SUPPORTING INFORMATION

Construction of Covalent Membrane Protein Complexes and High-throughput Selection of Membrane Mimics

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TABLE OF CONTENTS

Experimental details S2

Figure S1. Gel electrophoresis of αIIbβ3 TM dimerization reactions S5

Figure S2. Gel electrophoresis of αIIbβ3 TM dimerization reactions under different conditions S6

Figure S3. Terminal oxidation of β3(G690C) S7

Figure S4. Relative quantities of accumulated αIIbβ3 TM species S8

Figure S5. H-N spectra of αIIbβ3 TM in DHPC/POPC bicelles and SDS micelles S8

Figure S6. CD spectra of refolded off7-αIIb(A963C) and MBP-β3(G690C) S9

Figure S7. Template-assisted integrin αIIbβ3 TM dimerization S10

Figure S8. H-N spectra of covalently and non-covalently associated αIIbβ3 TM peptides S11

References S11

EXPERIMENTAL DETAILS

Protein expression and purification. All protein expression vectors were based on the commercial pET44 vector (Novagen, Inc.). To overexpress human integrin β3 residues Glu686-Phe727 incorporating C687S/G690C with a preceding tobacco etch virus (TEV) protease cleavage site (ENLYFQ|G), the original pET44 NusTag fusion protein was replaced with either *E. coli* maltose binding protein (MBP) or the third IgG binding domain of protein G (GB3), yielding vectors pET44-MBP-β3(G690C) and pET44-GB3-β3(G690C), respectively. To express human integrin αIIb residues Ala958-Pro998 incorporating A963C with the preceding TEV site, the previously used pET44-based vector[1] was amended to give pET44-GB3-αIIb(A963C). The GB3 fusion protein was also replaced with a gene coding for the His_6-tagged MBP-binding ankyrin repeat protein off7,[2] which was assembled from overlapping, synthetic oligonucleotides by PCR.[3] A third construct incorporating GB3 as second, N-terminal fusion protein was prepared as well, resulting in pET44-GB3-off7-αIIb(A963C).

Expression and purification of all constructs proceeded as described for pET44-GB3-αIIb.[1] In brief, gene expression was induced in *E. coli* BL21(DE3)pLysS,$T1^R$ cells (Sigma-Aldrich, Inc.) growing in M9 minimal medium at 37 °C by adding IPTG to a final concentration of 0.5 mM at A(600 nm)= 1.0. Cells were harvested after four hours and lysed by sonication in 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 300 mM NaCl, 100 mM SDS, 20 mM imidazole and 2 mM β-mercaptoethanol. The His_6-tagged proteins were purified by immobilized metal affinity chromatography (IMAC) using Ni^{2+}-based resin and eluted in 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 300 mM NaCl, 8 M urea, and 300 mM imidazole. Dialyzing against 50 mM Tris·HCl pH 8.0 and 0.5 mM EDTA overnight at 4 °C, allowed the refolding of fusion proteins in the absence of lipids/detergents as judged by CD spectroscopy (Figure S6). Specifically we note that, although folded MBP-β3(G690C) is soluble in aqueous solution, MBP refolding should be carried out at ≤2 μM to avoid protein aggregation and precipitation.[4] Protein concentrations were measured by UV spectroscopy.[5]

Analytical covalent dimer formation. For analytical assays, αIIb(A963C) and β3(G690C)-containing proteins were mixed in a volume of 100 μl at final concentrations of 10 μM each in 50 mM Tris·HCl, pH 8.0 and 0.5 mM EDTA. The solution contained the bicelles/micelles depicted in Figure 2/Figure S1 at a molar ratio of αIIb:β3:short-chain lipid/detergent:long-chain lipid of 1:1:2000:600 unless otherwise stated. For samples in organic solvents, proteins were prepared in CH_3CN/H_2O (1:1 v/v), 25 mM Hepes·NaOH, pH 7.4 or CH_3CN/H_2O/TFA (49.5:49.5:0.1 v/v) solution. Spontaneously formed cystines were reduced by adding tributylphosphine (TBP) or Tris(2-carboxyethyl)phosphine (TCEP) to final concentrations of 5 mM and incubating for 2 hr at 30 °C (Figure S1A). The use of either TBP or TCEP gave similar results (c.f. Figure 2A and Figure S4A). Disulfide bond formation was accelerated by adding the hydrophobic oxidant Cu^{2+}·(phenanthroline)$_2$ to 2.5 mM and was allowed to proceed at room temperature for 1 hours (c.f. Figure S2B). The Cu^{2+}·(phenanthroline)$_2$ complex was prepared freshly by mixing equal volumes of 200 mM $CuSO_4$ in water and 400 mM 1,10-phenanthroline in *N,N*-dimethylformamide.[6] To disintegrate the Cu^{2+}·(phenanthroline)$_2$ complex and block any remaining free cysteines, EDTA and N-ethylmaleimide (NEM) were added to final concentrations of 10 and 2 mM, respectively. Subsequently, SDS-PAGE was performed using 4-20% Tris-Hepes or 4-20% Tris-Tricine gels (Nusep, Inc.). Non-covalent dimer formation during SDS-PAGE is negligible (Figure S1A). Samples containing organic solvent were freeze-dried before performing SDS-PAGE.

Coomassie blue-stained gels were scanned and gel band intensities were integrated using the program Image J.[7] At identical protein concentrations, GB3-off7-αIIb(A963C) exhibited a 2.85-times higher staining intensity than GB3-β3(G690C) over the concentration ranges used in the present study (for example, Figure S1A). When considering dimer-staining intensities to be the linear sum of their constituent monomer intensities, staining intensities, I, relative to an equimolar amount of $1I_{\alpha}$ are $2.85I_{\beta}$, $0.5I_{\alpha\alpha}$, $1.43I_{\beta\beta}$ and $0.74I_{\alpha\beta}$. Obtained gel band intensities were converted to relative molar ratios accordingly (e.g., Figure 2A and Figure S1B). In dimerization reactions with only one GB3 fusion protein present on either the αIIb or β3 subunit, integrated gel band intensities were compared directly and are

expected to approximate the mass distribution of the GB3-tagged subunit (Figure 2B, Figures S1C-D and S4B).

Covalent dimer formation on a preparative scale. To construct disulfide-linked αIIb(A963C)-β3(G690C) on a preparative scale, GB3-αIIb(A963C) and GB3-β3(G690C) or, alternatively αIIb(A963C) and β3(G690C) derived from GB3 constructs, were mixed at final concentrations of 100 μM in 50 mM Tris·HCl, pH 8.0, 0.5 mM EDTA, 20 mM CHAPS, 6 mM DMPC solution. Subsequent to reduction with 1.25 mM TCEP, disulfide formation was assisted for 1 hr by adding Cu^{2+}·$(phenanthroline)_2$ to 3.125 mM. If applicable, GB3 fusion proteins were cleaved, following dialysis against 50 mM Tris·HCl, pH 8.0, 0.5 mM EDTA, 0.5 M urea, by TEV protease at a molar ratio of 1:50 in the presence of reduced and oxidized glutathione at 1 mM and 0.1 mM, respectively. Heterodimer was subsequently purified by reversed-phase HPLC as described previously for monomeric peptide.[1]

NMR spectroscopy. TROSY-HSQC spectra[8] were recorded at 28 °C with acquisition times of 85.4 and 71.7 ms in the $^1H^N$ and ^{15}N dimension, respectively, which avoids truncation artifacts for residues outside of the membrane (Figure 1A). ^{15}N longitudinal and transverse relaxation rates were measured[9] at 35 °C using a CPMG delay of 400 μs. Backbone H-N residual dipolar couplings, $^1D_{NH}$, were measured at 40 °C using the ARTSY scheme.[10] A negatively charged polyacrylamide gel[11,12] of 320 μl volume and 6 mm diameter was polymerized from a 4.0% w/v solution of acrylamide (AA), 2-acrylamido-2-methyl-1-propanesulfonate (AMPS) and bisacrylamide (BIS) with a monomer-to-crosslinker ratio of 63:1 (w/w) and a molar ratio of 95:5 of AA to AMPS. The dried gel was soaked with 320 μl of 0.3 mM $^2H/^{13}C/^{15}N$-labeled disulfide-linked αIIbβ3(A711P), 142 mM DHPC, 43 mM DMPC, 25 mM HEPES·NaOH, pH 7.4, 6% D_2O, 0.02% w/v NaN_3 solution. Upon transfer into an open-ended NMR tube,[13] a ^2H splitting of 0.4 Hz was observed. All experiments were recorded using a cryoprobe-equipped Bruker Avance 700 spectrometer and processed using the nmrPipe package.[14]

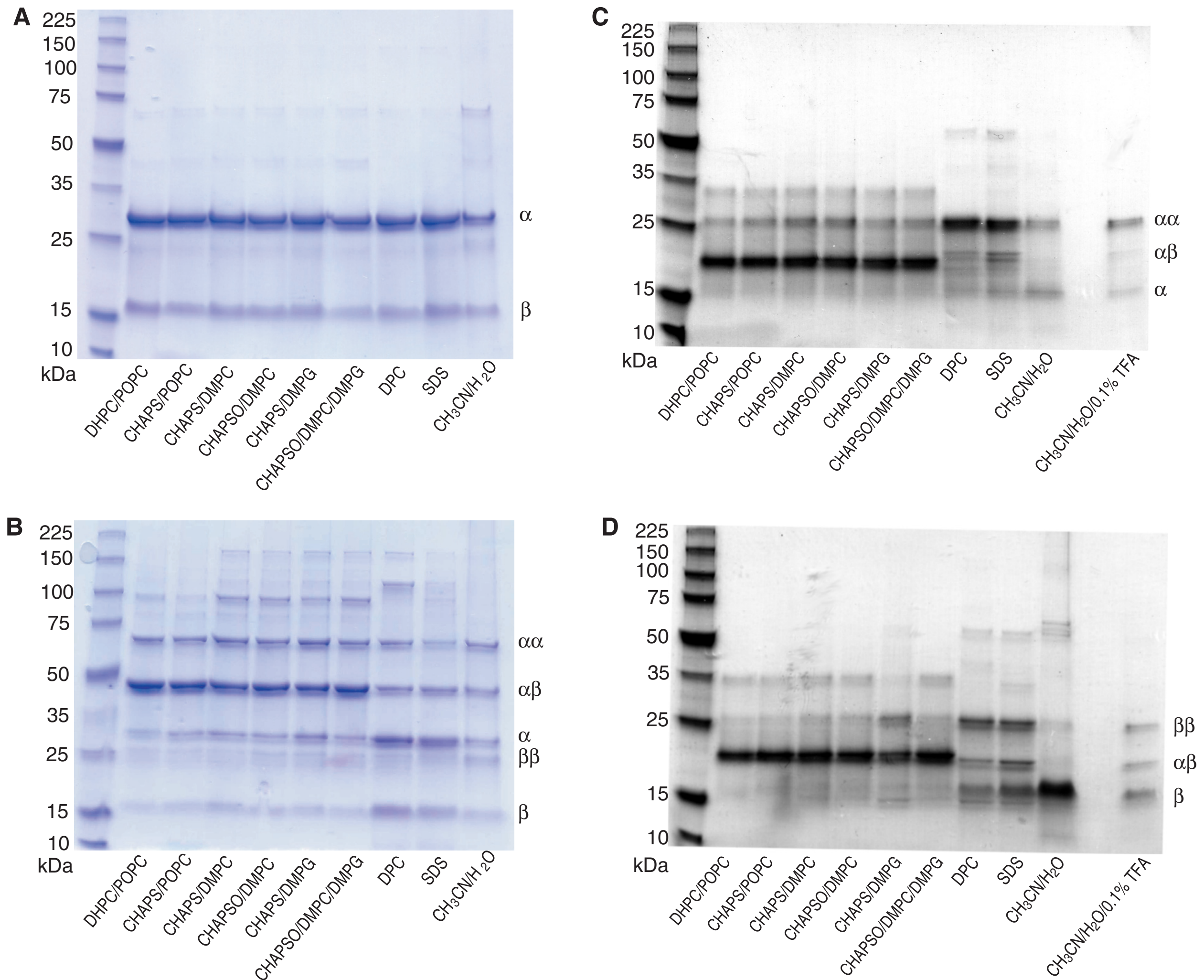

Figure S1. Gel electrophoresis of αIIbβ3 TM dimerization reactions. (**A**) GB3-off7-αIIb(A963C) and GB3-β3(G690C) proteins subsequent to reduction by tributylphosphine (TBP). (**B**) Dimer formation of GB3-off7-αIIb(A963C) and GB3-β3(G690C) as a function of membrane mimic. Shown is a 4-20% Tris-Hepes gels of reactions employing protein concentrations of 10 μM and a molar ratio of αIIb:β3:short-chain lipid/detergent:long-chain lipid of 1:1:2000:600. (**C-D**) Dimer formation of GB3-αIIb(A963C) and β3(G690C) or, alternatively, αIIb(A963C) and GB3-β3(G690C) as a function of membrane mimic. Shown are 4-20% Tris-Tricine gels of reactions employing protein concentrations of 10 μM and a molar ratio of αIIb:β3:short-chain lipid/detergent:long-chain lipid of 1:1:2000:600. Without GB3 present, monomeric and homodimeric β3(G690C) or αIIb(A963C) peptides ran as "smeared" bands, resulting from the immersion in and interference with migrating SDS micelles, that remained virtually undetectable.

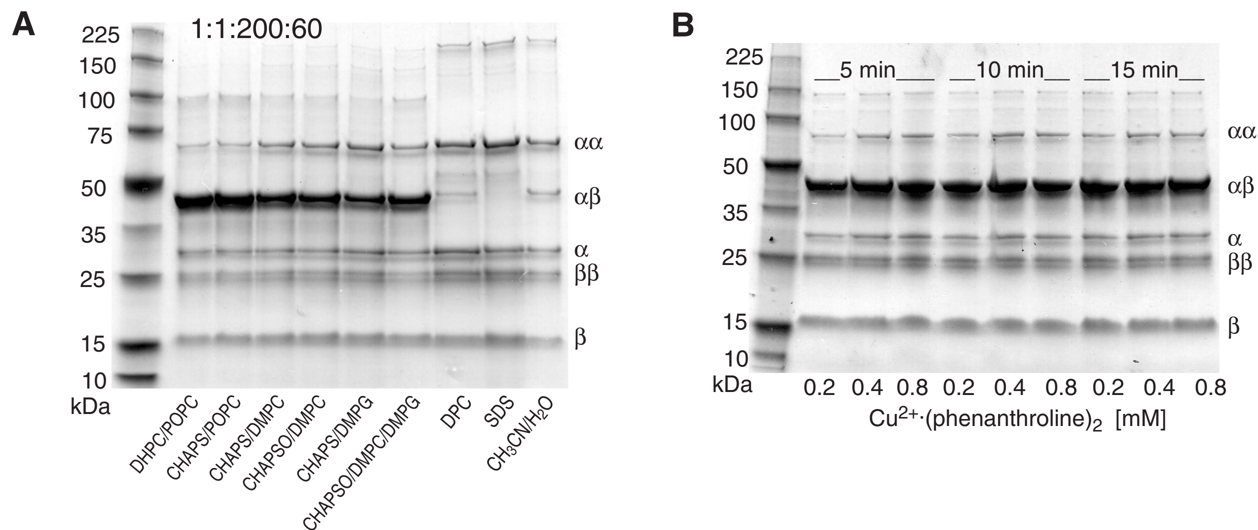

Figure S2. Gel electrophoresis of αIIbβ3 TM dimerization reactions at different conditions. (**A**) Identical conditions to Figure S1B were used except for protein concentrations of 100 μM at a molar ratio of αIIb:β3:short-chain lipid/detergent:long-chain lipid of 1:1:200:60. (**B**) The time and Cu^{2+}·(phenanthroline)$_2$ concentration dependence of covalent 10 μM GB3-off7-αIIb(A963C) and 10 μM GB3-β3(G690C) dimerization in 20 mM DHPC/6 mM POPC was examined. Proteins were reduced with 1 mM TCEP and oxidized with Cu^{2+}·(phenanthroline)$_2$ as indicated.

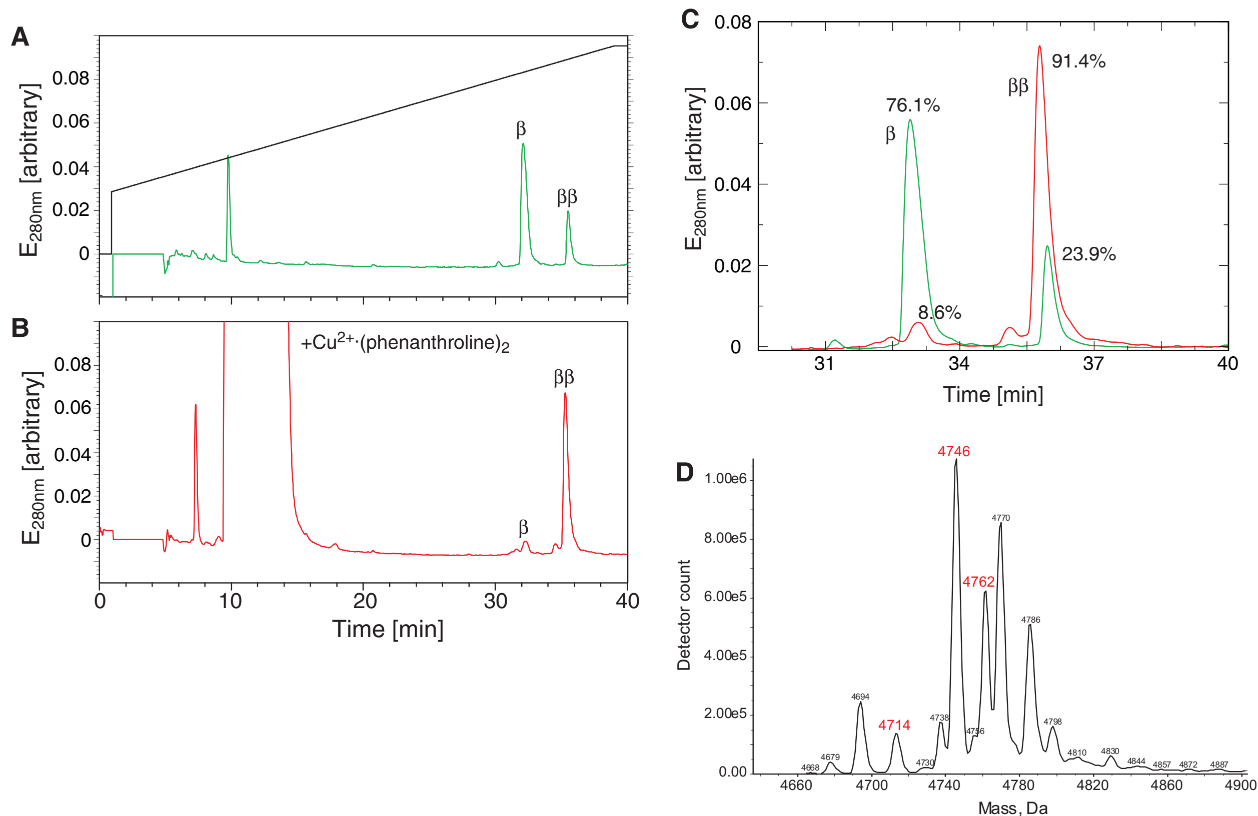

Figure S3. Terminal oxidation of β3(G690C). (**A-B**) 100 µM β3(G690C) was reduced by 1 mM TCEP in the presence of 200 mM SDS for 2 hr. Subsequently, disulfide bond formation was allowed to take place either spontaneously (panel A) or assisted by 5 mM Cu^{2+}·(phenanthroline)$_2$ (panel B). After incubating for 1 hr, 10 mM EDTA was added and HPLC was performed using a Agilent Zorbax 300SB-C18 column (4.6x250mm). A linear gradient, ranging from 30% to 100% of buffer B (49.5% n-propanol, 49.5% CH_3CN, 0.1% TFA) was performed over a period of 40 min. Buffer A consisted of 0.1% TFA. (**C**) Integration of peak areas of eluted monomer and dimeric β3(G690C) peptides suggested that 8.6% of total β3(G690C) peptide was incapable of forming dimer. (**D**) Mass reconstruction from electrospray mass spectrometry of the remaining β3(G690C) monomer peak upon Cu^{2+}·(phenanthroline)$_2$ oxidation. For the intact sulfhydryl of Cys690, a mass of 4714 Da would be expected and was observed, but dominating were oxidized forms. Oxidation of Cys690 was therefore terminal with respect to forming a disulfide bond.

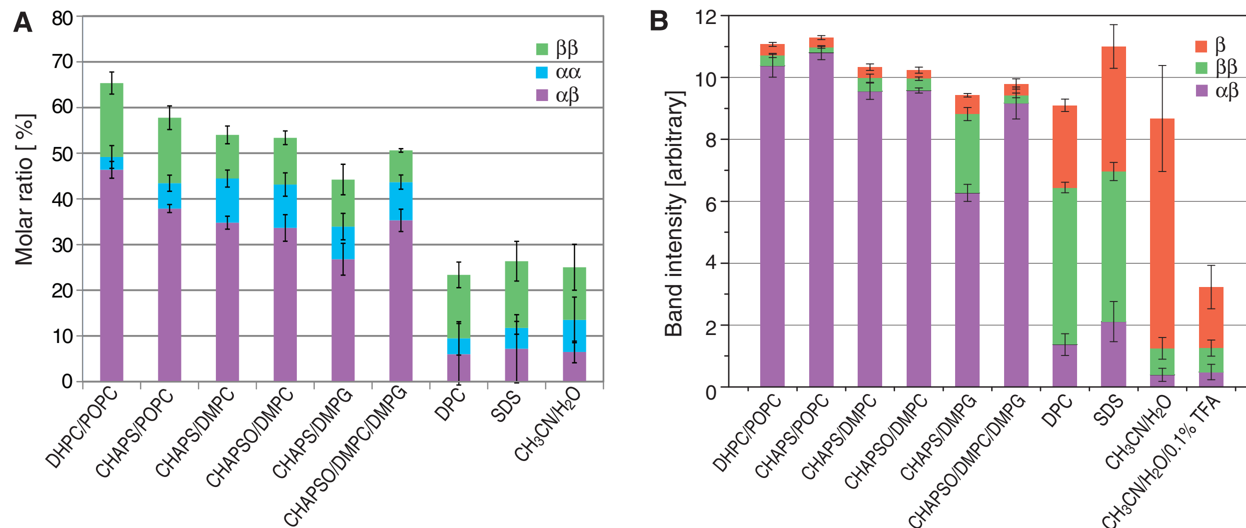

Figure S4. Relative quantities of accumulated αIIbβ3 TM species as a function of membrane mimic. (**A**) Dimeric species obtained with GB3-off7-αIIb(Ala963Cys) and GB3-β3(Gly690Cys) proteins and the reducing agent TCEP (Tris(2-carboxyethyl)phosphine) instead of TBP (c.f. Figure 2A). The size of each color-coded bar denotes its molar ratio among α, β, αα, ββ and αβ species. For visual clarity, explicit bars were omitted for α and β. (**B**) Species obtained with αIIb(Ala963Cys) and GB3-β3(Gly690Cys) proteins and TCEP. The size of each color-coded bar approximates the mass distribution of GB3-tagged β3 among β, ββ and αβ species. DHPC/POPC and CHAPS/POPC bicelles, which were indistinguishable within experimental uncertainties, were identified as best membrane mimics. In all experiments the molar ratio of αIIb:β3:short-chain lipid/detergent:long-chain lipid was 1:1:2000:600 with protein concentrations of 10 µM. Error bars denote the standard error of the mean of three experiments.

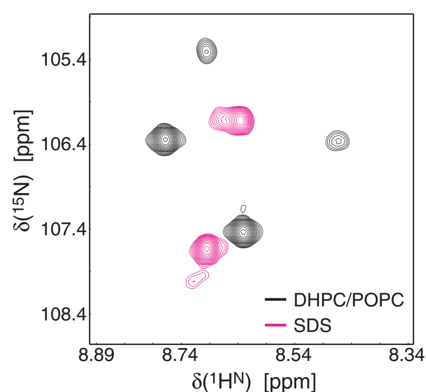

Figure S5. H-N correlation spectra of non-covalently associated integrin αIIbβ3 TM domains in membrane mimics. Comparison of DHPC/POPC bicelles with SDS micelles at relative contour levels of 1:1.61. Peptide concentrations of 0.1 mM were employed with a molar ratio of αIIb:β3:short-chain lipid/detergent:long-chain lipid of 1:1:2000:600. Spectra were recorded using $^{2}H/^{15}N$-labeled peptides in 25 mM HEPES·NaOH, pH 7.4 solution at a ^{1}H frequency of 700 MHz at 28 °C.

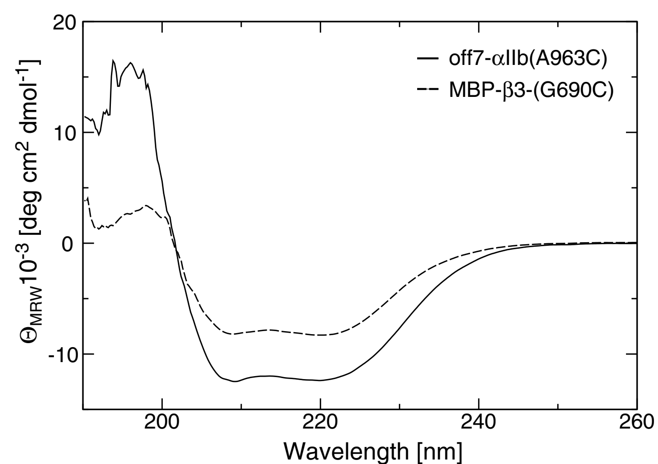

Figure S6. Characterization of refolded off7-αIIb(A963C) and MBP-β3(G690C). CD spectra of refolded off7-αIIb(A963C) and MBP-β3(G690C) in 10 mM KH_2PO_4/K_2HPO_4, pH 7.4. Protein concentrations of 10 μM were employed. Distinct minima at 208 and 222 nm indicate predominantly helical folds in accordance with MBP and off7 structures (Figure S7B).

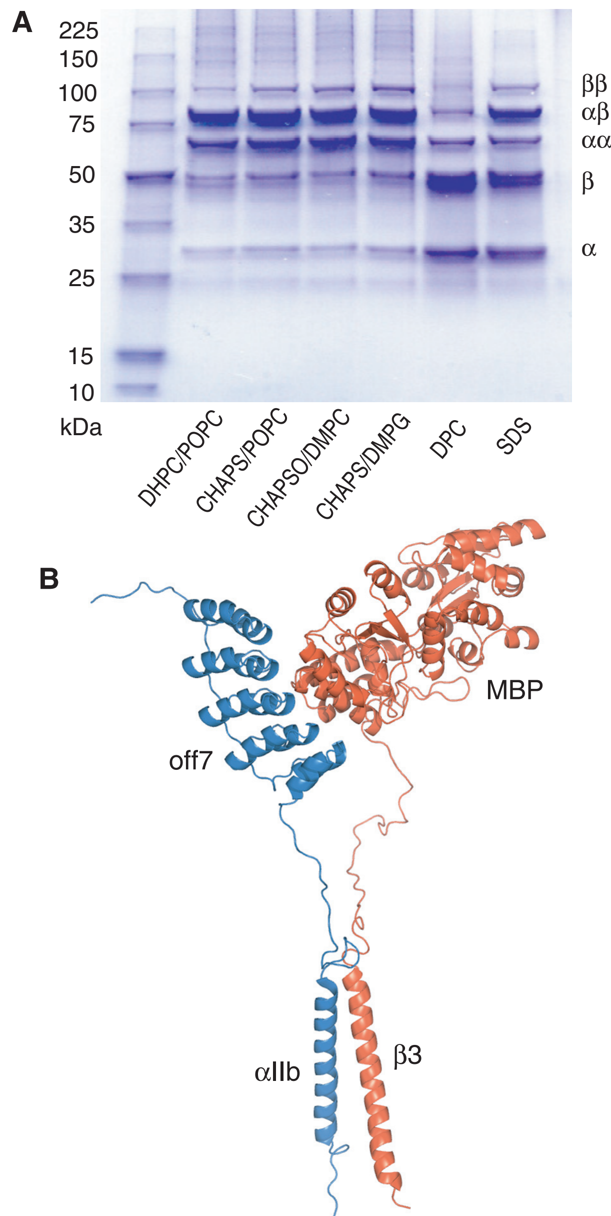

Figure S7. Template-assisted integrin αIIbβ3 TM dimerization. The engineered ankyrin repeat protein off7 (17 kDa) was evolved to bind maltose binding protein (MBP, 41 kDa) and does so with a K_D of 4.4×10^{-9} M in aqueous solution.[2] Since MBP is also a common fusion protein, although its larger size relative to GB3 means a substantial unproductive incorporation of isotope labeled metabolites, we tested whether heterodimization could be assisted by MBP-off7 complex formation. (**A**) Dimerization reactions of GB3-off7-αIIb(A963C) and MBP-β3(G690C) as a function of membrane mimic; compare to Figure S1B. Shown is a 4-20% Tris-Hepes gel of dimerization reactions at a molar ratio of αIIb:β3:short-chain lipid/detergent:long-chain lipid of 1:1:2000:600. Protein concentrations of 10 μM were employed. It is noted that MBP and off7 lack cysteine residues. (**B**) Structural model of the off7-αIIb/MBP-β3 complex constructed from PDB entries 1svx and 2k9j using linker segments, encompassing TEV cleavage sites, of random conformation. The model was prepared using the program Modeller.[15]

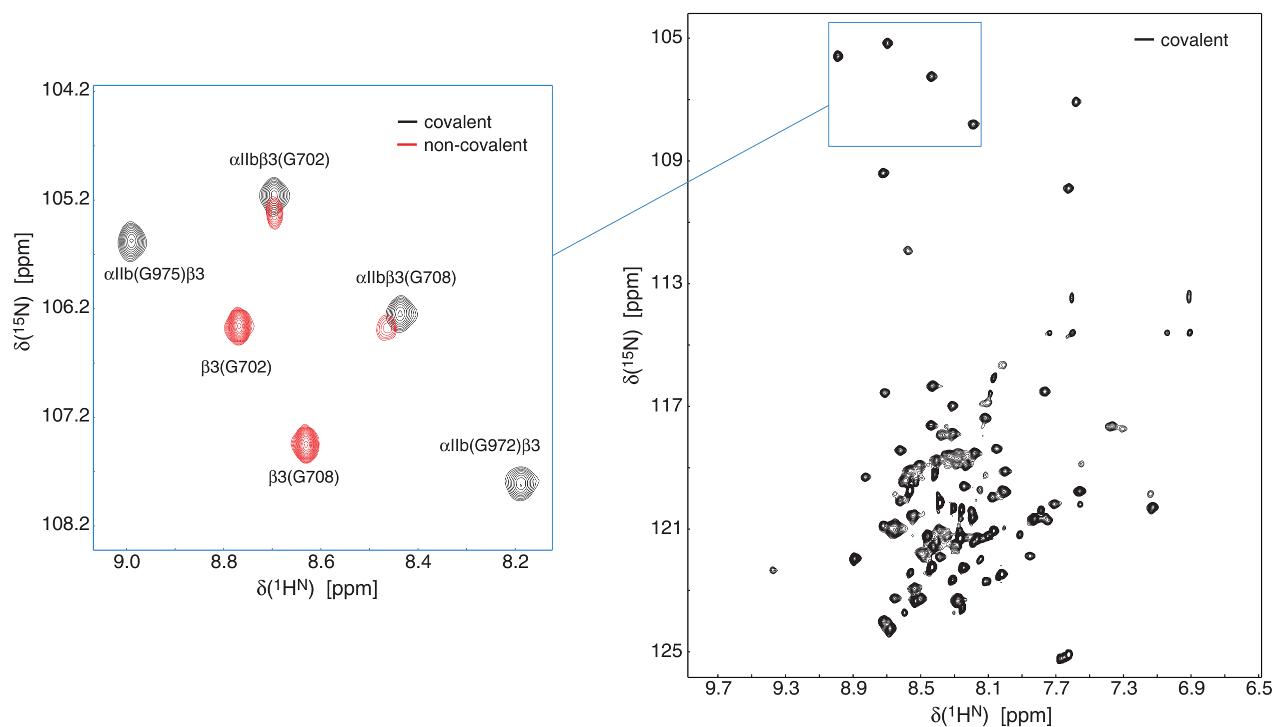

Figure S8. Comparison of H-N correlation spectra of covalently and non-covalently associated αIIbβ3 TM peptides. ^{1}H/^{14}N-labeled αIIb and ^{2}H/^{15}N-β3 TM peptides or ^{2}H/^{15}N-labeled, covalently linked αIIb(Ala963Cys)-β3(Gly690Cys) peptides were reconstituted at concentrations of 0.1 mM each in 200 mM DHPC, 60 mM POPC, 25 mM HEPES·NaOH, pH 7.4. Spectra were recorded at 700 MHz and 28 °C.

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