Energy Landscapes Differences among Integrins Establish the Framework for Understanding Activation

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Supplementary Text

Affinity of soluble $\alpha_4\beta_1$ for FITC-LDVP by FP from saturation binding

Fitting the measured FP (FP_{obs}) and soluble $\alpha_4\beta_1$ concentration ($[\alpha_4\beta_1]_{\text{tot}}$) data at fixed labeled probe concentration ($[\text{L}]_{\text{tot}} = 1\,nM$) to Eq. S1 yielded binding affinity to the probe ($K_{\text{d}}^{\text{app}}$), FP of the free probe (FP_{L}) and FP of the $\alpha_4\beta_1$ bound probe ($FP_{\alpha_4\beta_1\cdot\text{L}}$).

$$FP_{\text{obs}} = FP_{\text{L}} + \frac{[\text{L}]_{\text{tot}} + K_{\text{d}}^{\text{app}} + [\alpha_4\beta_1]_{\text{tot}} - \sqrt{\left([\text{L}]_{\text{tot}} + K_{\text{d}}^{\text{app}} + [\alpha_4\beta_1]_{\text{tot}}\right)^2 - 4[\text{L}]_{\text{tot}}[\alpha_4\beta_1]_{\text{tot}}}}{2[\text{L}]_{\text{tot}}} \cdot \left(FP_{\alpha_4\beta_1\cdot\text{L}} - FP_{\text{L}}\right) \tag{S1}$$

Affinity of intact $\alpha_4\beta_1$ for FITC-LDVP by FACS from saturation binding

For intact $\alpha_4\beta_1$, cells at fixed density were incubated with 0.005–100 nM FITC-LDVP and indicated Fabs at 22 ° C for 4 hr and subjected to flow cytometry. Binding of FITC-LDVP was measured as mean fluorescence intensity (MFI). Using L to denote FITC-LDVP, at equilibrium:

$$\alpha_4\beta_1 + \text{L} \rightleftharpoons \alpha_4\beta_1\cdot\text{L} \qquad K_{\text{d}}^{\text{app}} = \frac{[\alpha_4\beta_1][\text{L}]}{[\alpha_4\beta_1\cdot\text{L}]} \tag{S2}$$

$$[\alpha_4\beta_1]_{\text{tot}} = [\alpha_4\beta_1] + [\alpha_4\beta_1\cdot\text{L}] \tag{S3}$$

$$[\text{L}]_{\text{tot}} = [\text{L}] + [\alpha_4\beta_1\cdot\text{L}] \tag{S4}$$

where $[\text{L}]_{\text{tot}}$ is the total concentration of FITC-LDVP, $K_{\text{d}}^{\text{app}}$ is the apparent binding affinity of $\alpha_4\beta_1$ for FITC-LDVP, and $[\alpha_4\beta_1]_{\text{tot}}$ is the total concentration of $\alpha_4\beta_1$ on cell surface. Solve Eq. S2–S4 for $[\alpha_4\beta_1\cdot\text{L}]$:

$$[\alpha_4\beta_1\cdot\text{L}] = \frac{[\text{L}]_{\text{tot}} + K_{\text{d}}^{\text{app}} + [\alpha_4\beta_1]_{\text{tot}} - \sqrt{\left([\text{L}]_{\text{tot}} + K_{\text{d}}^{\text{app}} + [\alpha_4\beta_1]_{\text{tot}}\right)^2 - 4[\text{L}]_{\text{tot}}[\alpha_4\beta_1]_{\text{tot}}}}{2} \tag{S5}$$

Therefore, the measured MFI (MFI_{obs}) is

$$\begin{aligned} MFI_{\text{obs}} &= \frac{[\alpha_4\beta_1\cdot\text{L}]}{[\alpha_4\beta_1]_{\text{tot}}} \cdot MFI_{\text{max}} \\ &= \frac{[\text{L}]_{\text{tot}} + K_{\text{d}}^{\text{app}} + [\alpha_4\beta_1]_{\text{tot}} - \sqrt{\left([\text{L}]_{\text{tot}} + K_{\text{d}}^{\text{app}} + [\alpha_4\beta_1]_{\text{tot}}\right)^2 - 4[\text{L}]_{\text{tot}}[\alpha_4\beta_1]_{\text{tot}}}}{2[\alpha_4\beta_1]_{\text{tot}}} \cdot MFI_{\text{max}} \end{aligned} \tag{S6}$$

where MFI_{max} is the MFI when all $\alpha_4\beta_1$ on cell surface are bound with FITC-LDVP.

At a fixed cell density D_{cell}, the total concentration of $\alpha_4\beta_1$ on cell surface $[\alpha_4\beta_1]_{\text{tot}}$ can be expressed as:

$$[\alpha_4\beta_1]_{tot} = \frac{D_{cell} N_{percell}}{N_A} \tag{S7}$$

where $N_{percell}$ is the receptor number per cell, and N_A is the Avogadro constant.
Substituting Eq. S7 to Eq. S6 gives

$$MFI_{obs} = \frac{N_A[L]_{tot} + N_A K_d^{app} + D_{cell} N_{percell} - \sqrt{\left(N_A[L]_{tot} + N_A K_d^{app} + D_{cell} N_{percell}\right)^2 - 4 N_A [L]_{tot} D_{cell} N_{percell}}}{2 D_{cell} N_{percell}} \cdot MFI_{max} \tag{S8}$$

For saturation binding in the presence of saturating open-stabilizing Fabs 9EG7 and HUTS4, global fitting of MFI_{obs} and $[L]_{tot}$ data at different fixed cell density D_{cell} to Eq. S8 yield MFI_{max}, $N_{percell}$ and K_d^{app},wherein K_d^{app} represent the intrinsic affinity of EO, K_d^{EO}. For saturation binding under other conditions, fixing the determined $N_{percell}$ in Eq. S8 and fitting MFI_{obs} and $[L]_{tot}$ data at a certain fixed cell density D_{cell} yield MFI_{max} and K_d^{app}. In absence of Fab or in the presence of extension-stabilizing Fab, K_d^{app} represents the K_d^{ens}. In presence of saturating closure-stabilizing Fabs, K_d^{ens} is calculated from K_d^{app} following the following equation:

$$K_d^{ens(BC+EC)} = \frac{P^{\alpha_4\beta_1\cdot Fab} K_d^{ens(Basal)} K_d^{app(C\ Fabs)}}{K_d^{ens(Basal)} - \left(1 - P^{\alpha_4\beta_1\cdot Fab}\right) K_d^{app(C\ Fabs)}} \tag{S9}$$

Affinity of intact $\alpha_4\beta_1$ for macromolecule fragments from competitive binding

Intrinsic ligand-binding affinity of $\alpha_4\beta_1$ EO conformation for macromolecule fragment of VCAM, fibronectin and MAdCAM was determined by competing with 1 nM FITC-LDVP binding on cell surface under saturating open-stabilizing Fabs. We describe the displacement of the fluorescent probe FITC-LDVP by the unlabeled macromolecule fragment by the decreased MFI (MFI_{obs}):

$$MFI_{obs} = \frac{MFI_0}{[Competitor]_{tot}/IC_{50} + 1} \tag{S10}$$

where MFI_0 observed MFI in absence of competitor, $[Competitor]_{tot}$ is the competitor concentration, and IC_{50} is the concentration of competitor that displaces half of the labeled ligand. Fitting MFI_{obs} and $[Competitor]_{tot}$ to Eq. S10 yield IC_{50} and MFI_0. Intrinsic binding affinity of EO to the macromolecule fragment, $K_d^{EO:competitor}$ was calculated as:

$$K_d^{EO:competitor} = \frac{IC_{50}}{1 + [L]_{tot}/K_d^{EO:L}} \tag{S11}$$

where $[L]_{tot}$ is the total fixed concentration of FITC-LDVP used, and $K_d^{EO:L}$ is the intrinsic affinity of EO conformation for FITC-LDVP, which is determined from saturation binding on cell surface.

Calculation of probability of each conformational state under basal condition

The experimentally determined ligand-binding affinities $K_d^{ens(Basal)}$ (measured in the absence of Fab), K_d^{EO}, $K_d^{ens(EC+EO)}$ and K_d^{EC} relate to the probabilities P^{BC}, P^{EC} and P^{EO} for ectodomain and intact receptor according the following equations:

$$P^{EO} = \frac{K_d^{EO}\left(K_d^{ens(BC+EC)} - K_d^{ens(Basal)}\right)}{K_d^{ens(Basal)}\left(K_d^{ens(BC+EC)} - K_d^{EO}\right)} \tag{S12}$$

$$P^{EC} = \frac{K_d^{ens(BC+EC)}\left(K_d^{ens(BC+EC)} - K_d^{ens(Basal)}\right)\left(K_d^{ens(EC+EO)} - K_d^{EO}\right)}{K_d^{ens(Basal)}\left(K_d^{ens(BC+EC)} - K_d^{EO}\right)\left(K_d^{ens(BC+EC)} - K_d^{ens(EC+EO)}\right)} \tag{S13}$$

$$P^{BC} = \frac{K_d^{ens(BC+EC)}\left(K_d^{ens(Basal)} - K_d^{ens(EC+EO)}\right)}{K_d^{ens(Basal)}\left(K_d^{ens(BC+EC)} - K_d^{ens(EC+EO)}\right)} \tag{S14}$$

Likewise, for headpiece

$$P^{O} = \frac{K_d^{O}\left(K_d^{C} - K_d^{ens(Basal;\,HP)}\right)}{K_d^{ens(Basal;\,HP)}\left(K_d^{C} - K_d^{O}\right)} \tag{S15}$$

$$P^{C} = \frac{K_d^{C}\left(K_d^{ens(Basal;\,HP)} - K_d^{O}\right)}{K_d^{ens(Basal;\,HP)}\left(K_d^{C} - K_d^{O}\right)} \tag{S16}$$

Calculation of free energy of each conformational state under basal condition

For ectodomain and intact receptor, using EO as the reference state ($\Delta G^{EO} = 0$), the relative free energies of the BC and EC states, ΔG^{BC} and ΔG^{EC}, are related to the probabilities (populations) P^{BC}, P^{EC} and P^{EO} through the Boltzmann distribution (as also shown in Fig. 1B):

$$\Delta G^{BC} = -RT\ln\left(\frac{P^{BC}}{P^{EO}}\right) = -RT\ln\frac{K_d^{ens(BC+EC)}\left(K_d^{ens(BC+EC)} - K_d^{EO}\right)\left(K_d^{ens(Basal)} - K_d^{ens(EC+EO)}\right)}{K_d^{EO}\left(K_d^{ens(BC+EC)} - K_d^{ens(Basal)}\right)\left(K_d^{ens(BC+EC)} - K_d^{ens(EC+EO)}\right)} \tag{S17}$$

$$\Delta G^{EC} = -RT\ln\left(\frac{P^{EC}}{P^{EO}}\right) = -RT\ln\frac{K_d^{ens(BC+EC)}\left(K_d^{ens(EC+EO)} - K_d^{EO}\right)}{K_d^{EO}\left(K_d^{ens(BC+EC)} - K_d^{ens(EC+EO)}\right)} \tag{S18}$$

Likewise, for headpiece, using O as the reference state ($\Delta G^{O} = 0$)

$$\Delta G^{C} = -RT \ln\left(\frac{P^{C}}{P^{O}}\right) = -RT \ln \frac{K_d^{C}\left(K_d^{ens(Basal;\ HP)} - K_d^{O}\right)}{K_d^{O}\left(K_d^{C} - K_d^{ens(Basal;\ HP)}\right)} \tag{S19}$$

Integrin conformational equilibria regulation by intracellular and extracellular signaling event

We examine how the intracellular adaptor binding and exertion of cytoskeletal force regulate integrin activation, *i.e.* regulating the probability of high affinity EO conformation. In presence of intracellular adaptor and extracellular ligand, the three integrin conformational states can each potentially be in four different binding states: free, adaptor-bound, ligand-bound, and adaptor and ligand-bound. Free energies (ΔG^{i}) of these twelve states were calculated as shown in following table. Briefly, the ΔG^{i} of the unbound states were determined experimentally. Adaptor or ligand binding lowers the energy of the specific conformational state by the stabilizing term $-RT \ln (C/K_d)$, where R is gas constant, T is temperature, C is the concentration of the active intracellular adaptor or extracellular ligand concentration, and K_d is the dissociation constant between adaptor or ligand and the integrin conformational state(s) for which it is specific. In presence of force, for states bound to both adaptor and extracellular ligand, the force-dependent stabilizing term $-F \cdot N_A \cdot \Delta x / m$ was added, where N_A is the Avogadro number, F is force, Δx is the change in distance between the states, and m is the constant, 4184, to convert energy unit from J/mol to kcal/mol. The statistical weight of each state (S^{i}) was calculated according to $S^{i} = \exp(-\Delta G^{i}/(RT))$. The probability of each state (P^{i}) was calculated as its statistical weight over the partition function (Q), wherein Q which is the sum of the statistical weights of all states in the ensemble, $Q = \sum S^{i}$.

Free and bound states	Free energy ΔG^{i}(kcal/mol)
BC	ΔG^{BC}
EC	ΔG^{EC}
EO	0
adaptor bound BC	$\Delta G^{BC} - RT \ln\left(C_{ada} / K_{d,\ ada}^{BC}\right)$
adaptor bound EC	$\Delta G^{EC} - RT \ln\left(C_{ada} / K_{d,\ ada}^{EC}\right)$
adaptor bound EO	$-RT \ln\left(C_{ada} / K_{d,\ ada}^{EO}\right)$
ligand bound BC	$\Delta G^{BC} - RT \ln\left(C_{lig} / K_{d,\ lig}^{BC}\right)$
ligand bound EC	$\Delta G^{EC} - RT \ln\left(C_{lig} / K_{d,\ lig}^{EC}\right)$
ligand bound EO	$-RT \ln\left(C_{lig} / K_{d,\ lig}^{EO}\right)$
adaptor and ligand bound BC	$\Delta G^{BC} - RT \ln\left(C_{ada} / K_{d,\ ada}^{BC}\right) - RT \ln\left(C_{lig} / K_{d,\ lig}^{BC}\right)$
adaptor and ligand bound EC	$\Delta G^{EC} - RT \ln\left(C_{ada} / K_{d,\ ada}^{EC}\right) - RT \ln\left(C_{lig} / K_{d,\ lig}^{EC}\right) - F \cdot N_A \cdot \Delta x^{E} / m$
adaptor and ligand bound EO	$-RT \ln\left(C_{ada} / K_{d,\ ada}^{EO}\right) - RT \ln\left(C_{lig} / K_{d,\ lig}^{EO}\right) - F \cdot N_A \cdot \left(\Delta x^{E} + \Delta x^{O}\right) / m$

$$P^{i} = \frac{\exp(-\Delta G^{i}/(RT))}{\sum \exp(-\Delta G^{i}/(RT))} \tag{S20}$$

EC_{50} of Fabs/ligands for integrin

For determining EC_{50} values for conformation-specific Fabs or ligands (titrator), we made the assumption that the change in FP/MFI was directly proportional to the increase in concentration of Fab/ligand-bound open $\alpha_4\beta_1$. Therefore, data were fit to a dose response curve:

$$FP_{\text{obs}} = FP_0 + \frac{FP_{\text{sat}} - FP_0}{EC_{50}/[\text{Titrator}]_{\text{tot}} + 1} \tag{S21}$$

$$MFI_{\text{obs}} = MFI_0 + \frac{MFI_{\text{sat}}}{EC_{50}/[\text{Titrator}]_{\text{tot}} + 1} \tag{S22}$$

where FP_0 and MFI_0 are the FP and MFI without added Fab, respectively, FP_{sat} and MFI_{sat} are plateau values of FP and MFI, respectively, at high titrator concentration, and EC_{50} is the titrator concentration at the inflection point where half-maximum change in FP or MFI was observed. The titrator's K_{d} for $\alpha_4\beta_1$ is approximated by EC_{50}.