Supplementary Data S1

Derivation of the ΔG^{aa}_{app} scale

The fraction of unglycosylated molecules is < 0.15 for all constructs, and has been ignored in the calculations. The fraction $f'_{2g} = C_{2g}/(C_{1g} + C_{2g})$ (where C is the pixel-count for the band in question) of doubly glycosylated molecules varies from 0 for H-segments such as GGPGALAALALAALAALALAALAGPGG that are fully integrated as a transmembrane segment, to 0.86 for H-segments such as GGPGDKQEGEWPTGLRLSRIGGIGPGG (corresponding to residues 304-322 in the translocated P2 domain of Lep) that are fully translocated across the membrane, reflecting the fact that a lumenally exposed glycosylation site is only modified in ~95% of the molecules. To correct for this, the values used in the calculation of ΔG_{app} were normalized:

$f_{1g} = (f'_{1g} - 0.13)/0.87)$; $f_{2g} = 1 - f_{1g}$

As the quantitation is maximally sensitive for H-segments with ΔG_{app} values close to zero ($p \approx 0.5$ in Fig. 1D), we balanced, for each kind of residue, the contribution from the central residue by varying the number of Leu residues until an H-segment with ΔG_{app} in the range [-1.2, 1.2] kcal/mol was found. Since, as shown in the main text, there is an appreciable positional variation in ΔG_{app} for different H-segments of a given overall amino acid composition, the approach taken has been to calculate individual ΔG^{aa}_{app} values in a step-wise fashion by comparing H-segments with as similar sequences as possible. Values were calculated to two decimal places and rounded off to one decimal place when discussed in the text.

1. $\Delta G^{Leu}_{app} = -0.55$ kcal/mol; $\Delta G^{Ala}_{app} = 0.11$ kcal/mol
These values were obtained from Eq. 1 in the main text and the assumption $\Delta G^{flank}_{app} = 0$.

2. $\Delta G^{Ile}_{app} = -0.60$ kcal/mol
This value was obtained from ΔG^{Leu}_{app} by adding the mean $\Delta\Delta G^{Leu\rightarrow Ile}_{app} = -0.05$ kcal/mol for the following pairs of H-segments:

GGPGAAAALAAAALAAAALAAAAGPGG
GGPGAAAALAAAAIAAAALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Ile}_{app} = -0.06$ kcal/mol

GGPGAAAALALALPLALALAAAAGPGG
GGPGAAAALALALPIALALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Ile}_{app} = 0.14$ kcal/mol

GGPGAAAALALALNLALALAAAAGPGG
GGPGAAAALALALNIALALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Ile}_{app} = 0.01$ kcal/mol

GGPGAAAALALALHLALALAAAAGPGG
GGPGAAAALALALHIALALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Ile}_{app} = -0.15$ kcal/mol

GGPGAAAALALALRLALALAAAAGPGG
GGPGAAAALALALRIALALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Ile}_{app} = -0.14$ kcal/mol

GGPGAAAALALALELALALAAAAGPGG
GGPGAAAALALALEIALALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Ile}_{app}$ = -0.06 kcal/mol

GGPGAAAALALALQLALALAAAAGPGG
GGPGAAAALALALQIALALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Ile}_{app}$ = -0.13 kcal/mol

3. ΔG^{Phe}_{app} = -0.32 kcal/mol
This value was obtained from ΔG^{Leu}_{app} by adding the $\Delta\Delta G^{Leu\rightarrow Phe}_{app}$ = 0.23 kcal/mol for the following pair of H-segments:

GGPGAAAALAAAALAAAALAAAAGPGG
GGPGAAAALAAAAFAAAALAAAAGPGG

4. ΔG^{Val}_{app} = -0.31 kcal/mol
This value was obtained from ΔG^{Leu}_{app} by adding the $\Delta\Delta G^{Leu\rightarrow Val}_{app}$ = 0.24 kcal/mol for the following pair of H-segments:

GGPGAAAALAAAALAAAALAAAAGPGG
GGPGAAAALAAAAVAAAALAAAAGPGG

5. ΔG^{Cys}_{app} = -0.13 kcal/mol
This value was obtained from ΔG^{Leu}_{app} by adding the mean $\Delta\Delta G^{Leu\rightarrow Cys}_{app}$ = 0.42 kcal/mol for the following pairs of H-segments:

GGPGAAAALAAAALAAAALAAAAGPGG
GGPGAAAALAAAACAAAALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Cys}_{app}$ = 0.39 kcal/mol

GGPGAAAALALAALAALALAAAAGPGG
GGPGAAAALALAACAALALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Cys}_{app}$ = 0.45 kcal/mol

6. ΔG^{Met}_{app} = -0.10 kcal/mol
This value was obtained from ΔG^{Leu}_{app} by adding the $\Delta\Delta G^{Leu\rightarrow Met}_{app}$ = 0.45 kcal/mol for the following pair of H-segments:

GGPGAAAALAAAALAAAALAAAAGPGG
GGPGAAAALAAAAMAAAALAAAAGPGG

7. ΔG^{Trp}_{app} = 0.30 kcal/mol
This value was obtained from ΔG^{Leu}_{app} by adding the mean $\Delta\Delta G^{Leu\rightarrow Trp}_{app}$ = 0.84 kcal/mol for the following pairs of H-segments:

GGPGAAAALAAAALAAAALAAAAGPGG
GGPGAAAALAAAAWAAAALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Trp}_{app}$ = 1.03 kcal/mol

GGPGAAAALALAALAALALAAAAGPGG
GGPGAAAALALAAWAALALAAAAGPGG $\Delta\Delta G^{Leu\rightarrow Trp}_{app}$ = 0.66 kcal/mol

8. ΔG^{Thr}_{app} = 0.52 kcal/mol

This value was obtained from ΔG^{Ala}_{app} by adding the $\Delta\Delta G^{Ala\rightarrow Thr}_{app} = 0.41$ kcal/mol for the following pair of H-segments:

GGPGAAAALALAAAAALALAAAAGPGG
GGPGAAAALALAATAALALAAAAGPGG

9. $\Delta G^{Tyr}_{app} = 0.68$ kcal/mol
This value was obtained from ΔG^{Trp}_{app} by adding the $\Delta\Delta G^{Trp\rightarrow Tyr}_{app} = 0.38$ kcal/mol for the following pair of H-segments:

GGPGAAAALALAAWAALALAAAAGPGG
GGPGAAAALALAAYAALALAAAAGPGG

10. $\Delta G^{Gly}_{app} = 0.74$ kcal/mol
This value was obtained from ΔG^{Ala}_{app} by adding the $\Delta\Delta G^{Ala\rightarrow Gly}_{app} = 0.63$ kcal/mol for the following pair of H-segments:

GGPGAAAALALAAAAALALAAAAGPGG
GGPGAAAALALAAGAALALAAAAGPGG

11. $\Delta G^{Ser}_{app} = 0.84$ kcal/mol
This value was obtained from ΔG^{Ala}_{app} by adding the $\Delta\Delta G^{Ala\rightarrow Ser}_{app} = 0.73$ kcal/mol for the following pair of H-segments:

GGPGAAAALALAAAAALALAAAAGPGG
GGPGAAAALALAASAALALAAAAGPGG

12. $\Delta G^{Asn}_{app} = 2.05$ kcal/mol
This value was obtained from ΔG^{Ala}_{app} by adding the $\Delta\Delta G^{Ala\rightarrow Asn}_{app} = 1.94$ kcal/mol for the following pair of H-segments:

GGPGAAAALALAALAALALAAAAGPGG
GGPGAAAALALALNAALALAAAAGPGG

13. $\Delta G^{His}_{app} = 2.06$ kcal/mol
This value was obtained from ΔG^{Asn}_{app} by adding the mean $\Delta\Delta G^{Asn\rightarrow His}_{app} = 0.01$ kcal/mol for the following pairs of H-segments:

GGPGAAAALALALNLALALAAAAGPGG
GGPGAAAALALALHLALALAAAAGPGG $\quad \Delta\Delta G^{Asn\rightarrow His}_{app} = 0.08$ kcal/mol

GGPGAAAALALALNIALALAAAAGPGG
GGPGAAAALALALHIALALAAAAGPGG $\quad \Delta\Delta G^{Asn\rightarrow His}_{app} = -0.07$ kcal/mol

14. $\Delta G^{Pro}_{app} = 2.23$ kcal/mol
This value was obtained from ΔG^{Asn}_{app} by adding the mean $\Delta\Delta G^{Asn\rightarrow Pro}_{app} = 0.18$ kcal/mol for the following pairs of H-segments:

GGPGAAAALALALNLALALAAAAGPGG
GGPGAAAALALALPLALALAAAAGPGG $\quad \Delta\Delta G^{Asn\rightarrow Pro}_{app} = 0.11$kcal/mol

GGPGAAAALALALNIALALAAAAGPGG
GGPGAAAALALALPIALALAAAAGPGG $\Delta\Delta G^{Asn\rightarrow Pro}_{app}$ = 0.24 kcal/mol

15. ΔG^{Gln}_{app} = 2.36 kcal/mol
This value was obtained from ΔG^{Asn}_{app} by adding the mean $\Delta\Delta G^{Asn\rightarrow Gln}_{app}$ = 0.31 kcal/mol for the following pairs of H-segments:

GGPGAAAALALALNLALALAAAAGPGG
GGPGAAAALALALQLALALAAAAGPGG $\Delta\Delta G^{Asn\rightarrow Gln}_{app}$ = 0.38kcal/mol

GGPGAAAALALALNIALALAAAAGPGG
GGPGAAAALALALQIALALAAAAGPGG $\Delta\Delta G^{Asn\rightarrow Gln}_{app}$ = 0.24 kcal/mol

16. ΔG^{Lys}_{app} = 2.71 kcal/mol
This value was obtained from ΔG^{Asn}_{app} by adding $\Delta\Delta G^{Asn\rightarrow Lys}_{app}$ = 0.66 kcal/mol for the following pair of H-segments:

GGPGAAAALALALNLALALAAAAGPGG
GGPGAAAALALALKLALALAAAAGPGG $\Delta\Delta G^{Asn\rightarrow Lys}_{app}$ = 0.66kcal/mol

17. ΔG^{Arg}_{app} = 2.58 kcal/mol
This value was obtained from ΔG^{Lys}_{app} by adding the $\Delta\Delta G^{Lys\rightarrow Arg}_{app}$ = -0.13 kcal/mol for the following pair of H-segments:

GGPGAAAALALALKLALALAAAAGPGG
GGPGAAAALALALRLALALAAAAGPGG

18. ΔG^{Glu}_{app} = 2.68 kcal/mol
This value was obtained from ΔG^{Lys}_{app} by adding the $\Delta\Delta G^{Lys\rightarrow Glu}_{app}$ = -0.03 kcal/mol for the following pair of H-segments:

GGPGAAAALALALKLALALAAAAGPGG
GGPGAAAALALALELALALAAAAGPGG

19. ΔG^{Asp}_{app} = 3.49 kcal/mol
This value was obtained from ΔG^{Lys}_{app} by adding the $\Delta\Delta G^{Lys\rightarrow Asp}_{app}$ = 0.78 kcal/mol for the following pair of H-segments:

GGPGAAAALALALKLALALAAAAGPGG
GGPGAAAALALALDLALALAAAAGPGG

Supplementary Data S2

Additivity of ΔG^{aa}_{app} values

The H-segments below allow $\Delta\Delta G_{app}$ values to be calculated from both single- and double-residue replacements. The first column shows the H-segment, the second the corresponding ΔG_{app} value, and the third column the $\Delta\Delta G_{app}$ value per residue.

Gly->Ala	**ΔG_{app} kcal/mol**	**$\Delta\Delta G_{app}$ kcal/mol**
GGPGAAAALALAAGAALALAAAAGPGG	0.16	
GGPGAAAALALAAAAALALAAAAGPGG	-0.46	-0.62
GGPGAAAALAGAALAAGALAAAAGPGG	1.20	
GGPGAAAALAAAALAAAALAAAAGPGG	-0.06	-0.63
Trp->Ala		
GGPGAAAALALAAWAALALAAAAGPGG	-0.41	
GGPGAAAALALAAAAALALAAAAGPGG	-0.46	-0.05
GGPGAAAAAAAWLLWAAAAAAAAGPGG	0.88	
GGPGAAAAAAAAALLAAAAAAAAGPGG	0.79	-0.04
Tyr->Ala		
GGPGAAAALALAAYAALALAAAAGPGG	-0.03	
GGPGAAAALALAAAAALALAAAAGPGG	-0.46	-0.43
GGPGAAAAAAAYLLLYAAAAAAAGPGG	0.97	
GGPGAAAAAAAALLLAAAAAAAAGPGG	0.05	-0.46
Ser->Ala		
GGPGAAAALALAASAALALAAAAGPGG	0.27	
GGPGAAAALALAAAAALALAAAAGPGG	-0.46	-0.73
GGPGAAAAAASLLALLSAAAAAAGPGG	0.90	
GGPGAAAAAALLAAALLAAAAAAGPGG	-0.66	-0.78

Supplementary Data S3

Membrane integration *in vivo*

The Lep constructs containing the H-segments listed in Table I below were expressed in BHK cells as described in Methods. ΔG_{app} values were calculated in the same way as for the *in vitro* measurements, but without the correction for inefficient glycosylation applied to the *in vitro* data. Table I, column 4 gives the *in vivo* ΔG_{app} values estimated from the linear correlation ΔG_{app}(*in vivo*) = 1.54 ΔG_{app}(*in vitro*) + 0.52 kcal/mol (see Fig. 2B in the article). The transition from a non-inserted to a membrane-inserted form takes place over a slightly narrower range of Ala→Leu replacements *in vivo*, and the zero-point is displaced 0.52 kcal/mol compared to the *in vitro* results.

Table I

H-segment	*in vitro* ΔG_{app} (kcal/mol)	*in vivo* ΔG_{app} (kcal/mol)	*in vivo* ΔG_{calc} (kcal/mol)
GGPGAAAAAAAAAAAAAAAAAGPGG	0.79	1.06	1.74
GGPGAAAALAAAALAAAALAAAAGPGG	-0.06	0.81	0.43
GGPGAAAALALAAAAALALAAAAGPGG	-0.46	-0.14	-0.19
GGPGAAAALALAALAALALAAAAGPGG	-1.07	-1.51	-1.13
GGPGAAAALALAAGAALALAAAAGPGG	0.16	0.55	0.77
GGPGAAAALALALGLALALAAAAGPGG	-1.52	-1.85	-1.82
GGPGAAAALALAAWAALAAAAAAGPGG	0.07	0.97	0.63
GGPGAAAALALALWAALALAAAAGPGG	-1.07	-1.06	-1.13
GGPGAAAALALALPLALALAAAAGPGG	-0.17	0.47	0.26
GGPGALAALALALPLALALAALAGPGG	-1.26	-1.85	-1.43
GGPGAAAALALALKLALALAAAAGPGG	0.38	1.16	1.11
GGPGALAALALALKLALALAALAGPGG	-0.81	-0.26	-0.73
GGPGALAALALALDAALALAALAGPGG	0.34	1.28	1.04
GGPGALAALALLLDLALALAALAGPGG	-0.55	-0.44	-0.33

Supplementary Data S4

Analysis of flanking residues

H-segments used to analyse the role of flanking residues.

H-segment	ΔG_{app} kcal/mol
3L/16A series	
GGPGAAAALAAAALAAAALAAAAGPGG	-0.06
GGGPGAAAALAAAALAAAALAAAAGPGGG	0.12
GGGGPGAAAALAAAALAAAALAAAAGPGGGG	0.03
GGGGGPGAAAALAAAALAAAALAAAAGPGGGGG	-0.12
GGGGGGPGAAAALAAAALAAAALAAAAGPGGGGGG	-0.23
NNPNAAAALAAAALAAAALAAAANPNN	0.63
4L/15A series	
GGPGAAAALALAAAAALALAAAAGPGG	-0.46
NNPNAAAALALAAAAALALAAAANPNN	-0.21