Supplementary Note 1.

Cell-cell fusion assay. CHO cells (a gift from J. M. Coffin) were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO2, except where noted otherwise. Cells were not tested for Mycoplasma contamination. Plasmids pPEP98, pPEP99, pPEP100, and pPEP101 encode HSV-1 (strain KOS) gB, gD, gH, and gL genes, respectively, in a pCAGGS vector and were gifts from P. G. Spear. Plasmids pCAGT7 (carrying the T7 polymerase gene) and pT7EMCLuc (carrying the firefly luciferase gene) were also gifts from P. G. Spear. Plasmid pSC386 carrying the herpesvirus entry mediator (HVEM) gene and the pCAGGS vector were gifts from G. H. Cohen and R. J. Eisenberg. Cell fusion was measured by using the luciferase assay[27]. CHO cells were seeded into 6-well and 24-well plates and transfected the next day at 70-90% confluence using Lipofectamine 2000. Target cells in the 6-well plate were transfected with 1.6 μg pSC386 (HVEM) DNA and 0.4 μg pT7EMLuc (firefly luciferase) in 1 ml Optimem with 5 μl Lipofectamine 2000 per well. Effector cells in 24-well plates were transfected with 80 ng each pCAGT7 (T7 polymerase), pPEP99 (HSV-1 gD), pPEP100 (HSV-1 gH), and pPEP101 (gL), plus 80 ng of either pCAGGS (empty vector), pPEP98 or pRC30 (WT HSV-1 gB), or a single cysteine gB mutant in 200 μl Optimem and 1 μl Lipofectamine 2000 per well. After 4 hours, the target cells were washed with phosphate-buffered saline (PBS), treated with trypsin, and co-cultured with effector cells at a 1:1 ratio for 16 hrs. Cells were then washed with 1 ml PBS per well and lysed with 200 μl/well of 1x lysis buffer (Promega) and either frozen at -80°C and assayed at a later time or assayed immediately. Luciferase production for each sample was assayed by measuring luminescence on a BioTek plate reader after adding 100 μl of substrate (Promega). After the pCAGGS background was subtracted from all samples, the light output of each mutant was expressed as a percentage of the matched WT gB construct (pPEP98 or pRC30). Every mutant was tested in at least two biological replicates, each consisting of three technical replicates. Values reported here represent average fusion activity of each biological replicate relative to WT gB (100% fusion). Error bars show one standard deviation from the biological replicate average.

Supplementary Table 1. Inter-protomer contacts in TMD and CTD

Protomer B		Protomer C		Distance, Å
LEU 788	CD2	LEU 783	CG	3.82
ALA 791	CB	GLY 787	O	3.35
	O	ALA 790	CB	3.86
	CB	ALA 791	N	3.63
			CA	3.74
			CB	3.53
PHE 792	CE2	LEU 783	CD1	3.86
	CZ			3.5
	CE1	GLY 787	CA	3.4
			N	3.82
PHE 795	CB	ALA 790	O	3.94
			CB	3.75
	CG			3.88
	CD1		O	3.92
	N	ALA 794	CB	3.89
	CA			3.72
VAL 798	CG1	TYR 797	CB	3.71
			CG	3.35
			CD1	3.66
			CD2	3.53
	CG2		CB	3.96
			CG	3.95
			CD2	3.53
LEU 801	CD1	LEU 801	CD1	3.87
	O	LEU 809	CD2	3.31
GLN 802	N	TYR 797	OH	3.54
	CA			3.31
	CB			3.66
PRO 805	CB	LEU 809	CD1	3.81
			CD2	3.74
	CG		CD1	3.82
			CD2	3.53
MET 806	CG	TYR 810	OH	3.35
	SD			3.71
TYR 810	CE1	MET 850	O	3.87

TYR 810	CZ			3.67
	OH		C	3.43
			O	2.73
			CB	3.84
	CE1	VAL 853		3.95
		SER 854	N	3.59
			CA	3.75
			CB	3.93
			OG	3.02
	CZ			3.13
	OH			2.67
	CA	GLU 857	CD	3.75
			OE1	3.43
			OE2	3.49
	C		CD	3.79
			OE1	3.00
			OE2	3.96
	O		OE1	3.56
	CB		OE2	3.69
	CG			3.72
	CD1			2.89
	CE1			3.79
PRO 811	N	GLU 857	CD	3.7
			OE1	2.81
	CA			3.46
	C			3.15
	CG			3.52
	CD		CD	3.63
			OE1	3.11
LEU 812	N	GLU 857	CD	3.42
			OE1	2.26
	CA			3.08
	C			3.57
	CB		CG	3.65
			CD	3.75
			OE1	3.19
		GLU 860	CD	3.97
			OE1	3.4
	CG		CG	3.93

LEU 812			CD	3.42
			OE1	3.31
			OE2	3.81
	CD1		CD	3.6
			OE1	3.82
			OE2	3.47
		LYS 864	CE	3.96
			NZ	3.18
THR 813	CG2	VAL 853	CG1	3.52
	N	GLU 857	CD	3.89
			OE1	3.09
	CG2		CD	3.47
			OE1	3.49
			OE2	3.24
LYS 815	NZ	LYS 864	NZ	2.87
GLU 845	OE2	LYS 839	CD	3.25
			CE	3.29
			NZ	3.98
MET 846	SD	ALA 843	CA	3.95
			CB	3.8
	CE	MET 846	CG	3.75
			CB	3.79
			CE	3.81
ILE 847	CG2	MET 806	CE	3.61
		TYR 810	CB	3.75
			CG	3.93
			CD2	3.9
		THR 813	OG1	3.64
	CD1			3.91
	CG2	THR 814		3.75
ARG 848	CG	THR 813	O	3.91
TYR 849	OH	ASP 834	O	3.88
	CE1	ASP 836		3.4
			N	3.95
	OH			3.15
			CA	3.72
			CB	3.23
	CD1	LYS 839		4.00

TYR 849	CE1			3.67
	CE2			3.9
	CZ			3.61
	O	LEU 840	CD1	3.57
	CD1		N	3.71
			CA	3.91
MET 850	C	MET 806	CG	3.82
	CB		CE	3.91
	CE	ILE 847	CG1	3.61
		MET 850	SD	3.74
ALA 851	CA	ASN 804	OD1	3.97
	CB			3.86
	N	MET 806	CG	3.56
			CB	3.98
	CA		CG	3.66
			CB	3.78
	CB			3.57
		THR 814	CG2	3.85
VAL 853	CG2	LEU 840	CD1	3.53
	CB	ILE 847		3.89
	CG1			3.58
	CG2			3.82
SER 854	CB	ASN 804	CA	3.55
			OD1	3.66
	OG			3.95
	CB	PRO 805	CD	3.78
	OG			3.32
			CG	3.88
ARG 858	NE	SER 803	CB	3.31
			CA	3.63
			C	3.44
	CZ		CB	3.63
			C	3.85
			OG	3.91
	NH2		CB	3.26
			CA	3.91
			C	3.37
			O	3.39

ARG 858			OG	3.08
	NE	ASN 804	N	3.1
			CA	3.77
			CB	3.33
	CZ		N	3.75
			CB	3.37
	NH2		N	3.64
			CB	3.33

Supplementary Table 2. Mutations and their predicted effect on structure. Interactions <4Å that may be disrupted by the specified mutation are listed in reference to "protomer B". Mutations identified in HSV-2 are identified with an "*". Further residue contact details can be found in Supplementary Fig. 5 and Supplementary Table 1. A truncation resulting from a naturally occurring frameshift, rather than being engineered, is marked with a "#".

Hyperfusogenic Point Mutations			
Mutation	**Isolated (I) or Engineered (E)**	**Location in the CTD**	**Possible effect on the structure**
R796C[2]	E	TMD, next to R800, may interact with headgroups	Elimination of basic charge and possible disruption of membrane interactions
R800W[2]	E	TMD, next to R796, may interact with headgroups	Elimination of basic charge and possible disruption of membrane interactions
P805A*[3]	E	start of h1a, invariant residue	Disruption of h1 structure and thus h1/h2 and h1/tmd packing
Y810A*[3]	E	follows h1a, sc makes HB with sc of S854 and mc of M850 in C	Disruption of HBs at the h1/h2 interface
T813I[2]	E	h1b, sc has numerous weak interactions with h2 of A and C	Disruption of h1/h2 packing
L817H[4] or L817P[5] or E816/L817 2aa insertion[6]	I/E	end of h1b	Unclear
D836A*[3]	E	h2, conserved among alphas, contributes negative charge to the inner face, sc makes HBs to A838 and K839 in B.	Unclear, possible decrease in CTD base negative charge, disruption of h1/h2 packing, and h1b to h2 linker structure
Y849A*[7]	E	h2, sc makes HB with mc of D834 and mc of D836 in C	Disruption of HBs at the h2/h2 interface
V853A*[3]	E	h2, sc makes hydrophobic contacts with h1 region of A and h2 of C	Disruption of h1/h2 packing
S854F[8]	I	h2, sc makes HB with sc of Y810 of A and weak interactions with h1a of C	Disruption of HBs at the h1/h2 interface
A855V[4,9]	I	h2, no contacts of note	Unclear, potential increase in hydrophobicity
E857D*[10]	I	h2, sc makes HBs with mc	Disruption of HBs at the

		of L812 and T813 of A	h1/h2 interface
R858H[6,11] or R858C[2]	I/E	h2, poor density for sc, sc makes weak interactions with tmd-h1a connector of C, may interact with headgroups	Elimination of basic charge and possible disruption of membrane interactions, disruption of h2/TMD/h1 interface
T859P*[12]	I	h2, no contacts of note	Proline would disrupt the h2 helix thereby disrupting h1/h2 and h2/tmd packing
K864A/K865 A/K866A[13]	E	C terminus of h2, may interact with headgroups	Elimination of basic charge and possible disruption of membrane interactions

Limited surface expression mutations			
Mutation	**Isolated (I) or Engineered (E)**	**Location in the CTD**	**Possible effect on the structure**
L801A*[3]	E	TMD, faces CTD symmetry axis, mc interacts with h1a of C and sc interacts with equivalent residues on A and C	Disruption of tmd/h1 and threefold tmd interactions
M806A*[3]	E	h1a, faces CTD symmetry axis, sc contacts h1a-h1b connector of C and h2 of A	Disruption of h1/h1 and h1/h2 interactions
L809A*[3]	E	h1a, faces CTD symmetry axis, contacts tmd and h1a of A	Disruption of h1/tmd and h1/h1 interactions
P811A*[3]	E	precedes h1b, invariant residue	Disruption of h1 structure and thus h1/h2 packing
M846A*[3]	E	h2, faces CTD symmetry axis, sc interacts with equivalent residues on A and C	Disruption of threefold h2 interaction
L852A*[3]	E	h2, no known contacts	Unclear

Slow rate-of-entry mutation			
Mutation	**Isolated (I) or Engineered (E)**	**Location in the CTD**	**Possible effect on the structure**
A851V[2]	I	h2, mc and sc interact weakly with h1a and h1b of C	Stabilization of h1/h2 packing

C-terminal truncations				
Mutation	**Expression**	**gB-null virus complementation**	**Fusion phenotype**	**Possible effect on the structure**
HSV-1 gB810[6]	ND	No	No fusion	Loss of h1b, h2, and h3; loss of CTD core
HSV-1 gB838[6]	ND	No	No fusion	Loss of h2 and h3; misfolded CTD core
HSV-1 gB840[14]	ND	No	ND	Loss of h2 and h3; misfolded CTD core
HSV-1 gB849[6]	ND	No	No fusion	Loss of h3, partial loss of h2; misfolded CTD core
HSV-1 gB851[6,15] HSV-2 gB851[7]	Reduced/ Reduced	No/ No	No fusion/ No fusion	Loss of h3, partial loss of h2; misfolded CTD core
HSV-1 gB855[14] HSV-2 gB855[7]	ND/ >WT	No/ Poor	ND/ Syncytial, hyperfusogenic	Loss of h3, partial loss of h2; misfolded CTD core in HSV-1 but not HSV-2
HSV-2 gB858[7]	>WT	Yes	Syncytial, hyperfusogenic	Loss of h3, partial loss of h2
HSV-1 gB863[6]	ND	Yes	Reduced	Loss of h3, partial loss of h2
HSV-2 gB866[7]	>WT	Yes	Syncytial, hyperfusogenic	Loss of h3
HSV-1 gB868 [15,16]	WT	ND	Hyperfusogenic	Loss of h3
HSV-2 gB870[12]#	WT	ND	Syncytial	Loss of h3
HSV-2 gB874[7]	>WT	Yes	Syncytial, hyperfusogenic	Loss of h3
HSV-1 gB876[14-16]	WT	Yes	Syncytial, hyperfusogenic	Partial loss of h3
HSV-2 gB879[7]	>WT	Yes	Syncytial, hyperfusogenic	Partial loss of h3
HSV-2 gB884[7]	>WT	Yes	hyperfusogenic	Loss of unstructured C terminus
HSV-1 gB888[16]	ND	ND	WT	Loss of unstructured C terminus
HSV-2 gB894[7]	WT	Yes	WT	Loss of unstructured C terminus

Supplementary References

1. Davis, I.W. et al. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res* **35**, W375-83 (2007).
2. Gage, P.J., Levine, M. & Glorioso, J.C. Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B. *J Virol* **67**, 2191-201 (1993).
3. Ruel, N., Zago, A. & Spear, P.G. Alanine substitution of conserved residues in the cytoplasmic tail of herpes simplex virus gB can enhance or abolish cell fusion activity and viral entry. *Virology* **346**, 229-37 (2006).
4. Engel, J.P., Boyer, E.P. & Goodman, J.L. Two novel single amino acid syncytial mutations in the carboxy terminus of glycoprotein B of herpes simplex virus type 1 confer a unique pathogenic phenotype. *Virology* **192**, 112-20 (1993).
5. Diakidi-Kosta, A., Michailidou, G., Kontogounis, G., Sivropoulou, A. & Arsenakis, M. A single amino acid substitution in the cytoplasmic tail of the glycoprotein B of herpes simplex virus 1 affects both syncytium formation and binding to intracellular heparan sulfate. *Virus Res* **93**, 99-108 (2003).
6. Cai, W.H., Gu, B. & Person, S. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J Virol* **62**, 2596-604 (1988).
7. Fan, Z. et al. Truncation of herpes simplex virus type 2 glycoprotein B increases its cell surface expression and activity in cell-cell fusion, but these properties are unrelated. *J Virol* **76**, 9271-83 (2002).
8. Walev, I., Lingen, M., Lazzaro, M., Weise, K. & Falke, D. Cyclosporin A resistance of herpes simplex virus-induced "fusion from within" as a phenotypical marker of mutations in the Syn 3 locus of the glycoprotein B gene. *Virus Genes* **8**, 83-6 (1994).
9. Haanes, E.J., Nelson, C.M., Soule, C.L. & Goodman, J.L. The UL45 gene product is required for herpes simplex virus type 1 glycoprotein B-induced fusion. *J Virol* **68**, 5825-34 (1994).
10. Muggeridge, M.I. Characterization of cell-cell fusion mediated by herpes simplex virus 2 glycoproteins gB, gD, gH and gL in transfected cells. *J. Gen. Virol.*, 2017-2027 (2000).
11. Bzik, D.J., Fox, B.A., DeLuca, N.A. & Person, S. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. *Virology* **137**, 185-90 (1984).
12. Muggeridge, M.I., Grantham, M.L. & Johnson, F.B. Identification of syncytial mutations in a clinical isolate of herpes simplex virus 2. *Virology* **328**, 244-53 (2004).
13. Rogalin, H.B. & Heldwein, E.E. The interplay between the HSV-1 gB cytodomains and the gH cytotail during cell-cell fusion. *J Virol* (2015).
14. Baghian, A., Huang, L., Newman, S., Jayachandra, S. & Kousoulas, K.G. Truncation of the carboxy-terminal 28 amino acids of glycoprotein B specified by herpes simplex virus type 1 mutant amb1511-7 causes extensive cell fusion. *J Virol* **67**, 2396-401 (1993).
15. Silverman, J.L., Greene, N.G., King, D.S. & Heldwein, E.E. Membrane Requirement for Folding of the Herpes Simplex Virus 1 gB Cytodomain Suggests a Unique Mechanism of Fusion Regulation. *Journal of virology* **86**, 8171-84 (2012).
16. Foster, T.P., Melancon, J.M. & Kousoulas, K.G. An alpha-helical domain within the carboxyl terminus of herpes simplex virus type 1 (HSV-1) glycoprotein B (gB) is

associated with cell fusion and resistance to heparin inhibition of cell fusion. *Virology* **287**, 18-29 (2001).