Single Molecule Adhesion Measurements Reveal Two Homophilic Neural Cell Adhesion Molecule Bonds with Mechanically Distinct Properties*

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Neural cell adhesion molecule (NCAM) is a cell surface adhesion glycoprotein that plays an important role in the development and stability of nervous tissue. The homophilic binding mechanism of NCAM is still a subject of debate on account of findings that appear to support different mechanisms. This paper describes single molecule force measurements with both full-length NCAM and NCAM mutants that lack different immunoglobulin (Ig) domains. By systematically applying an external, time-dependent force to the bond, we obtained parameters that describe the energy landscape of NCAM-NCAM bonds. Histograms of the rupture forces between the full-length NCAM extracellular domains revealed two binding events, one rupturing at higher forces than the other. These bond rupture data show that the two bonds have the same dissociation rates. Despite the energetic and kinetic similarities, the bond strengths differ significantly, and are mechanically distinct. Measurements with NCAM domain deletion mutants mapped the weaker bond to the Ig1–2 segment, and the stronger bond to the Ig3 domain. Finally, the quantitative agreement between the fragment adhesion and the strengths of both NCAM bonds shows that the domain deletions considered in this study do not alter the intrinsic strengths of either of the two bonds.

The neural cell adhesion molecule (NCAM) is one of the most abundant cell surface adhesion molecules in the peripheral and central nervous systems (1). NCAM helps to regulate the development of the nervous system, neurite growth and extension, and cell migration (2). The three major isoforms of NCAM differ only in their membrane anchors. Two transmembrane isoforms differ in their cytoplasmic tail length, NCAM A (180 kDa) and NCAM B (140 kDa), and one membrane-bound form is attached via a glycosylphosphatidylinositol anchor, NCAM C (120 kDa). The extracellular segments contain two fibronectin type III repeats and five tandem immunoglobulin (Ig) domains. The Ig domains are the active participants in the homophilic binding of NCAM (1, 3). NMR spectroscopy and x-ray crystallography revealed the structures of Ig domains 1, 2, and 3. The structures show that all three domains similarly comprise two β sheets connected by a cysteine bridge (3–5).

The homophilic binding between NCAM extracellular domains is of considerable interest, because of the importance of this association in neural development. Three different, apparently contradictory homophilic binding mechanisms were proposed, based on structural and biochemical data. In the first proposed mechanism all five Ig domains interdigitate to form a complex that is stabilized mainly by the autologous association of antiparallel Ig3 domains (Fig. 1a). Bead aggregation studies with different NCAM fragments suggested further contributions from adhesion between Ig1 and Ig5, and between Ig2 and Ig4 (6). Cell-substrate binding assays with NCAM domain deletion mutants that lacked specific Ig domains showed that the removal of Ig3 abolished bead aggregation and cell adhesion (7). Further studies mapped the adhesive site on the Ig3 domain to a decapetide (7–9). Dynamic light scattering showed, however, that the isolated Ig3 module does not dimerize in solution, contradicting the previously proposed Ig3 self-association mechanism (5). However, the possible interaction of the Ig3 domain with both Ig domains 1 and 2 was suggested by experiments investigating neurite outgrowth in the presence and absence of the third Ig domain (5).

A second proposed mechanism involves adhesion via a double reciprocal dimerization between Ig1 and Ig2 domains (3, 10, 11) (Fig. 1b). In this case, both NMR spectroscopy (11) and surface plasmon resonance (4, 10) did not detect the self-association of the Ig3 domains, but instead detected interactions between Ig1 domains 1 and 2. Crystallographic data also supports the antiparallel dimerization of these two domains (12). Their removal, however, failed to abrogate NCAM-mediated cell adhesion.

The third model postulates a zipper mechanism, and was suggested by the crystal structure of the Ig1–3 fragment (5). In this case, the Ig12 segment forms a lateral, cis NCAM dimer (formed from two molecules on the same cell surface). The structure also suggested two trans bonds (Fig. 1c): namely, one involving an antiparallel association between domains Ig2 and Ig3 and another involving the association of domains Ig3 and Ig1 from opposing cell surfaces. This model differs from either of the other two proposed binding mechanisms.

Recent measurements with a surface force apparatus (SFA) and surface plasmon resonance (13) provided direct evidence that both the first and second binding mechanisms can account for NCAM adhesion. The SFA, a force measurement technique that quantifies the force between two surfaces as a function of the separation distance within ±1 Å, showed that opposing full-length NCAM ectodomains form two bound states at different intersurface distances, but with similar adhesion energies. NCAM deletion mutants lacking various Ig domains mapped these binding interactions to the Ig12 and Ig3 domains (13).

The NCAM binding mechanism may also depend on the growth...
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FIGURE 1. Representations of the proposed homophilic binding mechanisms of NCAM, depicting both (a) the self-association of the Ig3 domains and (b) the double reciprocal dimerization of domains Ig1 and Ig2. The proposed zipper model (c) consists of dimers, linked via the Ig12 domains, forming reciprocal Ig2–3 bonds (left panel) or Ig1–3 and Ig2–2 bonds (right panel).

stage of the organism. It is known that, in the embryonic stage of development, NCAM is post-translationally modified with two polysialic acid chains on the fifth Ig domain. Recent force measurements suggest that the excluded volume of the polymer and associated steric repulsion impedes the close apposition of opposing NCAM extracellular domains (14), thereby weakening the interaction between NCAMs on opposing cells. In the adult, the polysialic acid chains are not added, allowing for stronger adhesion by NCAM and other adhesion proteins on the same cell membrane. This tighter binding may keep cells stationary for tissue stability and memory purposes (15).

To independently test the different binding models and to quantify differences in the strengths and kinetics of individual NCAM bonds, we conducted single molecule force probe measurements with both full-length adult NCAM and NCAM deletion mutants, which lacked different Ig domains. The deletion mutants used in this study lacked Ig domains 1 and 2 (NCAM ΔIg12) and Ig domain 3 (NCAM ΔIg3). We used the atomic force microscope (AFM) to probe the individual protein–protein bonds (16). In these experiments the protein was covalently attached to both the AFM probe tip and a second test surface. The resulting force histograms indicated the presence of two, independent homophilic NCAM bonds. Specific tip velocities controlled the rate of bond loading. Determinations of the rupture force as a function of the loading velocity revealed similarities and differences in the properties of the two bound states. In particular, the bond kinetics are the same, within error, but the mechanical strengths differ. Thus, two bonds can have different mechanical strengths even though they may have similar bond energies and kinetic rates. Finally, comparison of the binding strengths of NCAM fragments with the full-length protein show that the intrinsic properties of both NCAM bonds are unaffected by the domain deletions used in this study.

EXPERIMENTAL PROCEDURES
Materials and Methods

Fig. 2 is a schematic of the covalent attachment of the protein to both the AFM tip and surface. A modification of the procedure described above. First a chromium adhesion layer with an approximate thickness of 30 Å was thermally deposited at a rate of about 0.2 Å/s. This was followed by the deposition of a gold layer of 800 Å at a rate of about 1.2 Å/s. Tips and substrates were rinsed thoroughly with ethanol, dried with argon, and placed directly into an ethanolic thiol solution containing 1 mM 1,8-octanedithiol (Aldrich) and 10 mM 6-mercaptohexan-1-ol (Aldrich) for ~18 h, to yield a 1:20 molar ratio. Tips and samples were rinsed with ethanol, dried with argon, and placed into a phosphate buffer solution containing 1 mg/ml of poly(ethylene glycol)-α-maleimide, ω-N-hydroxysuccinimide ester (NHS-PEG-MAL) (Shearwater Corp., Huntsville, AL). The maleimide group from the poly(ethylene glycol) spacer interacts with exposed thiols on the 1,8-octanedithiol monolayer at the surface, whereas the free N-hydroxysuccinimide group on the poly(ethylene glycol) spacer reacts with free amine groups on the NCAM protein, which is added later. This amine reactive chemistry tethers the proteins at random surface amines. Although the proteins are not oriented on the surface as in previous studies (14), the long poly(ethylene glycol) tether allows the proteins relatively free orientational mobility, so that they are not immobilized in fixed random orientations. The aqueous buffer used as the solvent for this and the following solutions contained 50 mM NaH₂PO₄ (Fisher Scientific), 100 mM NaCl (J. T. Baker Chemical Co.), and 1 mM EDTA (Fisher Scientific) and was brought to pH 7.4 by adding 1 M NaOH (Fisher Scientific). After 20 min, the tips and samples were rinsed with buffer and immediately mounted on the AFM stage. The modified gold substrate was placed on the AFM stage and sandwiched under an O-ring and Teflon cell, which contained ~1.5 ml of a solution containing 0.06 mg/ml NCAM in the aqueous buffer described above. Both tip and substrate were incubated with the protein for 90 min. The cell was then flushed 10 times with buffer solution, while keeping the tip and sample submerged, to remove any non-adsorbed protein prior to conducting the force measurements.

Both full-length NCAM 120 extracellular domain and two mutated forms of NCAM with deleted Ig domains were used in this study. All
measurements were performed with soluble NCAM 120, or its fragments, engineered with a C-terminal oligohistidine tag. The latter was not used for immobilization in these studies. The soluble protein was expressed in Chinese hamster ovary cells (13). Technical details concerning the plasmid construction, cell transformations, and the purification and characterization of full-length NCAM, NCAM ΔIg12, and NCAM ΔIg3 were described previously (13). The sequence map showing the locations of the regions deleted in the two domain deletion mutants is shown in Fig. 4.

AFM Set Up—All force probe measurements were obtained with a commercial AFM apparatus (Pico AFM, Molecular Imaging) using a commercial controller and data acquisition electronics (Digital Instruments). All experiments were performed at room temperature. Cantilever force constants were determined by the thermal fluctuation method (18), and were 0.28–0.35 N/m. Loading rates for the NCAM experiments ranged from 200 to 10000 pN/s, with 2000 force extension curves obtained per loading rate. The loading rate was calculated by multiplying the tip velocity (frequency times distance traveled per cycle) by the slope of the experimentally measured force-distance curve just prior to rupture, $k_s$.

Analysis

Each measured force curve was analyzed separately via a scripted analysis program written for Labview® (National Instruments) (19). Fig. 5 shows typical force extension curves without (Fig. 5a) and with (Fig. 5b) a bond rupture event. The occurrence of a binding event causes the tip to move with negative deflection. The measured tip deflection for each rupture event was multiplied by the cantilever force constant to obtain the rupture force. Force measurements were binned into histograms for each loading rate to show the frequency of events versus the rupture force. The bin sizes of the histograms were determined from the error propagated for each particular measurement, and depended on both the error in the spring constant and the tip fluctuation (20). Bin sizes from 6 to 10 pN were typical for measurements with loading rates ranging from 200 to 1500 pN/s, whereas for higher loading rates (1500–10000 pN/s) they were 10–15 pN. Histograms were analyzed by both the dynamic force spectroscopy (DFS) method (21–23) and the full microscopic theory (FMT) (24). We previously used both methods to analyze the force extension curves measured with the AFM (16). The following sections briefly recapitulate these analyses.
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![Potential energy diagram for a bond subject to a mechanical force.](image)

**FIGURE 6.** Potential energy diagram for a bond subject to a mechanical force. The solid line represents the intermolecular potential in the absence of an externally applied force. The dashed line shows the net potential experienced by the bond under an applied force. (The figure was adapted from Refs. 22 and 23.) $E_s$ corresponds to the energy difference between the unperturbed bound and free states, $E_p$ is the difference between the energy at the transition state and the potential minimum, and $\Delta E_b$ is the energy difference between $E_b$ and $E_p$.

**DFS Analysis**—DFS is based on the physical scenario illustrated in Fig. 6, a and b. In Fig. 6a, the potential energy diagram describing the intermolecular bond contains a single barrier between the bound and free (dissociated) states. In this case, the application of an external force, $F$, tilts the energy landscape and creates a transient capture well. If the force applied is great enough, this well can be deeper than the minimum $F_d$ at the transition barrier.

The energy, $E_p$, required for a bond to move from its bound state to a free state along the potential energy landscape is then lowered by an amount equal to this mechanical energy $E_F$ for a bond. This increases the flux of states from the bound state over the transition barrier to the unbound state, and thereby increases the rate of dissociation.

**DFS Direct Fit Method (DFS-DF)**—The equation describing the rate coefficient for the dissociation of a bond in the presence of an external force, $F$, is given by (25),

$$k = k_0 \exp[\beta F x^t]$$

(Eq. 1)

where $k_0$ is the intrinsic rate constant, $x^t$ is the distance from the free energy minimum to the transition barrier, $\beta = k_BT$, where $k_B$ is the Boltzmann constant, and $T$ is the absolute temperature. For a bound state confined by a single barrier, the probability distribution that describes the rupture force $p(F)$, based on the DFS model, is as follows.

$$p(F) = \frac{\beta k_0}{k_s \nu} \exp \left[ \beta F x^t - \frac{k_0}{k_s \nu} (e^{(\beta F x^t)} - 1) \right]$$

(Eq. 2)

Here, $k_s$ is the effective force constant defined above and $\nu$ is the tip velocity. This equation can be modified for histograms containing two peaks because of two independent binding events (26) as in the following.

$$p(F) = p(F, k_0, 1, x_1^t) + p(F, k_0, 2, x_2^t)$$

(Eq. 3)

Histograms for each loading rate, $\nu$, were fit by nonlinear least-squares to Equations 2 or 3, as necessary, with Igor Pro 4 software (WaveMetrics Inc.). We thus obtained the best fit parameters for $k_0$ and $x^t$ values for one or two peaks at each loading rate. We refer to this approach as the DFS-DF method.

To verify the best model of the data, we compared the fits obtained with different forms of Equation 3. The simplest case assumed a single distribution, i.e. Equation 2. For both $x_1^t$ and $x_2^t$ were set to 0. We also considered the case of two peaks in which $k_{0,1} = k_{0,2}$. For some data sets, we included a third distribution function. We distinguished between different models of the data on the basis of the $\chi^2$ values of the fits, standard deviations of the fitted parameters, and ANOVA.

**DFS Most Probable Force Method (DFS-MPF)**—An alternative method of determining $x^t$ and $k_0$ based on the DFS model involves constructing plots of the most probable force $F_m$ versus the logarithm of the loading rate, $F_m$ is the maximum of $p(F)$ obtained from fits of Equations 2 and 3 to the histograms of rupture frequency as a function of rupture force. According to the DFS theory, for bound states confined by a single barrier, these plots are related to $x^t$ and $k_0$ by the following expression.

$$F_m = \frac{(k_BT/x^t)}{ln(k_1 / k_BT)}$$

(Eq. 4)

Thus, the slope of $F_m$ versus $ln(k_1 / k_BT)$ gives $x^t / k_BT$, which can be solved for $x^t$. Extrapolating the $F_m$ versus $ln(k_1 / k_BT)$ plot to $F_m = 0$ gives an expression for $k_0$.

$$k_0 = k_s \nu \frac{\exp(-E_0/k_BT)}{k_BT}$$

(Eq. 5)

where $k_0$ is the loading rate at zero force. We refer to this method as the DFS-MPF method.

An additional parameter can be obtained from values of $k_0$, obtained using either the DFS direct fit or the DFS most probable force methods. The energy barrier, $E_0$ (see Fig. 6a) is obtained from,

$$k_0 = 1/t_2 \exp(-E_0/k_BT)$$

(Eq. 6)

where $t_2$ is the diffusive relaxation time of the bond, and is assumed to be $10^{-9}$ s.

**Analysis Using the FMT**—The histograms for each loading rate were also analyzed with the FMT (24). According to this model, $p(F)$ is

$$p(F) = (k_1 / \nu) \frac{\exp(-S(t^* / k_0) / (k_0 \nu \xi \sqrt{2k}))}{k_0 \nu \xi \sqrt{2k}}$$

(Eq. 7)

where $t^* = (BF + k_0 \nu X^t) / k_0 \nu$, $S(t) = \exp \left[ -\frac{k_0 e^{-k_0 (x^t)^2 / 2}}{k_0 e^{k_0 (x^t)^2 / 2}} \right]$, and $\xi = \frac{k_0 \nu \xi^t}{2 k_0}$.

Here, $k_m$ is the molecular spring constant and $k = k_s + k_m$. For histograms containing two peaks such as in the data collected for the full-length NCAM, Equation 7 was modified according to Equation 3.

As described above, nonlinear least-squares fits of Equations 3 and 7 to the histograms measured at each loading rate was performed using Igor Pro 4 software (WaveMetrics Inc.), to obtain the best fit parameters for $k_m$, $k_0$, and $x^t$ at each loading rate. We refer to this approach as the “FMT direct fit” (FMT-DF). Different models were evaluated on the basis of the $\chi^2$ values of the fits, standard deviations of the fitted parameters, and ANOVA tests.

**RESULTS**

Measurements were performed using full-length NCAM and several mutated forms of the protein. We examined symmetric sample configurations, in which both the tip and substrate were modified with the same NCAM construct. We also conducted asymmetric measurements in which the tip and substrate were modified with different NCAM...
fragments. Control experiments with a modified tip without bound protein quantified the nonspecific adhesion.

Interactions between Full-length NCAM Extracellular Domains—Histograms of the rupture force for measurements between full-length NCAM on both the modified tip and substrate are given in Fig. 7, a–c. Loading rates are 781, 1152, and 7423 pN/s for histograms labeled a, b, and c, respectively. These histograms exhibit two peaks, with the second peak becoming more pronounced at higher loading rates. ANOVA tests and consideration of the standard deviations support the occurrence of two independent rupture events. This was also verified by using an alternative, more rigorous statistical analysis (26). The occurrence of two distinct peaks is indicative of either two independent binding events or the same bond exhibiting multiple states, a point that will be addressed later.

The dynamic force spectrum, that is, the plot of $F_{ma}$ versus the logarithm of the loading rate $\log(\kappa \nu)$, further supports the occurrence of two bond populations, as suggested by the histograms (Fig. 7d). The strengths of the two bonds (peaks 1 and 2) diverge with increasing loading rate. TABLE ONE summarizes the values for the fitted parameters that characterize the energy landscape of the bonds. The DFS-MPF analysis gives $x^T = 0.31 \pm 0.08$ nm and $f^T = 13 \pm 3$ pN for peak 1, whereas $x^Ig3 = 0.17 \pm 0.04$ nm and $f^Ig3 = 24 \pm 6$ pN for peak 2. Similar results were obtained with both the DFS-DF and FMT-DF analyses, where $x^Ig3$ and $f^Ig3$ values are 30–40% larger for peak 2 than for peak 1. ANOVA tests were performed on the two populations (two branches of the force spectra), and showed that, at a 95% confidence interval, there is a statistically significant difference between the fitted parameters for the two bound states.

The values for $k_0$ and $E_p$ for peaks 1 and 2 were, however, very similar, with $k_0$ ranging from 1 to 5 s$^{-1}$ and $E_p$ ranging from 19 to 20 kBT. The origin of this similarity may be in part because of the fact that $k_0$ is a prefactor to an exponential function. Better determination of $k_0$ would require less error in the histograms in Figs. 7–11, but this level of precision is apparently not available with our technology. Nevertheless, the bonds are energetically and kinetically very similar, so that the bond energies and kinetic rates alone cannot distinguish between the two bound states, nor identify the domains responsible for specific binding events.

Fig. 7d also shows the results of control measurements performed with tips that were not modified with protein. Fig. 7d shows that the rupture forces measured without NCAM on the tip are nearly a factor of two lower than those obtained with bound NCAM. Additionally, the percentage of binding events (number of events relative to the number of touches to the surface) obtained for the control (about 3%) was much lower than that obtained with the NCAM-derivatized tip (8–12%). Thus, the nonspecific adhesion is infrequent and the magnitude of the associated force is negligible compared with the protein-protein interactions.

Interactions between NCAM Ig3 Fragments, Symmetric Measurements—To map the binding events observed with the full-length NCAM onto the protein structure, we first performed measurements with NCAM lacking the Ig3 domain (NCAM Ig3). Prior surface force measurements indicated that the strongest homophilic NCAM bond requires the Ig3 domain. Symmetric measurements between NCAM Ig3 fragments gave the rupture force histograms shown in Fig. 8, a–c. These histograms exhibit a single peak at all loading rates, indicating that this fragment only forms one bond. The plot of the most probable rupture force $F_{ma}$ versus the logarithm of the loading rate also exhibits a single slope, further supporting the conclusion that this fragment forms only one bound state.

Fig. 8d overlays the NCAM Ig3 data onto the force spectra obtained with the full-length NCAM (Fig. 7d). The plot shows a clear correlation between the data obtained for NCAM Ig3 and peak 1 (Fig. 7d, lower branch), which corresponds to the weaker NCAM bond. The $f^*$ values obtained for peak 1 for full-length NCAM and NCAM Ig3 were, respectively, 13 ± 3 and 17 ± 4 pN, based on the DFS-MPF analysis. Similar agreement was also found with the parameters obtained by both the DFS-DF and FMT-DF analyses (TABLE ONE). ANOVA tests fur-
ther showed that the rupture force data obtained with NCAM Δlg3 was not statistically different from the peak 1 data obtained with the full-length NCAM. The tests did, however, show statistically significant differences between the NCAM Δlg3 data and the peak 2 data (Fig. 7d, upper branch).

Interactions between NCAM Δlg3 and NCAM, "Asymmetric" Measurements—The data in Fig. 9 exhibit tails into the higher force region, and the standard deviations in the most probable rupture forces are relatively large. These high force tails could be due to additional binding interactions. Alternatively, they could also be because of nonspecific binding and multipoint attachments. To improve the quality of the data obtained with the NCAM Δlg3 fragment, we carried out measurements with the tip modified with NCAM Δlg3 and the surface modified with full-length NCAM. In this case, one of the proteins was unaffected by the domain deletions and thus fully functional. This should reduce the frequency of nonspecific binding, and improve the signal-to-noise ratio. This approach had a similar effect in prior surface force measurements (13).

### TABLE ONE

| Experiment                  | $x'$  | $f'$  | $k_0$  | $E_b$  |
|-----------------------------|-------|-------|--------|--------|
| DFS most probable force     |       |       |        |        |
| NCAM peak 1                 | 0.3 ± 0.1 | 14 ± 6 | 3.2 ± 0.9 | 19.6 ± 0.5 |
| NCAM peak 2                 | 0.17 ± 0.04 | 24 ± 6 | 3.2 ± 0.9 | 19.6 ± 0.5 |
| NCAM Δlg3                   | 0.23 ± 0.06 | 17 ± 4 | 5 ± 1 | 19.1 ± 0.3 |
| NCAM Δlg3 asymmetric        | 0.3 ± 0.1 | 12 ± 7 | 2 ± 1 | 20.0 ± 0.4 |
| NCAM Δlg12                  | 0.26 ± 0.1 | 16 ± 7 | 2 ± 1 | 20.0 ± 0.4 |
| NCAM Δlg12 asymmetric       | 0.2 ± 0.06 | 21 ± 6 | 2.7 ± 0.8 | 19.7 ± 0.3 |
| DFS direct fit              |       |       |        |        |
| NCAM peak 1                 | 0.42 ± 0.09 | 10 ± 2 | 2 ± 1 | 20.5 ± 0.5 |
| NCAM peak 2                 | 0.32 ± 0.07 | 13 ± 3 | 2 ± 1 | 20.5 ± 0.5 |
| NCAM Δlg3                   | 0.3 ± 0.1 | 13 ± 5 | 4 ± 3 | 19.3 ± 0.4 |
| NCAM Δlg3 asymmetric        | 0.28 ± 0.06 | 14 ± 3 | 3 ± 2 | 19.6 ± 0.5 |
| NCAM Δlg12                  | 0.22 ± 0.04 | 19 ± 3 | 5 ± 2 | 19.1 ± 0.4 |
| NCAM Δlg12 asymmetric       | 0.2 ± 0.1 | 19 ± 9 | 3 ± 2 | 19.6 ± 0.5 |
| Full microscopic direct fit |       |       |        |        |
| NCAM peak 1                 | 0.4 ± 0.6 | 10 ± 15 | 1 ± 1 | 1665 ± 913 |
| NCAM peak 2                 | 0.37 ± 0.2 | 11 ± 6 | 1 ± 1 | 1665 ± 913 |
| NCAM Δlg3                   | 0.4 ± 0.2 | 10 ± 5 | 3 ± 14 | 331 ± 333 |
| NCAM Δlg3 asymmetric        | 0.33 ± 0.09 | 12 ± 3 | 2 ± 2 | 297 ± 107 |
| NCAM Δlg12                  | 0.3 ± 0.1 | 14 ± 5 | 3 ± 7 | 451 ± 369 |
| NCAM Δlg12 asymmetric       | 0.24 ± 0.07 | 17 ± 5 | 3 ± 3 | 698 ± 315 |

**FIGURE 8.** Histograms of the rupture force between NCAM Δlg3 fragments at different loading rates (a–d). Comparison of plots of the most probable rupture force $F_m$ versus the logarithm of the loading rate for both peaks 1 and 2 of the full-length NCAM with the force spectrum of the single NCAM Δlg3 peak (d).
As expected, the high force tails in Fig. 8 were noticeably reduced (Fig. 9). The standard deviations of the most probable rupture forces similarly decreased. The force spectrum in Fig. 9d mapped the bond to the lower branch of the force spectrum of the full protein. ANOVA tests showed that the populations were not significantly different when comparing data from asymmetric NCAM ΔIg3 to peak 1 (full-length NCAM). By contrast, the ANOVA test did show a statistically significant difference between NCAM ΔIg3 data and peak 2 within a 95% confidence interval.

Interactions between NCAM ΔIg12, Symmetric Measurements—To test the involvement of Ig domains one and two, we modified both the
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**FIGURE 11.** Histograms of the rupture force measured between NCAM and NCAM Δlg12 at different loading rates (a–c). Comparison of the most probable rupture force $F_m$ versus the logar-thith of the loading rate for the single NCAM versus NCAM Δlg12 bond with that of both peaks 1 and 2 of full-length NCAM (d).

Tip and surface with NCAM Δlg12. Rupture force histograms for these measurements are shown in Fig. 10, a–c. In contrast to studies with both the full-length NCAM and NCAM Δlg3, the histograms exhibit considerable tailing into the higher force region, which complicates the fit. Indeed, the error in the bond parameters determined with the DFS-MPF analysis of the data are large (~40–50% error) as shown in TABLE ONE. This large spread in the data could be because of nonspecific binding. The latter may be because of partially unfolded protein, if the truncation destabilized the fragment. Indeed, adhesion by this fragment measured in prior surface force measurements was much weaker than either of the two bound states between the full-length extracellular domains. Furthermore, circular dichroism spectra of this fragment exhibited slightly more random coil content than the full ectodomain (13). The corresponding dynamic force spectrum (Fig. 10d) shows no distinction between data obtained with NCAM Δlg12 and either peak 1 or 2 obtained with full-length NCAM.

**NCAM Versus NCAM Δlg1–2 Asymmetric Measurements**—As described above, we also conducted asymmetric measurements between NCAM Δlg12 fragments and the full-length ectodomain, to reduce the error in the bond strength measurements. Rupture force histograms for this asymmetric sample configuration are shown in Fig. 11, a–c. As expected, the amount of tailing, which we attributed to nonspecific interactions, decreased compared with the data measured only with NCAM Δlg12 fragments. The histograms exhibit a single distribution, indicative of the formation of a single NCAM-NCAM Δlg12 bond. The resulting dynamic force spectrum (Fig. 11d) shows a clear correlation between the asymmetric NCAM Δlg12 data and peak 2 (stronger bond) of full-length NCAM. The fitted values of $f^*$ obtained from both the data in Fig. 11d and peak 2 are $21 \pm 6$ and $24 \pm 6$ pN, respectively. Similar trends were observed with the DFS-DF and FMT-DF analyses. ANOVA tests showed that the data obtained from asymmetric NCAM Δlg12 measurements are not statistically different from peak 2. On the other hand, there is a statistically significant difference between the NCAM Δlg12 data and peak 1.

**Interactions between NCAM Δlg12 and NCAM Δlg3**—The third NCAM binding model (Fig. 1c) postulates that NCAM ectodomains also form adhesive Ig1-Ig3 and Ig2-Ig3 contacts. The latter are not predicted by either of the other two models. To test this, we measured binding between NCAM Δlg12 and NCAM Δlg3. These proteins could form both Ig1-Ig3 and Ig2-Ig3 bonds. However, in contrast to binding behavior in all other measurements except for the control data, the frequency of tip-surface binding was less than 3%. In addition, the rupture forces were well below the bond strengths measured with any of the other NCAM fragments investigated, and were comparable with the background levels determined in control measurements (see Fig. 7d).

**DISCUSSION**

These results show that the NCAM extracellular domains associate homophilically by two different adhesive interactions. The histograms shown in Fig. 6, a–c, obtained with full-length NCAM ectodomains clearly exhibited two peaks. At lower rupture forces, the distinction between the two peaks was less obvious, but the peak separation increased with increasing loading rate. This divergence is because of the different values of $x^*$, which affects the slopes of the dynamic force spectra in Fig. 6d. At lower loading rates, the bonds have similar strengths, bond energies, and unbinding rates (TABLE ONE). One of the important findings in this study is that, although the activation energies for unbinding $E_a$ and the intrinsic dissociation rates $k_d$ are, within error, the same, the two bonds exhibit different mechanical properties, as reflected by $x^*$. The significance of this result is that, as the rate of loading increases, one of the two bonds (upper branch) resists force more effectively (is stronger) than the other.

It is important to note that the fitted parameters obtained with the different analyses are very similar, and in several cases they agree quantitatively, within experimental error. The DFS-MPF and DFS-DF approaches agree, within error. The best fit parameters obtained for this model are therefore internally consistent. The standard deviations of the fitted parameters obtained with the FMT-DF are, however, much
higher. This is not unexpected, as an alternative analysis demonstrated that the microscopic spring constant $k_m$ is the least sensitive parameter, so that the associated errors in the fitted values are large (26).

We mapped the different binding interactions onto the protein structure by systematically testing NCAM variants lacking different Ig domains. The histograms of compiled rupture forces measured between identical NCAM $\text{Ig}3$ fragments exhibited a single peak, in contrast to the data obtained with the full-length protein. Importantly, the dynamic force spectrum (Figs. 8d and 9d) for the NCAM $\text{Ig}3$ measurements mapped onto the lower branch (weaker bond) of the force spectrum of the full-length NCAM. This indicates that $\text{Ig}3$ is required for the stronger NCAM bond (upper branch), whereas the remaining $\text{Ig}12$ domains mediate the weaker bond.

Similarly, the adhesion by NCAM lacking $\text{Ig}12$, but retaining $\text{Ig}3$, maps onto the higher force regime (stronger bond) between full-length NCAM extracellular domains (Figs. 10d and 11d). The absence of the lower branch of the dynamic force spectrum indicates that $\text{Ig}1$ domains 1 and 2 mediate the weaker protein-protein interaction. A similar conclusion can also be intimated from the parameters in TABLE ONE. Here $x^c$ (and therefore $f$) for NCAM $\text{Ig}3$ corresponds to peak 1 (weaker bond) of the full-length NCAM, whereas $x^c$ and $f$ obtained from the NCAM $\text{Ig}12$ asymmetric measurements map to peak 2 (stronger bond) of full-length NCAM. The ANOVA tests confirmed these conclusions at the 95% confidence level (13).

These findings agree qualitatively with recent results from SFA measurements (13). The SFA measurements also showed that NCAM can form either of two separate bound states at two distinct intersurface separations. The bond formed at intersurface distances that allow for complete interdigitation of the Ig domains was slightly stronger than the adhesion measured at a distance that permitted only the overlap of the outer NCAM domains (13). The fitted bond parameters from these AFM measurements also indicated that one NCAM bond is stronger than the other: namely, the value of the thermal force $f$ for peak 1 is about 1.5–1.8 times smaller than that of peak 2.

The measurements with the NCAM domain deletion mutants yielded similar insights into the regions of the NCAM structure responsible for these bound states. Prior NCAM binding models, which were based on cell adhesion measurements and on biochemical and structural data, suggested that the $\text{Ig}12$ domain mediates a trans adhesive bond through the double-reciprocal association of Ig domains 1 and 2. SFA measurements also confirmed that the outermost of the two trans bonds requires the $\text{Ig}12$ fragment (13). By contrast, the zipper model (Fig. 1c) postulates that the $\text{Ig}12$ region forms lateral dimers with proteins on the same cell surface (5). Importantly, because they lack the spatial information of the SFA, these single bond rupture measurements cannot distinguish between cis or trans binding. However, the vast majority of studies support the view that the $\text{Ig}12$ segment mediates adhesion between NCAMs on opposing cell surfaces.

It is possible that NCAM tethering at random amines could mask or alter NCAM binding sites. However, the qualitative agreement between the findings of this study and prior force measurements that were carried out with site selectively immobilized NCAM argues against this (14). Moreover, different bound states map to different protein domains, which is entirely inconsistent with a scenario in which a single site, altered by the attachment, exhibits two different strengths.

The correlation of the mechanically stronger bond with the presence of the $\text{Ig}3$ domain agrees with previous results, which show that cell adhesion and strong bead aggregation map to Ig3 (7–9, 27). By contrast, the zipper model postulated that NCAM forms Ig1-Ig3 and Ig2-Ig3 bonds (5). These AFM data show that the fragment lacking both Ig1 and Ig2 (NCAM $\Delta\text{Ig}1\text{Ig}2$) adheres, in contradiction to the zipper model. One might try to argue that adhesion between NCAM and NCAM $\Delta\text{Ig}1\text{Ig}2$ is because of an Ig1-Ig3 bond, which could form in the latter case. The evidence against this is as follows. First, the NCAM $\Delta\text{Ig}1\text{Ig}2$ fragments adhere to each other, and the prominent rupture force is the same as the peak measured between NCAM and NCAM $\Delta\text{Ig}1\text{Ig}2$. Second, the strengths of the single NCAM $\Delta\text{Ig}1\text{Ig}2$ versus NCAM $\Delta\text{Ig}1\text{Ig}2$ and NCAM versus NCAM $\Delta\text{Ig}1\text{Ig}2$ bonds are the same as that measured between the full-length proteins (Fig. 7, peak 2). Third, the $\Delta\text{Ig}1\text{Ig}2$ and $\Delta\text{Ig}3$ fragments do not adhere.

The absence of adhesion between $\Delta\text{Ig}1\text{Ig}2$ and $\Delta\text{Ig}3$ also indicate that Ig1-Ig5 and Ig2-Ig4 contacts, which were postulated to augment the Ig3-Ig3 bond (6), contribute little if any adhesive strength. Because the strengths of these fragments map onto the strengths of the full NCAM bonds, the lack of binding is not because of structural perturbations resulting from domain removal.

Atkins et al. (27) recently concluded that Ig3 only plays a structural role, by stabilizing the outer Ig12 segment. Our findings both that the NCAM $\Delta\text{Ig}1\text{Ig}2$ fragment adheres and that the full-length protein exhibits two bound states contradict the latter hypothesis. Our data show that domains lacking Ig1–2 do adhere.

Population average measurements such as cell adhesion, plasmon resonance, and SFA measurements do support the finding by Atkins et al. (27) that NCAM exhibits interdomain cooperativity. Removing Ig domains does reduce the population average adhesion (13). An interesting aspect of the AFM measurements reported here is that the dynamic force response, and hence the strengths of the individual fragments, map onto the force spectra of the two bound states of the full-NCAM ectodomain. In other words, the bonds formed by the individual fragments are as strong as the bonds formed by the intact ectodomain. This observation indicates that the protein modifications do not alter the intrinsic bond strength, but most likely affect the distribution of inactive and active conformations. Although the inactive conformations contribute to the average adhesion values measured with other techniques, they do not contribute to these single molecule measurements, that is, only the active proteins generate binding events such as in Fig. 2b.

Conclusion—These results demonstrate that the NCAM can form either of two homophilic bonds that involve different sets of Ig domains. The fitted bond parameters, which describe the energy landscape of this interaction show that, although their activation energies for unbinding and their dissociation rates are similar, the two bonds are mechanically distinct. The NCAM domain deletion mutants show that Ig12 is required for the weaker bond, and that Ig3 is required for the mechanically stronger bond.

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