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Single-molecule binding of CD44 to fibrin versus P-selectin predicts their distinct shear-dependent interactions in cancer

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Summary

P-selectin and fibrin(ogen) have pivotal roles in the hematogenous dissemination of tumor cells. CD44 variant isoforms, CD44v, have been identified as the major functional P-selectin ligands and fibrin receptors on metastatic colon carcinoma cells. The molecular recognition of CD44v by fibrin mediates firm adhesion at low shear, whereas CD44v–P-selectin binding supports transient rolling interactions at elevated shear stresses and low site densities of P-selectin. We used single-molecule force spectroscopy to provide a molecular interpretation for these two distinct adhesion events. The CD44v–P-selectin bond has a longer unstressed equilibrium lifetime, a lower reactive compliance and a higher tensile strength relative to the CD44v–fibrin bond. These intrinsic differences confer the ability to the CD44v–P-selectin pair to mediate binding at higher shear stresses. Increasing the duration of receptor–ligand contact (2–200 milliseconds) did not affect the micromechanical properties of the CD44v–P-selectin bond, but it increased the tensile strength and the depth of the free energy barrier of the CD44v–fibrin bond and decreased its reactive compliance. This bond strengthening at longer interaction times might explain why CD44v binding to immobilized fibrin occurs at low shear. Single-molecule characterization of receptor–ligand binding can predict the shear-dependent adhesive interactions between cells and substrates observed both in vitro and in vivo.

Key words: CD44, P-selectin, Fibrin(ogen), Force spectroscopy

Introduction

Mounting evidence suggests that P-selectin and fibrin(ogen) have crucial roles in the metastatic spread of tumor cells. P-selectin is a transmembrane glycoprotein that is expressed on the surface of activated endothelial cells and platelets. The most compelling evidence for the direct role of P-selectin in the metastatic process is the pronounced inhibition of metastasis in P-selectin-deficient mice compared with wild-type controls in a colon carcinoma model (Borsig et al., 2001; Kim et al., 1998). It is reported that platelets provide a protective shield by adhering to the tumor cell surface by a P-selectin-dependent mechanism, which masks them from the cytotoxic activity of the immune cells (Borsig et al., 2001). Along these lines, injection of O-sialoglycoprotease-treated colon carcinoma cells, which are devoid of selectin ligands, into wild-type mice results in a marked attenuation of platelet-tumor cell adhesive interactions and metastasis (Borsig et al., 2001).

Fibrinogen is a 340 kDa glycoprotein composed of two identical disulfide-linked subunits, each of which is formed by three distinct polypeptide chains, Aα, Bβ and γ-chains (Fig. 1A) (Weisel, 2005). Thrombin-mediated cleavage of the two N-terminal fibrinopeptides A and B [residues Aα(1–16) and Bβ(1–14), respectively] from the central region of fibrinogen results in fibrin formation (Weisel, 2005). The contribution of fibrin(ogen) to metastasis has been established by the use of fibrinogen-deficient mice, which exhibit markedly reduced metastatic potential over wild-type controls (Camerer et al., 2004; Palumbo et al., 2000). It is believed that platelet–fibrin(ogen) clots surrounding tumor cells protect them from immunological and physiological stresses in the bloodstream, and facilitate their lodging to the pulmonary vasculature (Nieswandt et al., 1999). This hypothesis is corroborated by in vivo data, which disclose that in mice lacking functional natural killer (NK) cells, fibrin(ogen) deficiency was no longer a significant determinant of metastatic potential (Palumbo et al., 2005).

We have recently identified CD44 variant isoforms (CD44v) as major functional P-selectin ligands (Hanley et al., 2006; Napier et al., 2007) and fibrin receptors (Alves et al., 2008; Alves et al., 2009) on metastatic colon carcinoma cells. CD44 is a type I transmembrane glycoprotein encoded by a single gene, and comprises at least 20 exons (Ponta et al., 2003). Exons 1–5, 16–18 and 20 are spliced together to form the smallest *CD44* transcript known as standard isoform (CD44s). At least ten exons (6–15; typically identified as v1–v10) can be alternatively spliced and inserted at a single site within the membrane proximal portion of the extracellular domain (Fig. 1B) to give rise to multiple variant isoforms of CD44 (Ponta et al., 2003).

In vitro assays reveal that CD44v binding to P-selectin mediates transient rolling interactions up to a wall shear stress level of 2 dyn/cm^2 (Hanley et al., 2006; Napier et al., 2007). In stark contrast, binding of CD44v to immobilized, monomeric fibrin mediates firm adhesion in the low shear regime (<1 dyn/cm^2) and at fibrin concentrations that are 100-fold higher than those of P-selectin (Alves et al., 2009; Hanley et al., 2006; Napier et al., 2007). Thus,

we aimed to provide a mechanistic interpretation at the molecular level for these two distinct adhesion events (i.e. rolling versus firm adhesion). Interestingly, in vivo studies reveal that selectins have a key role in the initial binding and subsequent lodging of metastatic tumor cells in target organs (Borsig et al., 2001; Kim et al., 1998), whereas fibrin(ogen) facilitates metastasis by mediating the sustained adhesion and stable implantation of tumor cells in the microvasculature (Palumbo et al., 2000; Palumbo et al., 2005). We hypothesize that intrinsic differences in the kinetic and micromechanical properties of individual bonds between CD44v and P-selectin, as opposed to fibrin, are responsible for their distinct shear-dependent adhesive interactions in vitro (Alves et al., 2008; Alves et al., 2009; Hanley et al., 2006; Napier et al., 2007) and in vivo (Palumbo et al., 2000; Palumbo et al., 2005).

To test our hypothesis, we used a micro-manipulation method based on single-molecule force spectroscopy (Hanley et al., 2003; Hanley et al., 2004). To this end, P-selectin or fibrin immobilized onto a cantilever tip was brought in contact for prescribed contact durations with either CD44v expressed on immobilized colon carcinoma cells or immunopurified CD44v incorporated into lipid vesicles and layered on a polyethyleneimine (PEI)-cushioned glass slide. The longer unstressed equilibrium lifetime, the lower reactive compliance, which refers to the susceptibility of bond rupture under force, and the higher tensile strength of CD44v–P-selectin versus CD44v–fibrin pair provide a molecular interpretation for the ability of the former pair to mediate binding at elevated shear stresses. We also found that increasing the receptor–ligand contact duration from 2 to 200 milliseconds does not affect the micromechanical properties of the CD44v–P-selectin bond at the single molecule level. By contrast, the tensile strength of the CD44v–fibrin bond increases with increasing contact durations. Inhibition of CD44v sulfation increases the tensile strength of the CD44v–fibrin bond, which then remains unaltered with increasing the contact duration. Taken together, these data suggest that the characterization of the biophysical properties of receptor–ligand pairs at the single-molecule level predicts the pattern of their shear-dependent binding interactions observed in vitro and in vivo.

Results

Here, we used force spectroscopy to provide a mechanistic interpretation at the single-molecule level for the two discrete CD44v-mediated adhesion events: rolling on P-selectin at high shear as opposed to firm adhesion on immobilized fibrin that occurs only in the low-shear regime. We have previously applied force spectroscopy to characterize the interactions between purified selectins immobilized on a cantilever tip and ligands expressed on whole cells (Hanley et al., 2003; Hanley et al., 2004). In view of recent observations showing that CD44v accounts for ~50% of LS174T colon carcinoma binding to P-selectin (Napier et al., 2007; Thomas et al., 2008), we developed a methodology to study binding interactions between purified molecules rather than whole cells. This approach enabled us to eliminate the potential contribution of unidentified P-selectin ligands other than CD44v on colon carcinoma cells. In this methodology, CD44 was immunopurified from LS174T colon carcinoma cells and reconstituted in 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC) lipid vesicles, and then layered on a PEI-cushioned glass slide (Fig. 1C). The hydrophobic interactions between the lipid and the transmembrane domain of CD44 allow its incorporation into the bilayer in the proper physiological orientation. Our experimental protocol has consistently generated spatially uniform DMPC lipid bilayers, as evidenced by staining with Nile Red (Fig. 2A).

To validate our methodology, we compared the kinetic and micromechanical properties of fibrin binding to CD44v-expressing LS174T colon carcinoma cells relative to immunopurified CD44v incorporated in lipid bilayers (Fig. 2B), because CD44v is the predominant fibrin receptor on LS174T cells, accounting for >85% of the binding to fibrin (Alves et al., 2009). In these experiments, we used a molecular force probe, which is a device that uses a small flexible cantilever that deflects in response to forces generated between the fibrin-coated cantilever tip and CD44v, either incorporated into lipid bilayers or expressed on LS174T cells, when repeatedly brought in contact for 20 milliseconds (Fig. 1C). As shown in representative force-distance traces, receptor–ligand unbinding at any given reproach velocity (5–25 μm/second) involved a single step rather than multiple force steps (Fig. 2C). To further ensure that most binding events were mediated by a single receptor–ligand pair, a low probability (Hanley et al., 2003) of binding (15–30%) was achieved by decorating the cantilever and lipid bilayers with dilute fibrin and CD44v concentrations, respectively. The specificity of CD44v–fibrin binding was demonstrated by incubating the CD44v-incorporated lipid vesicles with the function-blocking

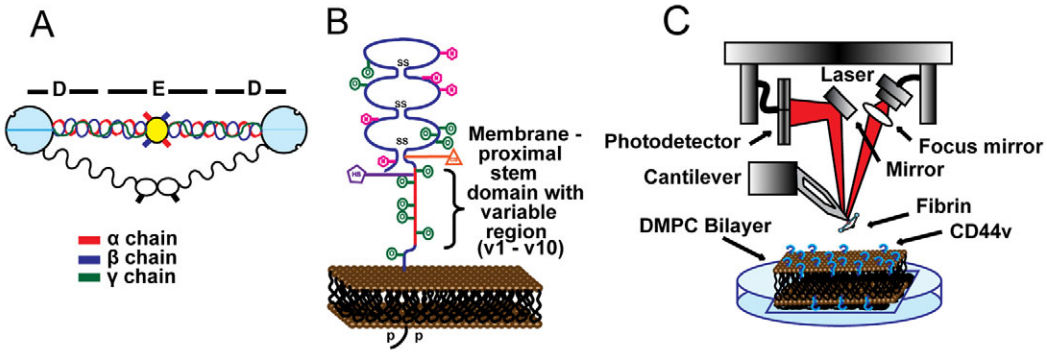

Fig. 1. Single-molecule force spectroscopy to probe the biomechanical properties of CD44v-fibrin bonds. (**A**) Schematic diagram of fibrinogen indicating the α-chain (red), β-chain (blue) and γ-chain (green). The central region E is a dimer formed by the N-terminal portions of all the six chains (yellow circle). The distal D regions are formed by the C-terminal of the Bβ- and γ-chains and a portion of the Aα chain. The two αC regions are made up of the C-terminal two-thirds of the Aα chains (black). (**B**) Schematic diagram of the variant isoform of CD44 (CD44v). Pink hexagons represent putative sites for N-linked glycosylation; green circles represent putative sites for O-linked glycosylation; SS, disulfide bond; P, serine phosphatases. (**C**) Schematic diagram of the molecular force probe (MFP) used to investigate the kinetic and micromechanical properties of receptor–ligand interactions at single-molecule resolution.

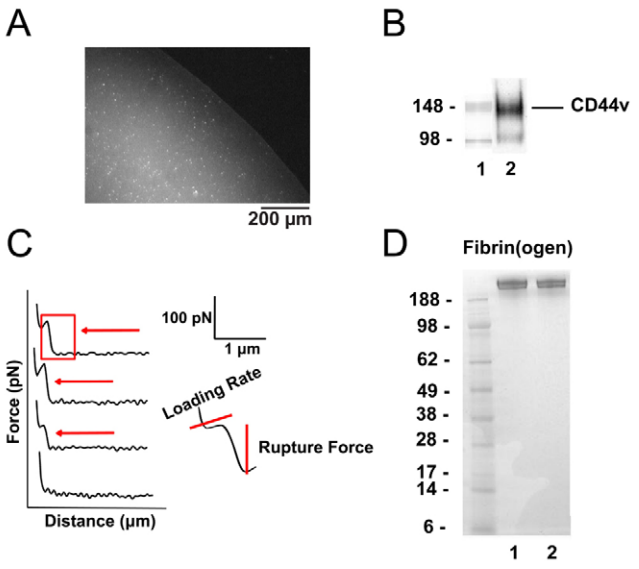

Fig. 2. Lipid bilayers and western blot images. (**A**) Image of fluorescently stained DMPC–CD44v lipid bilayer with Nile Red dye on PEI-coated glass substrate. (**B**) CD44v (lane 2) was immunoprecipitated from LS174T colon carcinoma cells and subjected to SDS-PAGE under reducing conditions, followed by western blotting using the anti-CD44 monoclonal antibody 2C5. (**C**) Typical force distance traces acquired from force spectroscopy experiments in which fibrin immobilized onto a cantilever tip was brought in contact hundreds of times with immunopurified CD44v incorporated into lipid vesicles and layered on a PEI-cushioned glass slide. The drop in the force indicates the rupture of a single CD44v–fibrin bond. (**D**) SDS-PAGE analysis of fibrinogen (lane 1) and fibrin (lane 2) under non-reducing conditions. The left lanes contain molecular weight markers (Seeblue Plus2, Invitrogen) with indicated molecular mass in kDa.

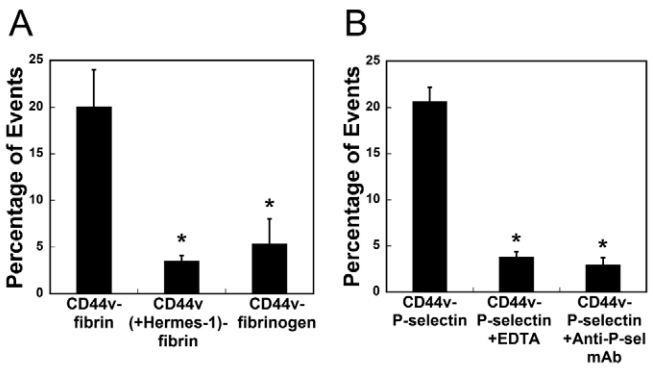

Fig. 3. Specificity of receptor–ligand binding. (**A**) Frequency of binding events between CD44v and fibrin or fibrinogen. Cantilever tips coated with 5 µg/ml fibrin or fibrinogen were brought into contact with a CD44v incorporated DMPC lipid bilayer. The frequency of binding between fibrin and CD44v was evaluated in the absence and presence of a function-blocking CD44 antibody Hermes-1. DMPC–CD44v lipid solutions were premixed with 20 µg/ml of the Hermes-1 monoclonal antibody just prior to the formation of bilayers on PEI-coated glass slides. Data represent the mean ± s.e.m. of n=3 independent experiments, where each experiment had at least 1200 approach/retract cycles. *P<0.05 with respect to CD44v–fibrin. (**B**) Frequency of binding events between CD44v and P-selectin in the absence and presence of the divalent cation chelator EDTA (5 mM final concentration) or the function-blocking anti-P-selectin monoclonal antibody AK4 (50 µg/ml). *P<0.05 with respect to CD44v–P-selectin.

anti-CD44 monoclonal antibody Hermes-1, which drastically reduced the frequency of binding events from ~20% down to <5% (Fig. 3A). We have previously reported that CD44v fails to bind to immobilized fibrinogen (Alves et al., 2009). Indeed, the probability of binding between CD44v and fibrinogen was at background levels (Fig. 3A). Addition of thrombin (2 U/ml) to fibrinogen-functionalized cantilevers results in generation of monomeric fibrin (Alves et al., 2009) and ~20% of successful adhesion events.

The average rupture force of the CD44v–fibrin bond grew linearly with the natural log of the loading rate for both the purified protein–protein (Fig. 4A) and cell–protein systems (supplementary material Fig. S1A). Moreover, the mean bond rupture forces at all loading rates were essentially identical for both systems (62 pN for CD44v–fibrin and 61 pN for LS174T CD44v–fibrin at 1000 pN/second). The Bell model parameters, namely the unstressed off-rate k°_{off} (second^{-1}) and reactive compliance x_{β} (nm), were extracted from least-squares fits of mean rupture force versus the logarithm of loading rate for fibrin binding to CD44v that was either incorporated into lipid bilayers or expressed on LS174T cells. The respective values for the off-rate and reactive compliance were similar for both the purified protein–protein and cell–protein systems (Fig. 4B,C). Taken together, the excellent agreement in the kinetic and micromechanical properties between both systems suggests that the purified protein–protein system consisting of CD44v reconstituted in a lipid bilayer is reproducible and reliable for studying the interactions between CD44v and its counter receptor at the single-molecule level.

We next characterized the binding of P-selectin to immunopurified CD44v. The specificity of this biomolecular interaction was first confirmed by the addition of EDTA (5 mM) to the glass slide, which nearly abrogated binding (Fig. 3B; supplementary material Fig. S1B). This finding is in accord with the Ca^{2+} dependence of CD44v–P-selectin binding (Hanley et al., 2006; Napier et al., 2007). Moreover, incubation of the P-selectin-coated cantilever tip with a function-blocking anti-P-selectin monoclonal antibody (AK4) resulted in a dramatic reduction in the binding frequency from 20% to <5% (Fig. 3B). In line with previously published work (Zhang et al., 2002), the magnitude of CD44v–P-selectin forces was drastically reduced in the presence of the anti-P-selectin monoclonal antibody (supplementary material Fig. S2A). Comparison of the Bell model parameters between the two systems revealed that off-rate and susceptibility of bond rupture under force were lower for the purified protein–protein rather than cell–protein system (Fig. 4B,C). The discrepancies in the values of off-rate and reactive compliance between the two systems can be attributed to the presence of yet unidentified selectin ligands on LS174T colon carcinoma cells. In light of our observations showing that CD44v is the major functional P-selectin ligand on LS174T cells (Napier et al., 2007), the other unidentified ligand(s) presumably possesses lower affinity for P-selectin, thereby contributing to a higher unstressed off-rate and reactive compliance for cell–protein system.

The CD44v–P-selectin bond displayed a higher tensile strength than that of CD44v–fibrin at all loading rates examined in this work (Fig. 4A). The markedly distinct forces required for rupturing the CD44v–P-selectin versus CD44v–fibrin bonds eliminate the possibility of CD44v extraction from the lipid bilayer. Moreover, previous work has shown that significantly higher forces (≥400 pN) are required for the extraction of integral membrane proteins

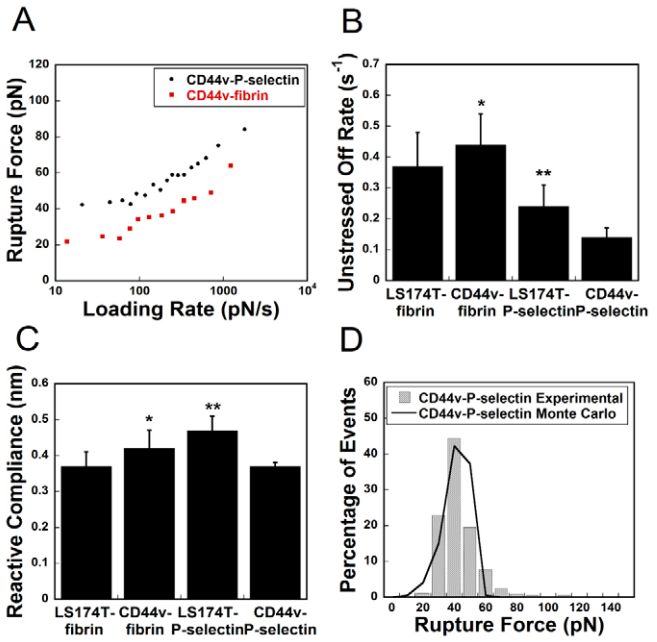

Fig. 4. Micromechanical and kinetic properties of cell–protein and protein–protein interactions at the single-molecule level. (**A**) Rupture force (pN) as a function of loading rate (pN/second), (**B**) unstressed off- rate (second^{-1}), and (**C**) reactive compliance (nm) for CD44v–P-selectin and CD44v–fibrin interactions. Data were acquired for a range of retraction velocities from 5 to 25 μm/second. Data represent mean ± s.d. of n=4–6 experiments, where each experiment had at least 1200 approach/reproach cycles. (**D**) Distribution of adhesion forces obtained experimentally (bars) or computed using a Monte Carlo simulation (line) based on Bell model kinetic parameters (see the Materials and Methods for details) for CD44v–P-selectin interactions. The retraction velocity was maintained at 20 μm/second. *P<0.05 with respect to CD44v–P-selectin. **P<0.05 with respect to CD44v–P-selectin and LS174T–fibrin.

(Afrin et al., 2003). The unstressed off-rate was significantly lower for the CD44v–P-selectin (0.14 second^{-1}) than the CD44v–fibrin (0.44 second^{-1}) bond, where modest differences were also noted for the reactive compliance (Fig. 4C). The Bell model parameters were validated for both receptor–ligand pairs by performing Monte Carlo simulations of bond rupture (Hanley et al., 2003; Hanley et al., 2004) under constant loading rates (Fig. 4D). Taken together, CD44v–P-selectin bond is mechanically stronger than that of CD44v–fibrin as evidenced by its higher tensile strength, lower susceptibility to rupture and longer unstressed bond lifetime. These results provide a mechanistic basis at the single-molecule level for the enhanced capacity of the CD44v–P-selectin rather than CD44–fibrin pair to mediate binding at higher shear stresses and low site densities.

To further substantiate these results, we evaluated the effect of contact duration between CD44v and P-selectin or fibrin on the modulation of their kinetic and micromechanical properties. Increasing the contact duration from 2 to 200 milliseconds did not affect the tensile strength of CD44v–P-selectin bond at the single-molecule level (Fig. 5A). In marked contrast, the mean rupture force of the CD44v–fibrin bond significantly increased from 32.1±1.4 to 39±1 pN (Fig. 5B). Calculations of the depth of the potential of the energy well describing the CD44v–fibrin interaction from rupture force distributions showed that it increased with increasing the contact duration from 5.9 k_BT to 8.2 k_BT, suggesting that the bond became four times [≈exp(8.2/5.9)] more stable at 200 milliseconds (Fig. 5C, Fig. 6). By contrast, the depth of the free energy barrier remained unchanged for the CD44v–P-selectin interactions (Fig. 5C). Moreover, the reactive compliance of the CD44v–fibrin bond, which describes the bond susceptibility under force, was much lower at 200 than at 2 milliseconds, whereas it was unaltered for the CD44v–P-selectin pair (Fig. 7A). It is noteworthy that the unstressed off-rate of the CD44v–fibrin bond dropped precipitously from 0.78 second^{-1} at 2 milliseconds to 0.14 second^{-1} at 200 milliseconds (Fig. 7B). By contrast, the CD44v–P-selectin bond presented only a modest change in the unstressed off-rate (from 0.19 to 0.10 second^{-1}) and unstressed bond lifetime (Fig. 7B,C). The increase in contact duration from 2 to 200 milliseconds did not affect the frequency of binding events (supplementary material Fig. S3A) or the percentage of single or multiple CD44v–fibrin bonds as estimated by Poisson distribution statistics (supplementary material Fig. S3B). Of note, intracellular contact durations of 2 and 200 milliseconds correspond to wall shear stresses of ~0.5 and 0.01 dyn/cm^2, respectively (Bongrad et al., 1988). Cumulatively, this analysis clearly reveals that the CD44v–fibrin single bond stabilizes with increasing interaction times, as evidenced by its decreased unstressed off-rate and reactive compliance and its increased tensile strength and depth of the free energy barrier. This bond maturation at the single-molecule level might offer a molecular interpretation for the stable adhesion mediated by CD44v-bearing cells or microspheres to high densities of immobilized monomeric fibrin at low shear stresses (Alves et al., 2009) and correspondingly high contact durations.

Treatment of immunopurified CD44v with chondroitinase ABC, which degrades all forms of chondroitin sulfate (CS) and dermatan sulfate (DS) (Clark et al., 2004) essentially abolished the frequency of binding events between CD44v and fibrin at all contact durations (Fig. 8A), indicating the crucial roles of CS and/or DS in CD44v–fibrin molecular recognition. To further investigate the contribution of sulfated glycosaminoglycans to CD44v–fibrin binding, LS174T colon carcinoma cells were treated with sodium chlorate (60 mM) before immunoprecipitation of CD44v. As we recently showed (Alves et al., 2009), this treatment blocks the incorporation of sulfate groups to CD44v. Although there was no significant increase in the frequency of binding events upon blocking sulfation on CD44v, this intervention increased the tensile strength of CD44v–fibrin bond which then remained unaltered at all tested contact durations (Fig. 8B). Altogether, these data suggest that unsulfated or undersulfated stretches of CS and/or DS glycosaminoglycans are responsible for the enhanced tensile strength of CD44v–fibrin bonds.

Discussion

P-selectin and fibrin(ogen) have key roles in the metastatic dissemination of tumor cells. Microscopic observations of tumor cells, including colon carcinoma, trapped in the lung vasculature disclose their intimate association with platelets (mainly via P-selectin) (Borsig et al., 2001; Laubli et al., 2006) and fibrin(ogen) (Crissman et al., 1988; Im et al., 2004), which facilitate blood-borne metastasis. It is believed that platelet and fibrin(ogen) clots surrounding tumor cells provide a protective mask against the immunological and physiological stresses in the bloodstream, and facilitate their lodging to new metastatic sites (Nieswandt et al., 1999). In vivo studies have established that the initial seeding and

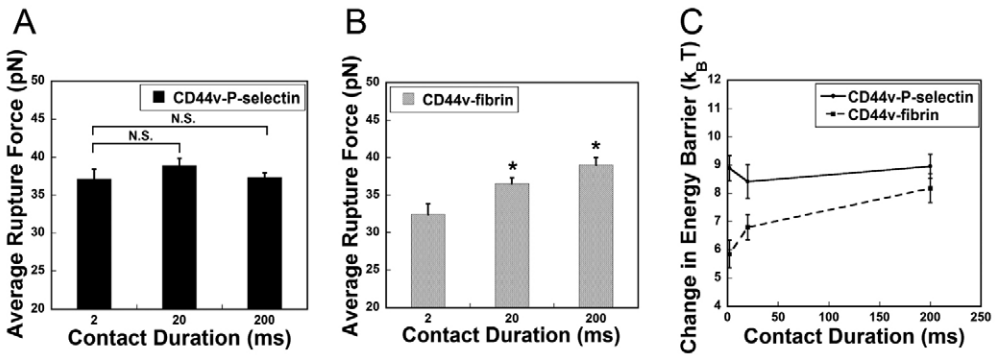

Fig. 5. Effects of contact duration on rupture force and depth of free energy barrier of CD44v–P-selectin and CD44v–fibrin bonds. Average rupture force for (**A**) CD44v–P-selectin and (**B**) CD44v–fibrin binding as a function of contact duration. Data represent mean ± s.e.m. of n=6 independent experiments. NS, no statistical significance. *P<0.01 with respect to 2 milliseconds data. (**C**) Depth of the free energy barrier (k_BT) as a function of contact duration. Data represent mean ± s.d. of n=6 independent experiments. Data were acquired with a retract velocity of 20 μm/second.

subsequent lodging of metastatic colon carcinoma cells in target organs is markedly attenuated in P-selectin-knockout mice compared with wild-type controls (Borsig et al., 2001; Kim et al., 1998). In contrast to selectins, fibrinogen does not have a role in the initial binding and seeding of tumor cells within the pulmonary vasculature (Palumbo et al., 2000). Instead, it appears to facilitate metastasis by mediating the sustained adhesion and survival of tumor cells in the shear environment of target organs (Palumbo et al., 2000; Palumbo et al., 2005). We have recently discovered that CD44v is the major functional P-selectin ligand (Hanley et al., 2006; Napier et al., 2007) and fibrin receptor (Alves et al., 2008; Alves et al., 2009) on metastatic colon carcinoma cells. Interestingly, *in vitro* studies reveal that CD44v binding to P-selectin mediates transient tethering and rolling interactions at elevated shear stresses (Hanley et al., 2006; Napier et al., 2007), whereas CD44v binding to immobilized, monomeric fibrin supports firm adhesion at low shear (~0.5 dyn/cm^2). Therefore, delineating the biophysical nature of these distinct CD44-dependent adhesion events to P-selectin versus fibrin at the single-molecule level will enhance our understanding of the metastasis process, and might provide new avenues for research to combat this disease.

We have used force spectroscopy to study the binding interactions between purified molecules rather than whole cells, thereby enabling us to eliminate the potential contribution of unidentified P-selectin ligands other than CD44v to this process. In these experiments, CD44 immunopurified from metastatic LS174T colon carcinoma cells was incorporated into DMPC lipid vesicles in the proper orientation, and layered on a PEI-cushioned glass. The PEI cushion accommodates the inversely oriented CD44 molecules between the bilayer and glass slide, thus ensuring that the bilayer is not disrupted (Sarangapani et al., 2004). Unlike unsaturated or tissue-derived lipids, such as egg phosphatidylcholine (Sarangapani et al., 2004), which are hygroscopic as powders and are subject to hydrolysis and oxidation more readily, DMPC saturated lipids are very stable, and the reconstituted vesicle solution can be used for up to one month. This protocol enabled us to obtain spatially uniform DMPC–CD44 lipid bilayers, which displayed reproducible binding probabilities everywhere along the surface when probed with P-selectin- or fibrin-functionalized cantilever tips. By carefully controlling the concentration of CD44 incorporated into the lipid bilayer, we limited the probability of binding to <30%, thereby ensuring that most binding events were mediated by a single receptor–ligand pair (supplementary material Fig. S3B). The excellent agreement in the kinetic and micromechanical properties of fibrin binding to immunopurified CD44 incorporated in lipid bilayers versus CD44-expressing LS174T colon carcinoma cells validates the use of the purified protein system for studying receptor–ligand interactions at the single-molecule level. ERM proteins crosslink the actin cytoskeleton to CD44 (Ponta et al., 2003). Lantrunculin (0.1 μM), a compound that prevents actin polymerization by irreversibly binding to actin monomers (Yarmola et al., 2000), failed to impair the frequency of binding events (supplementary material Fig. S1B) and the tensile strength (supplementary material Fig. S1C) of the CD44v–fibrin bond, suggesting that actin cytoskeleton is not involved in the regulation of CD44v–fibrin binding. Similar

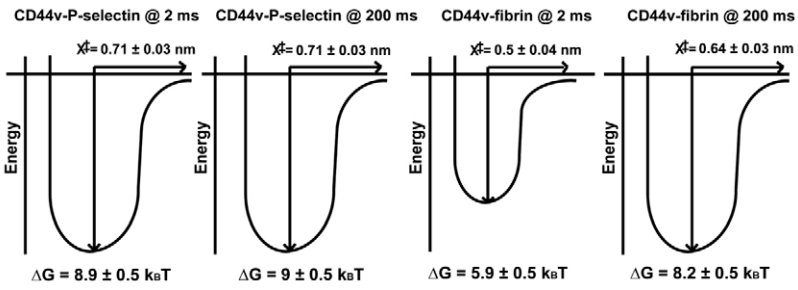

Fig. 6. CD44v–fibrin bond maturation. Schematic diagram showing the change in the depth of the free energy barrier for CD44v–P-selectin and CD44v-fibrin bonds with increasing contact duration. The CD44v–fibrin, but not CD44v–P-selectin, bond undergoes stabilization with increasing contact time.

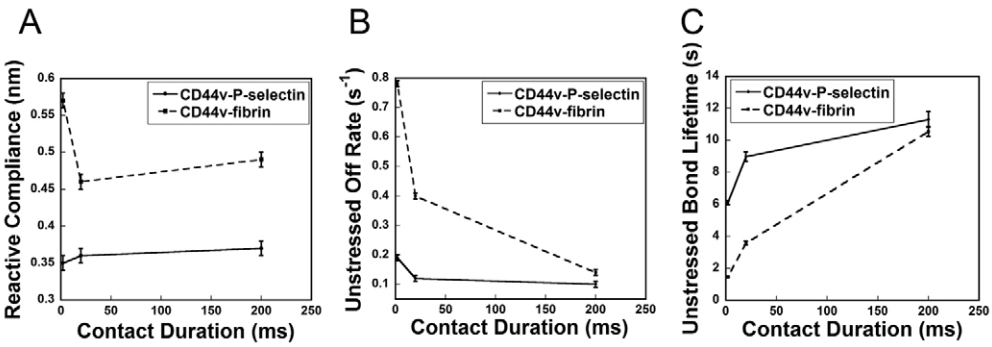

Fig. 7. Effects of contact duration on the Bell model parameters of CD44v–P-selectin and CD44v–fibrin bonds. (**A**) Reactive compliance (nm), (**B**) unstressed off rates (second^{-1}) and (**C**) unstressed bond lifetime (seconds) as a function of the contact duration between receptor-ligand bonds. Data represent mean ± s.e.m. of n=6 independent experiments.

observations have been made for CD44v–P-selectin binding (Hanley et al., 2003).

We determined that the CD44v–P-selectin relative to the CD44v–fibrin bond has a longer unstressed lifetime, a lower susceptibility to bond rupture under force, a higher tensile strength and a deeper free energy well, which explain its ability to mediate binding at higher hydrodynamic forces. The loading rates examined in this work (50–2000 pN/second) fall within the physiological range (Tees et al., 2001), and were sufficient to document the differences in the kinetic and micromechanical properties of CD44v binding to fibrin versus P-selectin.

P-selectin binds CD44v through sialidase-sensitive O-linked glycans presented on CD44v that is independent of heparan, chondroitin and dermatan sulfates (Hanley et al., 2006; Konstantopoulos and Thomas, 2009; Napier et al., 2007). Hermes-1, an anti-CD44 monoclonal antibody, which exhibits function-blocking properties by recognition of the N-terminal hyaluronate binding domain of CD44, did not interfere with CD44v binding to P-selectin (supplementary material Fig. S2B), although it nearly abolished binding of CD44v to fibrin (Fig. 3A). The CD44-binding site is localized within the N-terminal portion of the fibrin β-chains, including amino acid residues (β15–66) (Alves et al., 2009). We have also demonstrated the crucial roles of CS and/or DS glycosaminoglycans in CD44v–fibrin molecular recognition. Interestingly, the CD44v–fibrin bond, but not the CD44v–P-selectin bond, strengthens at longer interaction times, which might offer a molecular interpretation for the CD44-dependent firm adhesion to immobilized fibrin in the low shear regime and correspondingly high contact durations. This bond strengthening detected between sulfated CS- and/or DS-bearing CD44v and fibrin with increasing contact duration might be attributed to segregation of charged residues of the fibrin β15–66 chain (Odrljin et al., 1996). Moreover, inhibition of sulfation on CD44v increases the CD44v–fibrin bond strength, which then remains unaffected with increasing interaction times. The removal of negatively charged sulfate groups from CD44v might facilitate the binding of non-sulfated or undersulfated stretches of CS and/or DS glycosaminoglycans to negatively charged regions of the fibrin molecule, thereby resulting in stronger binding even at the earliest contact duration.

P-selectin glycoprotein ligand-1 (PSGL-1) has been identified as the predominant high-affinity ligand for P-selectin on normal leukocytes. The P-selectin–PSGL-1 bond exhibits 'catch–slip' dissociation under force, in which bond lifetime is initially prolonged (catch) and then diminishes (slip) with increasing applied force (Evans et al., 2004; Marshall et al., 2003). The unstressed off-rate of P-selectin–PSGL-1 along the single pathway is 0.37±0.07 second^{-1}, whereas its reactive compliance is 0.23±0.01 nm, and displays a tensile strength of 70–150 pN for loading rates in the range of 300 to 30,000 pN/second, respectively (Evans et al., 2004). Comparison of the micromechanical properties of P-selectin binding to PSGL-1 relative to CD44v helps explain the more stable and slower rolling mediated by PSGL-1 compared with CD44 on P-selectin substrates in shear flow (McCarty et al., 2000). Force spectroscopy has been previously used to probe P-selectin binding to tetrasaccharide sialyl Lewis x (sLe^x) (Zhang et al., 2004). In a loading regime of 100–10,000 pN/second, Zhang and colleagues (Zhang et al., 2004) reported an unstressed off-rate of 0.3 second^{-1} and a reactive compliance of 0.45 nm along with a mean rupture force of ~50 pN for a loading rate of 1000 pN/second. The lower reactive compliance (0.37 nm) and unstressed off-rate (0.14 second^{-1}) along with the higher tensile strength (77 pN at 1000 pN/second) of the CD44v–P-selectin bond illustrate the higher affinity of immunopurified CD44 from metastatic LS174T colon carcinoma cells than tetrasaccharide sLe^x for P-selectin. This finding is attributed to the low affinity of selectins for isolated monocovalent sLe^x oligosaccharides.

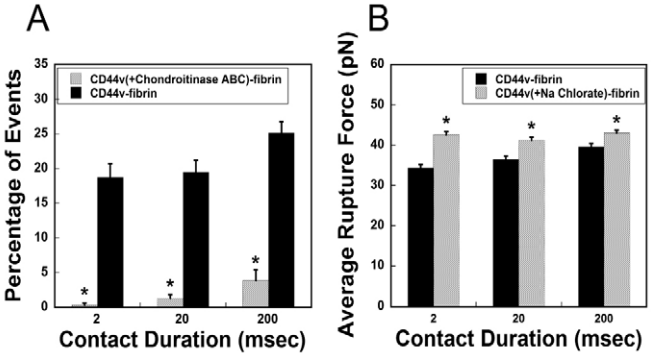

Fig. 8. Effect of glycosaminoglycans and sulfation on CD44v–fibrin binding. (**A**) Frequency of binding events between CD44v, either untreated or treated with chondroitinase ABC (1 U/ml), and fibrin as a function of contact duration. (**B**) Average rupture force of CD44v-fibrin bond as a function of contact duration. CD44v was immunopurified from LS174T cells cultured in medium containing either sodium chlorate (60 mM) to block sulfation or DPBS. Data represent mean ± s.e.m. of n=3 independent experiments performed at a retract velocity of 20 μm/second. *P<0.05 with respect to untreated control.

CD44 binding to immobilized monomeric fibrin mediates firm adhesion at low but not high shear. This might be ascribed to the CD44–fibrin bond strengthening at the single-molecule level with increasing receptor–ligand contact duration. In addition to single-bond maturation, we cannot rule out the contribution of the avidity effect to firm adhesion, especially in light of flow-based adhesion assays showing that binding of CD44 to immobilized fibrin under flow requires fibrin concentrations that are ~100-times higher than those of P-selectin. Firm adhesion mediated by other distinct receptor–ligand pairs might require significantly lower unstressed off-rates and reactive compliances, as has been reported for the high-affinity leukocyte-function-associated antigen-1 (LFA-1) to intercellular adhesion molecule-1 (ICAM-1) ($k°_{off}$=0.17 second^{-1} and x_β=0.21 nm) (Zhang et al., 2002).

In summary, single-molecule characterization of the biophysical properties of receptor–ligand pairs provides a mechanistic insight into the inherent dynamics of the shear-dependent receptor–ligand binding pertinent to cancer metastasis. These measurements enable us to predict the pattern of shear-dependent cell–substrate adhesive interactions observed in vitro and in vivo. Our findings offer a strong quantitative understanding of the interactions of CD44, which is aberrantly expressed on human metastatic tumor cells, with P-selectin and fibrin, which have vital roles in the facilitation of cancer metastasis.

Materials and Methods

Reagents and monoclonal antibodies

The fibrinogen (vWf, plasminogen and fibronectin free) was from Enzyme Research Laboratories (South Bend, IN). Thrombin, chondroitinase ABC (*Proteus vulgaris*) and sodium chlorate were from Sigma. The anti-human CD44 monoclonal antibody (2C5) was from R&D Systems (Minneapolis, MN). PEI was from Polysciences (Warrington, PA). DMPC was from Avanti Polar Lipids (Alabastar, AL).

Cell culture, whole-cell lysis and immunoprecipitation

The human LS174T colon adenocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in the recommended medium. CD44 was immunoprecipitated from LS174T cell lysates with the anti-CD44 monoclonal antibody 2C5 using recombinant Protein-G–agarose beads (Invitrogen) (Alves et al., 2009; Hanley et al., 2006; Napier et al., 2007).

Lipid bilayer preparation

To prepare lipid solutions, 8 mg DMPC were dissolved in 8 ml lipid buffer B [20 mM Tris-HCl, 50 mM NaCl, 1 mM $CaCl_2$, 0.1% (w/v) Triton X-100]. 100 µl CD44v at a concentration of 100 µg/ml were added to 400 µl lipid solution and incubated at 37°C for 2 hours (Erb and Engel, 2000). The lipid–protein solution was transferred to a 10 kDa MWCO dialysis cassette and dialyzed against 1 liter of lipid buffer A (20 mM Tris-HCl, 50 mM NaCl, 1 mM $CaCl_2$) three times for 12 hours each. Lipid–protein solutions were then stored at 4°C under argon for up to one month. To prepare bilayers, glass slides were cleaned with plasma oxygen for 5 minutes and immediately submerged in a solution of 100 p.p.m. PEI in 0.5 mM KNO_3 for 20 minutes before rinsing with deionized water and drying with nitrogen. The slides were further dried in a vacuum desiccator overnight before use. The PEI-coated slides were then incubated with a 4 µl droplet of the lipid-protein solution for 1–2 hours under a slightly dampened towel to prevent complete dehydration. The slides were then rinsed with Hank's Balanced Salt Solution (HBSS) and flooded with HBSS for use in the MFP experiments. In select experiments, the DMPC–CD44v solution was premixed with 20 µg/ml Hermes-1 anti-CD44 monoclonal antibody before incubation on the glass slide.

Treatment with inhibitors and enzymes

To degrade all forms of chondroitin sulfate and dermatan sulfate, CD44v-incorporated lipid bilayers were incubated for 1 hour at 37°C with 1 U/ml *Proteus vulgaris* chondroitinase ABC (Alves et al., 2009) before force spectroscopy measurements. To inhibit sulfation, CD44v was immunopurified from LS174T colon carcinoma cells, which were cultured for 48 hours at 37°C in medium containing 60 mM sodium chlorate (Alves et al., 2009). DPBS was used as diluent control.

SDS-PAGE and western blotting

Purified fibrinogen (Fig. 2D, lane 1) and freshly prepared monomeric fibrin (Fig. 2D, lane 2) were diluted with DPBS and NuPage LDS sample buffer and separated by gel electrophoresis using a 4–12% Bis-Tris NuPage gel (Invitrogen) under non-reducing conditions. The gel was stained with Gelcode Blue Coomassie reagent (Pierce) (Alves et al., 2008). Immunoprecipitated CD44v was resolved on a 4–20% Tris-HCl Criterion gel (Bio-Rad) using Tris-glycine running buffer under reducing conditions, transferred to Immuno-blot polyvinylidene diflouride membranes and stained with anti-CD44 monoclonal antibody (2C5) (Alves et al., 2009; Hanley et al., 2006; Napier et al., 2007).

Cantilever functionalization

To provide a surface that readily binds soluble proteins, MFP cantilevers (Veeco, Plainview, NY) were silanized with 2% 3-aminopropyltriethoxysilane (Hanley et al., 2003; Hanley et al., 2004). Cantilevers were functionalized with P-selectin–IgG Fc as previously described (Hanley et al., 2003; Hanley et al., 2004). For CD44v–fibrin interactions, the cantilevers were incubated in a 5 µg/ml solution of fibrinogen in D-PBS containing 50-fold molar excess of the crosslinker *bis*(sulfosuccinimidyl) suberate (BS^3; Pierce, Rockford, IL) for 30 minutes followed by quenching with Tris buffer. To form fibrin, the cantilever tips were incubated with 2 U/ml thrombin in DPBS for 2 hours at 37°C (Alves et al., 2008; Alves et al., 2009), followed by immersion in Tris buffer to block non-specific interactions.

Single-molecule force spectroscopy, data acquisition and analysis

Experiments were conducted using an MFP (Asylum Research, Santa Barbara, CA) (Hanley et al., 2003; Hanley et al., 2004). The softest triangular cantilever was calibrated using thermal noise amplitude and its deflection was measured by laser reflection onto a split photodetector. The distance between the cantilever and the DMPC–CD44v lipid bilayer was adjusted so that each approach cycle resulted in a slight depression force (~500 pN) before reproach (Hanley et al., 2004). The reproach velocity was varied from 5 µm/second to 25 µm/second, and the dwell time between the cantilever and the cell or bilayer was varied from 2 to 200 milliseconds. Rupture forces and corresponding loading rates were derived from force–distance traces using IgorPro 4.09 software (Wavemetrics, Lake Oswego, OR). Rupture force measurements were binned according to loading rate at increments of 50 pN/second up to 100 pN/second and at increments of 100 pN/second for rates greater than 100 pN/second (Bajpai et al., 2008; Bajpai et al., 2009). For each set of binned data, a mean adhesion force and loading rate were calculated and used to fit Bell model parameters. The Bell model parameters ($k°_{off}$ and x_β) were tabulated by a least-squares fit to the linear region of a graph of rupture force against the logarithm of loading rate (Hanley et al., 2003; Hanley et al., 2004). The measured rupture forces were binned using 10-pN-wide bins (Bajpai et al., 2008; Bajpai et al., 2009). The force distribution for a given reproach velocity was fitted using Hummer and Szabo's Model to determine the distance along the free energy surface from the well minimum to the energy at bond rupture ($x^\ddagger$) and the depth of the free energy barrier using Monte Carlo optimization methods (Hummer and Szabo, 2003).

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