Dynamic antibody binding properties in the pathogenesis of HIT

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Supplemental Material:

Measuring bimolecular PF4 binding to antibodies using optical trap-based force spectroscopy

Model system to study bimolecular protein-protein interactions using optical trap-based force spectroscopy

Figures 1S and 2S of the supplement illustrate a model system to study individual protein-protein interactions that permits the measurement of discrete rupture forces produced by covalently surface-bound interacting molecular pairs during repeated intermittent contact. Details of optical trap design and calibration, preparation of PF4-coated pedestals and antibody-coated beads, experimental procedure as well as data processing and analysis can be found in: Litvinov RI, Bennett JS, Weisel JW, Shuman H. Multi-step fibrinogen binding to the integrin (alpha)IIb(beta)3 detected using force spectroscopy. *Biophys J*. 2005;89(4):2824-2834.

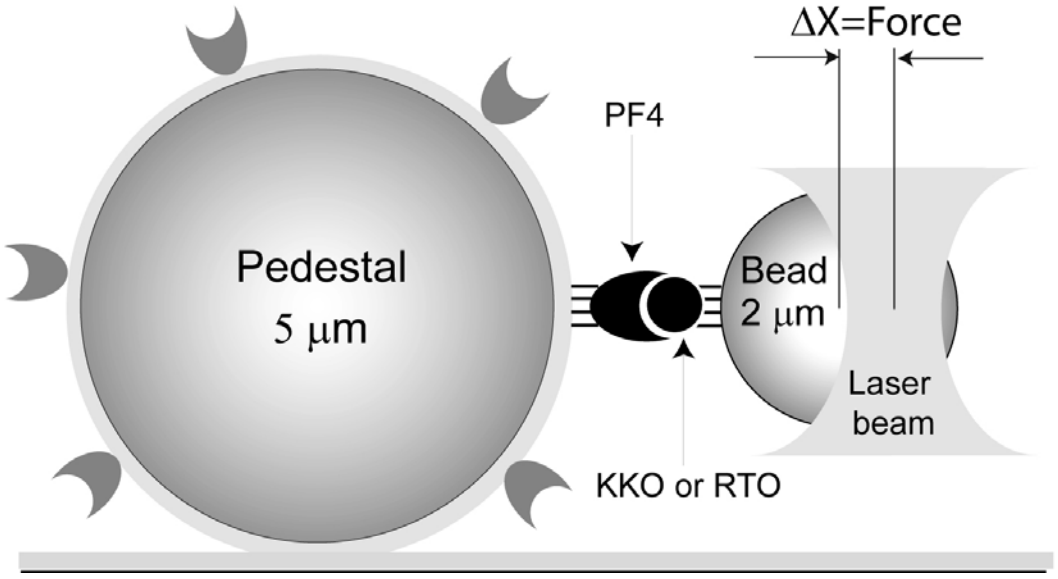

Figure 1S. Schematic drawing of the model system to study bimolecular protein-protein interactions using an optical trap. The latex bead coated with antibody is trapped near to the center of the laser beam while moving toward or away from the silica pedestal coated with polyacrylamide gel-bound PF4 molecules, touching it repeatedly. When the pedestal and the latex bead bind, the bead position remains nearly constant as the laser trap continues to move away from the pedestal. The force on the bead increases proportionally to the displacement of the laser focus (ΔX) from the bead.

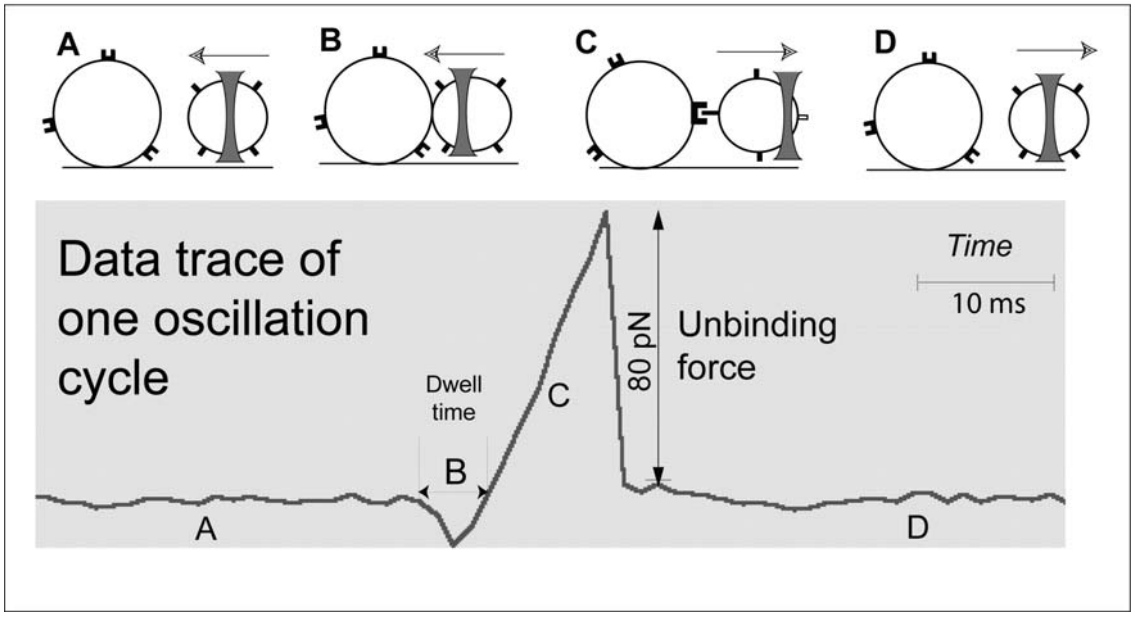

Figure 2S. Data trace for a typical interaction of two proteins as measured with the optical trap. The force that the optical trap exerts on the latex bead can be partitioned into four parts (*A–D*). The latex bead is trapped near the center of the laser beam while moving toward (*Upper A*) or away (*Upper D*) from the silica pedestal. (*Upper B*) At the moment of contact the pedestal stops the motion of the latex bead while the laser beam continues to move in the same direction (left). The optical trap exerts a negative, compressive force on the pedestal and latex bead. The trap motion then reverses, and the compressive force declines to zero. Peak B (*Lower*) represents a compressive force and dwell time between the surfaces. (*Upper C*) When the pedestal and latex bead bind, the bead position remains nearly constant as the laser trap continues to move to the right. The force on the bead increases in the positive direction almost linearly until the pedestal-bead bond is ruptured and the force rapidly returns to nearly zero. If no attachment occurs, there is no positive force.

Specific vs. nonspecific interactions in optical trap-based force spectroscopy

Since nonspecific interactions are a big concern in all surface-to-surface contact models, numerous control experiments were performed to distinguish between genuine Ab-PF4 binding and other attractive forces of a different physical nature. Sporadic surface adhesion events irrelevant to protein-protein interactions as well as optical artifacts observed with or without

trapped latex beads produced signals that appeared as forces below 10 pN (Litvinov et al., *Biophys J*. 2005;89(4):2824-2834). Accordingly, rupture forces in this range were not considered when the data were analyzed. The control experiments included untreated polyacrylamide coated pedestals in contact with BSA-coated beads, untreated beads vs. BSA-coated pedestals, and both interacting surfaces activated and coated with ethanolamine.

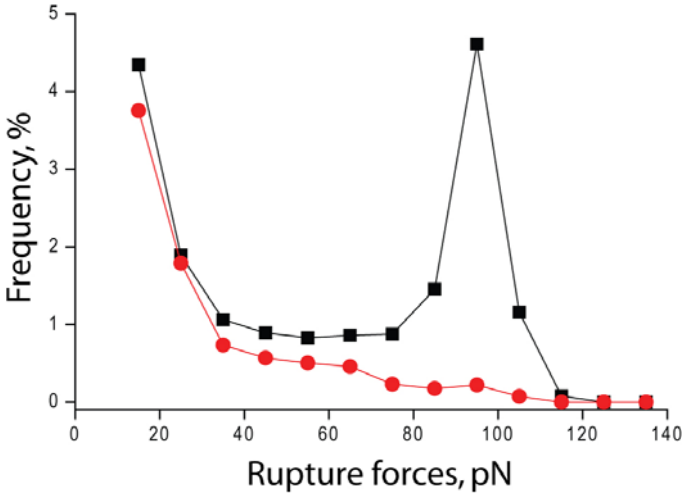

Figure 3S. Rupture force histograms of the interactions of surface-bound KKO antibodies with cross-linked PF4 in the absence (black squares) (6,400 contact cycles) and presence (red circles) (10,900 contact cycles) of 100 μg/ml free Fab KKO fragment.

To confirm the specificity of the rupture forces for KKO and RTO, experiments were performed in the presence of free antibodies or Fab fragments that should competitively inhibit binding of the antibody-coated microspheres. Figure 3 of the supplement shows a raw force curve reflecting the interactions of KKO-coated beads with cross-linked PF4 sitting on the pedestals and the same interactions in the presence of free Fab KKO fragment added at a saturating concentration. The results show that stronger forces >60 pN were almost fully eliminated by free Fab KKO, indicating that they reflect specific interactions between PF4 and KKO. The cumulative binding probabilities for KKO-PF4 interactions >60 pN in the absence and presence of free Fab KKO were 8.1±1.8% and 1.1±0.3%, respectively.

Single-molecule interactions in optical trap-based force spectroscopy

Another major issue is whether the measured forces really represent bimolecular Ab-PF4 binding. Usually two arguments have been used to support the view that individual interactions were indeed detected: first, when a limited fraction of touching cycles between the surfaces

resulted in binding, the bond number must be small, and, secondly, when histograms of the distribution of rupture forces were drawn, there appeared a series of quantized peaks that were multiples of a single value (Pierres, A., A.-M. Benoleil, and P. Bongrand. 1998. Studying receptor-mediated cell adhesion at the single molecule level. *Cell Adhes. Commun*. 5:375-395). Both arguments are applicable to our case.

If all the forces beyond 60 pN were to be summarized and compared to the total number of interaction cycles, their relative frequency would not exceed 10% for Ab-PF4 interactions. This low percentage means that an effective encounter between surface-bound KKO or RTO and PF4 molecules is really quite infrequent. This is due to low surface density of the reacting molecules (one protein molecule on beads is calculated to cover physically on average about 300 nm^2) and small contact area in one touching cycle (~450 nm^2). It is noteworthy that in order to precisely control the surface coverage of the antibodies and antigen and to make the results of force spectroscopy comparable, we used a reproducible immobilization protocol for PF4 on pedestals and identical coating protocols for KKO or RTO on beads, including the same initial concentration in the binding mixture with the same freshly activated surfaces.

In the case of multiple interactions, the histograms of the distribution of rupture forces should appear as a series of quantized peaks with probabilities inversely proportional to the number of bonds. However, apart from the non-specific interactions, we only observed a single well-defined peak in the force histograms, suggesting that this peak represents individual Ag-Ab bonds. Although unlikely, multiple interactions might possibly have taken place in some of our experiments, producing irreversible attachment events between an Ab-coated bead and a PF4-coated pedestal. The impossibility of detachment by pulling the bead off the sticky surface means that the attachment force is higher than the trap power, which is limited to the order of 140-150 pN in the optical trap. There might be immeasurable multiples of a single force with the attachment strength beyond the capability of the laser trapping power.

Another consideration is that if multiple bonds were formed during a contact, it is unlikely that all would be ruptured simultaneously; rather, it is more probable that they would be ruptured sequentially, so that multiple steps should have been observed during bond breakage. However, the rupture events we observed almost all occurred as a single step, at least to a time resolution of 0.5 ms (see Fig. 2S).