particular SecM AP used) is transformed into *E. coli*; expression of the construct is induced; and cells are briefly labeled with [35S]methionine, lysed, subjected to immunoprecipitation with a LepB antiserum and analyzed by SDS-PAGE (Fig. 1b and Supplementary Data Set 1). Constructs in which there is a strong external pulling force F on the nascent chain at the point when the ribosome reaches the last residue in the AP will yield mostly full-length protein, whereas if F is low there will be efficient translational arrest at the AP, thus yielding mostly the shorter, arrested form of the protein.

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Received 17 June 2014; accepted 26 November 2014; published online 5 January 2015; doi:10.1038/nsmb.2940
Figure 1: Negatively charged residues experience an electric pulling force during passage through the SecYEG translocon.
(a) Design of LepB constructs. A schematic picture of a translating ribosome bound to the SecYEG translocon and the sequence surrounding the nX stretch are shown below. Two engineered Asn-Ser-Thr acceptor sites for N-linked glycosylation (G1 and G2) are shown in red; these are not present in the constructs expressed in E. coli but are used in the experiment reported in d. (b) Pulse labeling of 5D constructs. AP and mut indicate, respectively, constructs with a functional SecM(Ms) AP and a nonfunctional mutant AP with the last proline residue mutated to alanine. (c) Fraction of full-length protein (fFL) plotted as a function of L + n for constructs with nX = 5N (yellow), 5Q (green), 5K (blue) and 5D (purple), and the SecM(Ms) AP. (d) Determination of the location of the 5D segment in the 5D, L + n = 48 construct by glycosylation mapping. Right, comparison of the results obtained for the 5D, L + n = 48 construct with previously determined glycosylation distances (described in main text). PTC, peptidyltransferase center. (e) fFL measured for a 5D construct with L + n = 48 residues (blue bars) and for a construct with L = 39 residues in which the nX stretch is replaced by a hydrophobic transmembrane segment of composition 6L, 13A (gray bars), at increasing concentrations of indole in the growth medium. The amount of nonprocessed pro-OmpA protein is also shown (red bars). Error bars, s.e.m. (n = 3 independent experiments).

Charged residues experience an electric pulling force
We obtained force profiles with the SecM(Ms) AP and nX = 5N, 5Q, 5K and 5D (Fig. 1c; summary of all experimental data in Supplementary Table 1). For 5N, 5Q and 5K, fFL was low for L + n < 50 residues and then increased slowly. The profile for 5D was dramatically different, with a very rapid increase from L + n = 45 residues to a first peak at L + n = 47–49 residues, a second peak at L + n = 53 residues and a slow decline. At L + n > 60 residues, all profiles merged. That the strong pulling force was seen only with the negatively charged 5D test segment suggests that it is electric in nature and is generated by the electrical component (∆Ψ) of the proton-motive force (PMF). We do not understand the reason for the relatively high fFL values seen for all constructs at L + n > 60 residues, but it is clearly not related to the charge characteristics of the nX stretch.

Unfortunately, there is no good method available to precisely map the location of the 5D stretch in the bacterial SecYEG channel at different linker lengths; instead, we applied the method of glycosylation mapping10–12 to the 5D, L + n = 48 construct translated in vitro in rabbit reticulocyte lysate in the presence of dog pancreas rough microsomes derived from the endoplasmic reticulum. In this case, there is no membrane potential that can act on charged residues in the nascent chain, but the overall structure of the ribosome-translocon complex and the geometry and relevant distances characterizing the nascent-chain ribosome-translocon conduit are highly conserved between bacteria and mammals13,14.

The method rests on the observation that the asparagine residue in an Asn-X-Thr/Ser) acceptor site for N-linked glycosylation (site G2 in Fig. 1a) in an extended nascent polypeptide chain must be ~65 residues away from the ribosomal peptidyl (P) site (or ~15 residues away from the N-terminal end of a membrane-integrated transmembrane segment) to reach the luminal active site of the oligosaccharyltransferase in the endoplasmic reticulum and become half-maximally glycosylated10–12. We engineered two glycosylation-acceptor sites (G1 and G2) into the 5D, L + n = 48 construct (Fig. 1a). We then translated mRNA truncated at the last proline codon in the AP in vitro

Figure 2: The electric force has both PMF-dependent and PMF-independent components and scales with the number of negatively charged residues in the nX stretch. (a) fFL plotted as a function of L + n for 5D constructs containing the SecM(Ms-Sup1) AP, measured in the absence (purple) or presence (blue) of 2 mM indole in the growth medium. fFL for 5N constructs obtained in the absence of indole is also shown (yellow). (b) Difference plots of the fFL profiles in a, representing the PMF-dependent component (blue) obtained by subtracting the 5D (+indole) profile from the 5D (−indole) profile and the PMF-independent component (red) obtained by subtracting the 5N profile from the 5D (+indole) profile. (c) fFL plotted as a function of L + n for 5D and 5N constructs containing the SecM(Ms-Sup1) AP. (d) fFL plotted as a function of L + n for nD and 5N constructs containing the SecM(Ms-Sup1) AP and with ten-residue-long (SG)X segments flanking the nX stretch. Error bars, s.e.m. (n = 3 independent experiments).

underlined), that requires a stronger tension in the nascent chain to reach a given fFL value3,8,9.

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in the presence of dog pancreas rough microsomes and [35S]methionine to generate a stalled ribosome–nascent chain complex that spans the membrane (Fig. 1a), then separated proteins by SDS-PAGE and determined the glycosylation status of the G1 and G2 sites from the intensities of the bands representing singly and doubly glycosylated product. The G1 site was kept in a fixed location and was always glycosylated, and we moved the G2 site stepwise relative to the 5D stretch in order to determine the location at which it is half-maximally glycosylated.

We found that the N-terminal end of the 5D stretch in the 5D, \( L + n = 48 \) construct is located 28 residues away from the G2-site asparagine and therefore that the total chain length between the P site and the asparagine is 28 + 48 = 76 residues at half-maximal glycosylation (Fig. 1d), results similar to those obtained previously for a highly charged 14K stretch engineered into a secretory mammalian protein. This places the 5D stretch in or near the cytoplasmic component of the pore ring and then translocates en bloc when the nascent-chain length reaches \( L + n = 46 \) residues.

To probe the PMF dependence of \( f_{FL} \) for the 5D construct, we grew E. coli cells in increasing concentrations of the uncoupler indole, a treatment that has been shown to reduce the PMF from its unperturbed value to essentially zero over a range of 1–5 mM indole (and to block cell division at >3 mM indole). At a fixed \( L + n = 48 \), \( f_{FL} \) was reduced from its maximal value to background levels over the same range of indole concentrations (Fig. 1e). Translocation of the SecA- and SecYEG-dependent outer-membrane protein OmpA was adversely affected only when the indole concentration was >3 mM, as assayed by the accumulation of the uncleaved precursor form (pro-OmpA). For a further control, we tested the effect of indole on

### Figure 3
A positively charged lysine residue placed N terminal to a 4D stretch reduces the electric pulling force. \( f_{FL} \) values are shown for 4D-SecM(Ms) constructs with asparagine, lysine, histidine and proline residues included in the indicated positions. In all cases, the number of residues between the indicated stretch of residues and the P site is \( L = 43 \) residues. Error bars, s.e.m. (\( n = 3 \) independent experiments).

### Figure 4
Physical model for translocation of charged residues (details in Supplementary Note). (a) The total electric potential (black) experienced by a charged residue has two components: the transmembrane potential \( \Delta V \) (blue) and the membrane surface potential \( V_s \) (red). The electric field strength (which is proportional to the force \( F \) acting on a point charge) is shown as a dashed line. The nascent polypeptide chain is modeled with the wormlike chain model. Approximate lengths of the ribosomal tunnel (yellow) and the translocon channel (gray) are indicated. (b) Simultaneous fitting of the experimental \( f_{FL} \) profiles for the (SG)5-nX-(SG)5-SecM(Ms) constructs and the nX-SecM(Ms-Sup1) constructs by local parameter optimization of six of the 13 parameters that describe the model (details in Supplementary Note). Results for the (SG)5-nX-(SG)5 set are shown (blue, experimental profiles; red, calculated profiles). Results for the other data sets are in Supplementary Figure 4. (c) Experimental \( f_{FL} \) values plotted against the predicted pulling force \( F \) for the (SG)5-nX-(SG)5-SecM(Ms) constructs. The solid line is a sigmoidal fit, \( f_{FL} = \frac{1}{1 + A e^{-\frac{L}{\sigma}}} \), with \( A = 5.2 \) and \( \sigma = 7.0 \) pN. (d) As in c, but for the SecM(Ms-Sup1) AP. The solid line is a sigmoidal fit, \( f_{FL} = \frac{1}{1 + A e^{-\frac{L}{\sigma}}} \), with \( A = 5.2 \) and \( \sigma = 11.8 \) pN.
A LepB construct in which the nX segment was replaced by a hydrophobic transmembrane segment of composition 6L, 13A, which we have previously shown to generate a strong pulling force at \( L = 39 \) residues, i.e., at the point when it is expected to integrate into the inner membrane\(^1\). In this case, there was no effect of indole on \( f_{FL} \) for concentrations \( \leq 3 \text{ mM} \). We conclude that the peak in the 5D force profile at \( L + n = 47–49 \) residues reflects an electric interaction between the negatively charged residues in the 5D stretch and the transmembrane potential across the SecYEG channel.

The force acting on the 5D test segment at \( L + n = 47–49 \) residues was strong enough to cause \( f_{FL} \) to saturate at \(~1.0\) (Fig. 1c). In order to increase the sensitivity of the assay in this region, we obtained force profiles for the 5D and 5N series of LepB constructs by using the stronger SecM(Ms-Sup1) AP (Fig. 2a). The overall shapes of the two force profiles were similar to those obtained with the SecM(Ms) AP, but the peak at \( L + n = 47–49 \) residues was better defined and was clearly distinct from the second peak at \( L + n = 52 \) or 53 residues. We also obtained a 5D-SecM(Ms-Sup1) force profile in the presence of 2 mM indole (Fig. 2a), i.e., at a concentration at which there is no effect on pro-OmpA processing but a strong effect on \( f_{FL} \) for the 5D-Sec(Ms) constructs (Fig. 1e). Remarkably, the peak at \( L + n = 47–49 \) residues was completely obliterated by indole, whereas the peak at \( L + n = 52 \) or 53 residues was hardly affected. This is even more clearly seen in Figure 2b, in which we isolated the effect of indole by subtracting the force profile of the 5D-Sec(Ms-Sup1) constructs obtained in the presence of indole from that obtained in its absence and also subtracted the 5N-Sec(Ms-Sup1) force profile from that of 5D-Sec(Ms-Sup1) obtained in the presence of indole. The PMF-dependent peak was strong and nearly symmetric around \( L + n = 48 \) residues, whereas the PMF-independent peak was of smaller magnitude and extended over the range \( L + n = 50–60 \) residues.

How does the force profile vary with the number of aspartate residues in the nD stretch? We obtained results with the SecM(Ms-Sup1) AP for \( n = 2–5 \) (Fig. 2c) and for \( n = 10 \) (Supplementary Fig. 2a). The magnitudes of both the \( L + n = 48 \) and \( L + n = 53 \) peaks scaled approximately with \( n \), as did the initial slope of the PMF-dependent \( L + n = 48 \) peak (Supplementary Fig. 3). As for the 5D stretch, 2 mM indole totally suppressed the first peak in the 10D force profile but reduced the second peak only marginally (Supplementary Fig. 2b).

In an attempt to further isolate the effect on \( f_{FL} \) of the nX stretch from influences of the surrounding sequence, we measured force profiles for nD stretches \( (n = 1–5) \) and a control 1N stretch embedded in an uncharged \((SG)_2-nX-(SG)_2\) segment, using the weaker SecM(Ms) AP (Fig. 2d). Overall, the force profiles were similar to those in Figure 2c, with a clear two-peak appearance. Again, the first peak was PMF dependent, and the second was not (Supplementary Fig. 2c,d). The profiles were shifted by about two residues toward higher \( L + n \) values, thus implying that the \((SG)_2\) linker attains a somewhat more compact conformation or makes a longer excursion in the space between the ribosome and the translocon\(^16\) than the linker used in Figure 2c.

Finally, we analyzed the effect on \( f_{FL} \) of a single positively charged lysine residue placed in different positions relative to a 4D stretch. For this experiment, we used the Sec(Ms) AP and fixed \( L \) at 43 residues. A single lysine residue placed five residues N terminal to the 4D stretch (thus entering the translocon ahead of the negatively charged residues) had no effect on \( f_{FL} \) (Fig. 3). In contrast, when the lysine residue was four, three, two or one residue upstream of the 4D stretch, \( f_{FL} \) was reduced from \( -0.85 \) to \(-0.60 \). Placing the lysine residue in the middle of the 4D stretch reduced \( f_{FL} \) to \(-0.70 \), but placing the lysine just C terminal to the 4D stretch had no effect on \( f_{FL} \). Placing either asparagine, histidine or proline in the middle of the 4D stretch also had no effect on \( f_{FL} \). This is the pattern expected if the long, positively charged lysine side chain is pushed back by the membrane potential toward the 4D stretch as it enters the SecYEG channel. That the neutralizing effect is visible already when the lysine residue is four residues upstream of the 4D stretch indicates that the NKNNN stretch has a compact conformation as it passes through the SecYEG channel, in contrast to the following 4D stretch that probably is in an extended conformation, as indicated by the lack of an effect on \( f_{FL} \) by the helix-breaking proline mutation.

A physical model of chain translocation
As a charged residue translocates through the ribosome and the translocon channel, it will minimally be subjected to interactions with the ribosomal tunnel (which is at a negative potential of \(~10–20\) mV relative to the cytoplasm\(^19\)), with the transmembrane electric potential \( \Delta \Psi \), with charged and polar residues in the translocon and with the membrane surface potential \( \Phi \). It will also need to pass through the relatively nonpolar interior of the translocon channel and, notably, through the hydrophobic ‘pore ring’. Because our constructs were specifically designed to probe electric forces acting on the nascent chain during its passage through the translocon, we asked whether our results could be explained by a physical model including only \( \Delta \Psi \) and \( \Phi \) (Supplementary Note); indeed, the experimental \( f_{FL} \) profiles could be reproduced with reasonable accuracy by a simple model (Fig. 4a) with parameters optimized over the experimental data (Fig. 4b and Supplementary Fig. 4). The first peak (at \( L + n = 50 \) residues) in the nD \( f_{FL} \) profiles was generated by the \( \Delta \Psi \), and the second peak (at \( L + n = 55 \) residues) was generated by the surface potential on the periplasmic side of the membrane. The model also correctly reproduced the increasingly steep rise in the \( f_{FL} \) profiles at \( L + n = 47 \) for increasing \( n \) values, but it failed in the region \( L + n > 60 \) residues because we did not attempt to model the nonelectrostatic forces apparently acting in this region (described above). Additional work probing both electrostatic and hydrophobic interactions between the nascent chain and the ribosome-translocon conduit will be required to substantiate and refine the model to a point that it can accurately predict force profiles for arbitrary nascent chain sequences.

The relation between the total electric force predicted by the model and the experimentally observed \( f_{FL} \) values for the \((SG)_2-nX-(SG)_2\) constructs with the Sec(Ms) AP (Fig. 4c) allows us to estimate forces (in pN) from measured \( f_{FL} \) values, not only for the nX constructs analyzed here but in general. A similar relation holds for the stronger Sec(Ms-Sup1) AP (Fig. 4d). The typical forces from the model (up to \(~40\) pN for the 5D stretch; Fig. 4d) compare favorably with previous measurements of, for example, the force in unfolding of titin domains (\(~100\) pN)\(^20\), the force exerted by a viral packaging motor (\(~50\) pN)\(^21\) or the force generated by the ribosome on the mRNA during the translocation step (\(~13\) pN)\(^22\).

DISCUSSION
Our findings highlight a hitherto-unappreciated aspect of membrane translocation dynamics: a strong electric force acting on the nascent polypeptide chain during its passage through the SecYEG translocon. Although our studies are confined to cotranslational translocation, there is no reason why post-translationally translocating chains should not experience the same kind of force. Fine-tuning of chain translocation dynamics by charged residues may have implications for, for example, cotranslational protein folding, membrane-protein biogenesis and regulatory processes involving translational arrest peptides\(^23\).
METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Foundation for Strategic Research, the European Research Council (ERC-2008-AdG 232648), the Swedish Cancer Foundation, the Swedish Research Council and the Knut and Alice Wallenberg Foundation to G.v.H., and by a grant from the Wenner-Gren Foundation to M.L.

AUTHOR CONTRIBUTIONS

N.I. contributed to the study design, the experimental work and the writing of the paper. R.H. contributed to the study design, the experimental and modeling work and the writing of the paper. M.L. contributed to the modeling work and the writing of the paper. G.v.H. contributed to the study design, the modeling work and the writing of the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Enzymes and chemicals. All enzymes were obtained from Thermo Scientific and New England BioLabs. Oligonucleotides were from Eurofins MWG Operon. [35S]methionine was from PerkinElmer. All other reagents were from Sigma-Aldrich.

DNA manipulations. All constructs were generated from the previously described pING1 plasmid carrying the lepB gene containing a 6L, 13A H-segment insert and the eight-residue SecM arrest peptide, HAPIRGSP, from M. succiniciproducens under the control of an arabinose-inducible promoter3. The plasmid was digested with SpeI and KpnI to release the 6L, 13A segment, and oligonucleotides corresponding to the various test residues (GG-nX-GG) were ligated in its place. Shorter linker lengths, L, between the test segments and the arrest peptide were generated by PCR with forward and reverse primers complementary to regions denoted by the right- or left-facing arrows in Supplementary Figure 1a. In order to generate constructs with the stronger HPPIRGSP and the nonfunctional HAPIRGS A arrest peptides, QuickChange site-directed mutagenesis was performed to convert the alanine to proline and proline to alanine, respectively (underlined).

Pulse-labeling analysis. E. coli MC1061 cells bearing the respective plasmids were grown overnight at 37 °C in M9 minimal medium supplemented with 19 natural amino acids (1 µg ml−1; no methionine), 100 µg ml−1 thiamine, 0.1 mM CaCl2, 2 mM MgSO4, 0.4% (w/v) fructose, and 100 µg ml−1 ampicillin. Cultures were back-diluted to OD600 = 0.1 and grown to OD600 = 0.35. LepB expression was induced with 0.2% (w/v) arabinose for 5 min. For cultures treated with indole, appropriate volumes of 1 M indole (in EtOH) were added to the cultures to final concentrations of 1, 2, 3, 4, and 5 mM indole 1 min before pulse labeling. EtOH was added to 0.5% (v/v) for mock-treated samples. Cells were then pulse labeled with [35S]methionine for 2 min at 37 °C before being added to an equal volume of 20% trichloroacetic acid (TCA). The samples were incubated on ice for 30 min and spun for 5 min at 20,800g at 4 °C. The pellet was washed with cold acetone, spun again for 5 min at 4 °C, and subsequently solubilized in Tris-SDS solution (10 mM Tris-Cl, pH 7.5, and 2% SDS) at 95 °C for 10 min. The samples were spun for 5 min at room temperature, and the lysate was used for immunoprecipitation with LepB and OmpA antisera. The samples were resolved by SDS-PAGE, and the was gel visualized with a Fuji FLA-3000 phosphorimager and ImageGauge V4.23 software. Quantification of protein bands was performed with QtiPlot 0.9.7.10. All experiments were repeated three times with independent culture incubations, and standard errors were calculated. Original images of autoradiographs used in this study can be found in Supplementary Data Set 1.

In vitro transcription and translation. In vitro transcription was performed with SP6 RNA polymerase according to the manufacturer’s protocol (Promega), with PCR products as templates for the generation of truncated nascent chains. RNA obtained was purified with the RNeasy Mini Kit (Qiagen). Translations were performed in a rabbit reticulocyte lysate system as described (Promega) for 15 min at 30 °C in the presence of 0.5 µl of dog pancreas rough microsomes and 1 µl of [35S]methionine (5 µCi). The reaction was stopped by the addition of an equal volume of sample buffer and treated with RNase A (200 µg ml−1) for 15 min at 30 °C before the samples were resolved by SDS-PAGE. All experiments were repeated three times with independent incubations, and standard errors were calculated.