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Published in:
Journal of Molecular Recognition

DOI:
10.1002/jmr.1108

Publication date:
2011

Document version
Early version, also known as pre-print

Citation for published version (APA):
Mechanically Enforced Bond Dissociation Reports Synergistic Influence of Mn$^{2+}$ and Mg$^{2+}$ on the Interaction Between Integrin $\alpha_7\beta_1$ and Invasin$^\dagger$

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Integrins require the divalent ions magnesium and manganese for ligand recognition. Here we mechanically enforced bond dissociation to explore the influence of these ions on the mechanical strength of the specific bond between $\alpha_7\beta_1$ integrin and its pathologically relevant ligand invasin. Upon addition of these cations to the measurement buffer, we observe a pronounced increase in the force necessary to separate integrin and invasin coated beads. Both ions were found to work synergistically. With free invasin in the measurement buffer we furthermore observe that competitive blocking of binding sites overrides the increase in binding strength of individual beads. We show that this is due to a very strong dependence of bond affinity on divalent ions. Our study illustrates the importance of divalent ions for the regulation of force transmission by integrin ligand bonds on the molecular level. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: mechanically enforced bond dissociation; biomembrane force probe; integrin; $\alpha_7\beta_1$; invasin; divalent ions

INTRODUCTION

Many pathogens trigger infections in animals by entering eukaryotic host cells and thus escaping from the host immune system. Among the bacteria to use this strategy are Yersinia pseudotuberculosis and Yersinia enterocolitica that utilize their surface protein, invasin, to firmly attach to integrin receptors on the cell surface. These heterodimeric trans-membrane receptors mediate cell–matrix interactions (Dedhar and Hanningan, 1996; Hughes and Pfaff, 1998; Cheresh, 2007). Ligation of invasin to integrins, such as laminin-binding integrins: $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$ (Hamburger et al., 1999) generates strong forces which together with integrin-triggered signaling events lead to the entrapment and uptake of the bacterium into the host cell (Isberg, 1989; Van Nhieu et al., 1996; Dersch and Isberg, 1999). The binding affinity of invasin to the respective integrins exceeds the bond affinities between integrins and their cognate extracellular matrix (ECM) ligands by two orders of magnitude (Eble et al., 1998, 2003; von der Mark et al., 2002). This enables invasin to compete successfully with the excess of ECM ligands for integrin binding (Van Nhieu and Isberg, 1991). Invasin can even interfere with the interaction of $\alpha_2\beta_1$, integrin with laminin-111, which exhibits one of the highest affinity constants for an integrin–ECM–protein interaction (Eble et al., 1998, 2003; von der Mark et al., 2002).

Integrins require divalent cations for ligand recognition and binding. A crystallographic study on $\alpha_5\beta_3$ revealed eight different divalent cation-binding sites (Xiong et al., 2001), but their mutual role on integrin structure, stability, and function is still not fully understood (Mould et al., 1995; Humphries, 2000; Humphries et al., 2003). Magnesium and calcium cations are the natural cofactors for the integrins (Smith et al., 1994). Moreover, affinity studies on a wide range of integrins, among them $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, and $\alpha_8\beta_2$ indicate that Mn$^{2+}$ cations are strong activators of integrin activity (Mould et al., 1995).

Different approaches have been applied previously to study the vital force-transmitting properties of integrins, among them atomic force microscopy, optical tweezers, and dynamic force microscopy (Weisel et al., 2003). However, most studies were performed on whole cells (Litvinov et al., 2002) in which the measured forces may be substantially influenced by the stiffness of the cytoskeleton or the plasma membrane tension. Only in a few cases forces were measured on the molecular level of integrins, by hydrodynamic flow measurements (Goldsmith...
et al., 2000) or by atomic force microscopy (Zhang et al., 2004). However, the important aspect of integrin force transmission property regulation by divalent ions has not yet been analyzed on the molecular level.

Here, we determine the forces necessary to break attachments between invasin and the \( \alpha_\beta_1 \) integrin to probe the effect of divalent ions on the mechanical strength of this bond. Measurements of mechanically enforced bond dissociation were performed as described by Merkel et al. (1999), using two beads, coated with invasin and \( \alpha_\beta_1 \) integrin, respectively. One bead is attached to an erythrocyte which serves as a tunable biomembrane force probe (BFP; Evans et al., 1995; Evans and Ritchie, 1997; Simson et al., 1998). The beads are brought together and pulled apart to associate and dissociate the binding partners while recording the rupture forces. Since both binding partners are immobilized on beads, our system resembles the in vivo situation in which both, integrin and invasin are membrane-anchored surface proteins of eukaryotic and bacterial cell, respectively. Although this immobilization may significantly reduce the lifespan of the specific bond between receptor and ligand (Nguyen-Duong et al., 2003), our conditions recapitulate the in vivo situation better than ELISAs or surface plasmon resonance measurements, in which only one of the two binding partners is immobilized. Invasin is pathologically highly relevant and has advantageous properties for our measurements: it is small, bears only one integrin binding site and has a very high binding affinity to \( \alpha_\beta_1 \) integrin. Moreover, we could take advantage of a soluble \( \alpha_\beta_1 \) integrin construct, which contains the ligand binding site but lacks the transmembrane domain (Eble and Berditchevski, 2002; Eble et al., 2003), thus avoiding the use of detergents which would damage the biomembrane probe. In this highly simplified system we could demonstrate on the molecular level that \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) enhance bond strength. Furthermore, our data indicate that these ions work in a synergistic manner and are sufficient to establish the basic mechanism of these regulatory processes.

MATERIALS AND METHODS

Materials

All experiments were conducted in a measurement buffer (30 mM Tris (Sigma) with the specified amounts of \( \text{MgCl}_2 \) (Sigma), or \( \text{MnCl}_2 \) (Merck, Darmstadt, Germany) and the amount of NaCl necessary to reach an osmolarity of 155 mOsm/l; pH 7.3). Buffers were filtrated by use of sterile filters directly after their preparation and again just before measurements. Melamine beads (Polybead cross-linked melamine particles, 10% solids (w/v) aqueous suspension; Polysciences, Warrington, PA, USA) with diameters of 2.55 and 3.27 \( \mu \text{m} \) were chosen for immobilization of the ligand and the receptor, respectively. The bigger bead, further referred to as the test bead, was functionalized with invasin and neutradvin. The latter protein enabled a firm attachment of the probe bead to a biotinylated erythrocyte via biotin–neutradvin bond.

Biomembrane force probe technique

A preswollen erythrocyte with a functionalized probe bead glued to its apex made up a BFP. The round shape of the erythrocyte, required for the proper force determination (Simson et al., 1998) was enforced by the osmolarity of the measurement buffer, which was set for our study at 155 mOsm/l. The erythrocyte was aspirated by a micropipette and its stiffness was tuned by adjusting the pipette suction pressure (Evans et al., 1995; Evans and Ritchie, 1997; Simson et al., 1998). The assembly of the BFP and the course of a force spectroscopy experiment are depicted and described in Figure 1.

If bonds are formed between integrin and invasin upon bead contact, a retraction of the test bead causes the erythrocyte to elongate and the probe bead to be displaced (see Figure 1). Membrane mechanics theory (Evans et al., 1995; Simson et al., 1998) provides a relation between the applied force and the observed probe bead displacement that is set by geometry and the applied suction pressure. The probe bead displacements were tracked by video microscopy. Many individual dissociation events were evaluated in dynamic yield force spectra, which were recorded at different binding conditions.

Proteins

The proteins were covalently anchored to a dextran matrix (Dextran 35-45 kDa; Sigma, St. Louis, MO, USA) attached to the melamine beads as specified in the next subsection. The dextran matrix mimics the glycocalyx of the cell thereby keeping attached proteins in an active state. The recombinant integrin \( \alpha_\gamma X_\beta_1 \) ectodomain, called soluble \( \alpha_\beta_1 \) integrin, and the integrin-binding competent C-terminal fragment of invasin, briefly called invasin, were produced as described previously.
(Leong et al., 1990; Eble et al., 1998, 2003; Dersch and Isberg 1999). Commercially obtained neutravidin (Pierce and Invitrogen, Carlsbad, CA, USA) was used at 60 μM in 10 mM sodium acetate buffer (pH 4.5) with 5% glycerol. In order to control the number of available binding sites the measurement buffer contained also a receptor blocking agent in defined concentrations. For this purpose we used invasin, further referred to as free invasin.

Covalent coupling of proteins to microbeads

We adjusted and optimized the immobilization of proteins on microbeads previously described in (Lofas and Johnsson, 1990; Johnsson et al., 1991) including some later adaptations (Strigl et al., 1999). The three main steps are bead dextranization, dextran carboxylation, and protein immobilization. The probe and test beads were treated in parallel except for the final step of protein immobilization. Both bead types were diluted to 2% solids (w/v) suspension in water, from which 50 μl were used for each preparation.

Dextran beads of both sizes were produced as in Strigl et al. (1999); washed in pure water and stored in 4 ml of phosphate buffered saline (PBS; 4.8 mM KH2PO4, 25.2 mM Na2HPO4, 121 mM NaCl (all Merck) in water; pH 7.4) at 4°C for up to 1 month. In the next step, carboxylation, reactive groups for protein attachment were generated and caused the microspheres to become negatively charged. For the carboxylation, performed as in Strigl et al. (1999), 1 ml of dextranized beads suspensions of an approximate concentration 0.025% (w/v) in PBS was used. The resulting electrostatic bead repulsion has previously been shown to conveniently reduce non-specific interactions (Strigl et al., 1999), but also the frequency of specific bindings. The binding frequency, or the probability of bond formation, is the ratio of the number of registered binding events to the total number of bead contacts. We found that at a carboxylation time of 20 min, the frequency of non-specific interactions is low (7%), whereas the frequency of specific bond formation is at an acceptable level (above 50%). Shorter carboxylation times resulted in a higher number of non-specific bonds upon inter-bead contact and were thus rejected. Carboxylation times above 20 min had a marginal influence on the non-specific binding frequency but considerably reduced the frequency of specific interactions. Hence, 20 min carboxylation time was chosen for our study. To estimate the binding frequency due to non-specific interactions, all receptors on the integrin beads were saturated with excess free invasin (1.5 μM) in the measurement buffer with 1 mM Mn2+ and 2 mM Mg2+.

The functionalization of carboxylated beads with proteins is a three-step procedure. First, the carboxyl groups of the dextran matrix were transformed into active esters using a mixture of N-hydroxysuccinimide (NHS, purity ≥99%; Fluka) and N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC, purity ≥97%; Fluka) (Strigl et al., 1999). Then the beads were washed twice in water (0°C), resuspended in 0.2 ml of ice-cold binding buffer (4.8 mM KH2PO4, 25.2 mM Na2HPO4, 33.4 mM NaCl (all Merck), 2 mM MgCl2 (Sigma) in water; pH 7.35), and sonicated for 60 s at 0°C. Immediately after this step, proteins (0.6 μM invasin and 1.2 μM neutravidin for the probe beads and 0.3 μM integrin for the test beads) dissolved in cold binding buffer were added, and both bead suspensions were rotated in the dark at 4°C for 10 min. Then the amino groups of the proteins and all remaining active ester groups were inactivated with 0.5 ml 0.1 M ethanolamine (purity ≥99%, Fluka) for 30 min. Finally, the beads were washed three times and stored in PBS at 4°C to be used within 1 week.

Biotinylation of erythrocytes

The erythrocytes freshly obtained from donors (8 μl), were washed1 twice in PBS at room temperature and three times in carbonate-bicarbonate buffer at 4°C (CBB; 0.1 M NaHCO3 dissolved in pure water and titrated to pH 8.3 with 0.1 M Na2CO3; all Merck). Subsequently, the pellet was treated with 1 ml of 0.5 mM Biotin-PEG-NHS 3400 (Nektar Therapeutics, San Carlos, CA, USA) in ice-cold CBB and rotated in the dark for 10 min. Finally, the cells were washed twice in PBS and stored for up to 7 h at 4°C in PBSA (PBS containing 1% (w/v) bovine serum albumin (BSA, purity ≥99%; Sigma–Aldrich, St. Louis, MO, USA)).

Experimental setup

The experiments were performed on the stage of a bright field light microscope (Axiovert 135TV, Carl Zeiss, Jena, Germany) equipped with a 40x/0.6 LD-Achromplan lens and a 1.6x optovar. The image was projected onto a scientific grade CCD camera (SensincamQE, PCO, Kehlheim, Germany) with external ventilator to reduce vibrations. The size of one square pixel corresponded to 105 nm in the object plane. Images of the force probe were collected with frame rates of up to 50 Hz and processed in a personal computer by a custom designed tracking program. The position of the probe bead was determined by a nonlinear least squares fit to a Gaussian function as described in Simson et al., 1998. Position detection was done in real time at an accuracy of 5 nm.

The micropipettes for the experiments were pulled using a Flaming Brown micropipette puller (P-97, Sutter Instrument Company, Novato, CA, USA) from borosilicate glass capillaries with 1 mm outer and 0.5 mm inner diameter (Hilgenberg, Malsfeld, Germany). The resulting glass microneedles were forged on a home built microforge (Zhelev et al., 1994). Obtained micropipettes had an inner diameter of 1.7–2.0 μm which was determined to an accuracy of 0.1 μm by measuring the maximum insertion depth of a conical glass needle that had been sputtered with gold and calibrated using a scanning electron microscope.

The force measurements were performed in a 3 mm high glass chamber with a total volume of 2 ml that was open on both sides for easy pipette access. The chamber was filled with measurement buffer containing a small amount of biotinylated erythrocytes and a few beads of both types. The chamber was cooled to 10°C by circulating thermostated water to minimize water evaporation and thereby changes in pressure and osmolarity.

Micropipettes filled with measurement buffer held the test bead and were hydraulically connected to a syringe for aspiration. The pipette for the BFP was connected to an accurate hydraulic pressure control system described in (Dieluweit et al., 2010). One micropipette was mounted on a water hydraulic micromanipulator (MHW-3, Narishige), the other on a mechanical three axis translation stage (M461-XYZ-M; Newport, Irvine, CA). The motion of the mechanical manipulator along the common axis of both pipettes was controlled by a piezoelectric micro-meter (Piezomike P-854, Physik Instrumente, Waldbronn,

1Erythrocytes were washed by 1 min centrifugation at 300 g and removal of the supernatant.
Data analysis

The force at bond rupture, here called yield force, was calculated from the maximum deflection of the probe bead during bond loading. The deflection was determined as the difference between the probe bead rest position and its recorded maximum position during piezo retraction (Figure 2). Maximum positions were extrapolated with half the piezo displacement between subsequent data points to avoid biasing due to the discreteness of data points. In order to remove the influence of membrane relaxation and to average out Brownian fluctuations, the rest position was calculated as the median of positions obtained in a 1 s time interval starting 0.5 s after bond rupture. An event was accepted as a binding event (99% confidence level) only if the maximum deflection exceeded three times the standard deviation $\delta$ of bead positions during this time interval. The standard deviation criterion effectively set a lower force limit of approximately 5 pN. Impingement forces were calculated from the backward probe deflection recorded during the 1 s long bead contact. In 91% of all registered contacts the beads were squeezed together with a force within the target interval 10–50 pN. In all measurements, the BFP spring constant was fixed at 200 pN/\(\mu\)m and the pulling rate (piezo speed) at 1.65 \(\mu\)m/s, which produced a force loading rate of 330 pN/s. Due to the elasticity of linkers and proteins, however, actual force loading rates were lower and varied from measurement to measurement with a standard deviation between 10 and 20%. The actual force loading rates were estimated from the slope of the rising flank in the tracking curve prior to a rupture.

In the result section, each complete measurement series is presented as a force histogram and the corresponding cumulative distribution function $\Phi(y)$:

$$\Phi_Y(y) = \frac{1}{N} \sum_{i=1}^{N} \theta(y - f_i)$$

where $N$ presents the total number of detected bonds, $f_i$ the set of measured yield forces, and $\theta$ is the Heaviside step function. The distribution functions were collectively cut-off at $F = 5$ pN to comply with the detection limitations set by the probe. In comparison to force histograms, distribution functions have the advantage that they do not rely on an (arbitrary) choice of a bin size. For the force histograms, the bin size was set to 10 pN. The median force was calculated for each spectrum.

For each measurement series we employed several bead combinations (on average 15). The overall standard deviation of the binding frequency was below 6% points, except for one case when it amounted to 8% points (the 5 mM Mn\(^{2+}\), 2 mM Mg\(^{2+}\) curve in Figures 3 and 4).

RESULTS

The regulatory role of Mn\(^{2+}\) and Mg\(^{2+}\) on the \(\alpha_3 \beta_1\) integrin–invasin interaction was tested under different experimental conditions, summarized in Table 1. The results are detailed in the following sections.

Influence of divalent cations on multiple bonds

We measured binding frequencies and yield forces for interactions between invasin and integrin under six different ionic conditions, determined by different concentrations of Mn\(^{2+}\) and Mg\(^{2+}\), in a measurement buffer without free invasin (Table 1). In the absence of free invasin, which would compete with bead-bound invasin for binding sites and thus reduce the number of available receptors (cf. next subsection), several integrin molecules from the test bead are expected to interact with invasin molecules on the probe bead, leading to the formation of multiple bonds. Detailed theoretical analyses of the force induced rupture of multiple bonds (Seifert, 2000, 2002; Erdmann and Schwarz, 2004) have shown that the scaling of rupture forces with the number of bonds depends on the details of the experiment and may vary from linear to logarithmic. Therefore an exact number of bonds sharing the load cannot be given.
The results are depicted in Figure 3 as histograms and also as cumulative distributions. In the absence of Mn$^{2+}$, an increase in the concentration of Mg$^{2+}$ leads to higher yield forces and binding frequency. This is in accordance with previous equilibrium studies (Mould et al., 1995) that indicated the necessity of magnesium ions for integrin function. A similar behavior was found for manganese ions, the concentration of which was varied at 0 mM Mg$^{2+}$ and at 2 mM Mg$^{2+}$. In both cases binding frequency and yield force were found to increase with Mn$^{2+}$ concentration. Interestingly, we observed significantly higher yield forces and binding frequencies in the simultaneous presence of both ions in a relatively low concentration [1 mM Mn$^{2+}$; 2 mM Mg$^{2+}$], as compared to the case when only one of these two ions was present at a time in higher concentration [0 mM Mn$^{2+}$; 5 mM Mg$^{2+}$] or [5 mM Mn$^{2+}$; 0 mM Mg$^{2+}$]. The highest force median (122 pN) and the highest binding frequency (75%) and thus the most favorable ionic conditions for ligand binding in our buffer system were observed when the buffer was containing 5 mM Mn$^{2+}$ and 2 mM Mg$^{2+}$. Thus, manganese ions were not only substituting for magnesium, but had a yield force-increasing effect on the $\alpha_7\beta_1$ integrin–invasin interaction. This shows that both ions act in a synergistic manner.

Figure 3. Yield force distributions measured for the $\alpha_7\beta_1$ integrin–invasin interaction in the absence of free invasin in the measurement buffer. The six series correspond to different concentrations of Mn$^{2+}$ and Mg$^{2+}$ within the buffer. (a): Histogram representation of the force distributions. Binding frequencies are printed beneath series labels, and force medians are shown as vertical bars. The histograms represent (from above) 263, 704, 234, 401, 489, and 373 individual rupture events. (b): Same data as in (a) but shown as cumulative distributions.

Figure 4. Yield force distributions measured for the $\alpha_7\beta_1$ integrin–invasin interaction at six different concentrations of free invasin in the measurement buffer. The divalent cation concentration was kept fixed at 5 mM Mn$^{2+}$ and 2 mM Mg$^{2+}$. (a): Histogram representation of the force distributions. The labels to the right designate the concentration of free invasin in the buffer and the binding frequency observed. The force median is represented by a vertical bar and a value next to it. The histograms represent (from above) 373, 293, 331, 275, 208, and 88 individual rupture events, respectively. (b): Same data as in (a) but shown as cumulative distributions.
Different concentrations of Mn2
and 341 individual rupture events. (b): Same data as in (a) but shown as cumulative distributions.

The results of the experiments are displayed in Figure 4. The median yield force in the absence of free invasin under these specific ionic conditions was found to be 122 pN. The addition of free invasin at low concentrations (3–6 nM) resulted in reduced yield forces as multiple bonds were suppressed. At higher concentrations (9–12 nM), the median yield force remained constant (28 pN) and any drop in binding frequency could be observed (40% at 9 nM; 25% at 12 nM free invasin). Accordingly, the single bond regime must have been reached at 9 nM invasin. A control study (last distribution in the Figure 5) gave a similar result with only a slight change of force median (26 pN vs. 28 pN) and binding frequency (42% vs. 40%). We thus conclude that for a measurement buffer containing 5 mM Mn2
 and 2 mM Mg2
, single bonds dominate the measured force spectra already at a free invasin concentration of 9 nM. These conditions were consequently adopted in the subsequent measurements of this study.

Further blocking with 2 μM free invasin, a concentration which should suffice to saturate close to all integrin receptors, enabled us to determine the background of weak, non-specific interactions at 5 mM Mn2
 and 2 mM Mg2
. We found a median force 14 pN and a binding frequency of 8%. The experiment was repeated at the same invasin concentration (2 μM) but at unfavorable ion conditions [0 mM Mn2
; 2 mM Mg2
]. In this case we observed (Figure 6) a significantly higher force median of 25 pN and binding frequency of 29%. Apparently, due to low integrin activity under these conditions, an efficient uptake of free invasin from the buffer, and thus an efficient blocking, was hampered. For the same reason, namely low receptor activity, the binding of bead-bound invasin to integrins was impaired.

### Table 1. Synopsis of median rupture forces. At 2 μM invasin concentrations, mostly non-specific interactions are probed, at 9 nM few to single (at 2 mM Mg2
 and 5 mM Mn2
) bonds and without invasin multiple bonds

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 (mM) | 2 μM | 9 nM | 0 nM |
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### Single bond regime

To reach the single bond regime, in which interbead attachments are mediated by one single \( \alpha_5\beta_1 \) integrin–invasin bond, we used a well-established strategy of reducing the number of available binding sites by adding free invasin as a receptor blocking agent (Strigl et al., 1999).

We explored the influence of free invasin in a buffer containing 5 mM Mn2
 and 2 mM Mg2
 because under these conditions integrins appeared most active (cf. previous subsection). The results of the experiments are displayed in Figure 4. The median yield force in the absence of free invasin under these specific ionic conditions was found to be 122 pN. The addition of free invasin at low concentrations (3–6 nM) resulted in reduced yield forces as multiple bonds were suppressed. At higher concentrations (9–12 nM), the median yield force remained constant (28 pN) and only a drop in binding frequency could be observed (40% at 9 nM; 25% at 12 nM free invasin). Accordingly, the single bond regime must have been reached at 9 nM invasin. A control study with a different batch of beads under the same ionic conditions (last distribution in the Figure 5) gave a similar result with only a slight change of force median (26 pN vs. 28 pN) and binding frequency (42% vs. 40%). We thus conclude that for a measurement buffer containing 5 mM Mn2
 and 2 mM Mg2
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Further blocking with 2 μM free invasin, a concentration which should suffice to saturate close to all integrin receptors, enabled us to determine the background of weak, non-specific interactions at 5 mM Mn2
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]. In this case we observed (Figure 6) a significantly higher force median of 25 pN and binding frequency of 29%. Apparently, due to low integrin activity under these conditions, an efficient uptake of free invasin from the buffer, and thus an efficient blocking, was hampered. For the same reason, namely low receptor activity, the binding of bead-bound invasin to integrins was impaired.

### Influence of divalent cations in the presence of 9 nM free invasin

The measurement series of Figure 3, except for the one conducted in the complete absence of divalent ions, were repeated in the presence of 9 nM invasin, see Figure 5. Again, Mn2
 ions were found to increase the yield forces in a dose-dependent manner. In contrast to the measurements conducted in the absence of free invasin, yield forces decreased at increased concentration of Mn2
 ions whenever the blocking agent was present. This effect was enhanced by a concomitant presence of Mn2
 and Mg2
 (Table 1). The lowest force median (26 pN) was reached at 5 mM Mn2
 and 2 mM Mg2
, i.e., exactly the conditions which were identified as the most favorable for ligand binding in the absence of free invasin (Figure 3). Evidently at these conditions integrins bound free invasin from the buffer most effectively, thereby maximally reducing the number of available binding sites for inter-bead connections.

![Figure 5. Yield force distributions measured for the \( \alpha_5\beta_1 \) integrin–invasin interaction in the presence of 9 nM free invasin. The five series correspond to different concentrations of Mn2
 and Mg2
 in the measurement buffer. (a): The histogram representations are based on (from above) 415, 344, 553, 350, and 341 individual rupture events. (b): Same data as in (a) but shown as cumulative distributions.](wileyonlinelibrary.com/journal/jmr)
The prefactor, reaction coordinate of the reaction (Evans and Ritchie, 1997). Between the bound state and the transition state along the respectively. (a): Histogram representation. (b): Cumulative distributions. In order to obtain a better understanding of the observed influence of Mn$^{2+}$ and Mg$^{2+}$ on the integrin–invasin bond strength, the applied blocking scheme is investigated here in more detail. Based on a simple chemical equilibrium, the fraction, θ, of surface bound integrin molecules not occupied by free invasin, i.e., the fraction of integrin molecules available for inter-bead attachments, is given by:

$$\theta = (1 + k_0 c)^{-1}. \tag{2}$$

Here $k_0$ denotes the dissociation constant of the integrin–invasin bond and $c$ the concentration of free invasin in the buffer. From equilibrium studies on integrins, e.g., Mould et al. (1995), we expect $k_0$ to increase along the conditions as listed from top to bottom in the legend of Figures 3 and 5. In addition, we would simultaneously expect a variation in the strength of single bonds.

From the theory of chemical reaction kinetics it was theoretically deduced (Bell, 1978) and later shown experimentally (Alon et al., 1995; Merkel et al., 1999) that the dissociation rate, $k$, of a single bond is exponentially enhanced by the application of a force, $F$.

$$k(F) = k_0 \exp \left( \frac{F}{F_0} \right) \tag{3}$$

$F_0$ denotes the characteristic force of the bond which is given by the ratio of thermal energy, $k_B T$, and the distance between the bound state and the transition state along the reaction coordinate of the reaction (Evans and Ritchie, 1997). The prefactor, $k_0$, denotes the dissociation rate of an unloaded bond between both beads. The latter rate is proportional to – but substantially higher than – the dissociation rate of an invasin–integrin complex where one of the binding partners is not connected to a bead (Nguyen-Duong et al., 2003).

From Equation (3) and straightforward assumptions on the kinetic process, the median rupture force of the bond system can be calculated to be (Evans and Ritchie, 1997; Strigl et al., 1999):

$$F_m = F_0 \ln \left( \frac{\ln 2}{k_0 F_0} \right). \tag{4}$$

where $f$ is the force loading rate. Most likely the on-reaction barely depends on divalent ions. Therefore, we expect $k_0$ to be proportional to $k_0^{-1}$ and consequently the yield forces of single bonds to increase with increasing $k_0$. This effect contributes to the increasing yield forces for invasin–integrin bonds without free invasin in the buffer, as seen in Figure 3. Moreover, a higher bond affinity will also result in a higher number of bonds formed in inter-bead attachments which further enhances the yield force increase as compared to the single bond case.

Interestingly, the increase in $k_0$ with divalent ion concentration does also explain the inverted effects of Mn$^{2+}$ and the combination of Mn$^{2+}$ and Mg$^{2+}$ ions on yield force distributions obtained in the presence of 9 nM free buffer invasin (Figure 5 compared with Figure 3). In this case, an increasing $k_0$ primarily results in a higher fraction of invasin-occupied integrin molecules on test beads, cf. Equation (2), and consequently in a lower density of binding sites that are available for inter-bead attachments. Therefore, whenever free invasin is available for the system, the average number of bonds connecting the two beads decreases when the ionic conditions for ligand binding become more favorable. This effect is much stronger than the simultaneous increase of yield force of each single bond because the number density varies algebraically with $k_0$ while the yield force of individual bonds only increases logarithmically.

The line of reasoning presented above explains the apparently opposing effects of Mn$^{2+}$ and Mg$^{2+}$ ions observed in the presence and in the absence of free invasin in the buffer; cf. Figures 3 and 5 and Table 1. The first distribution of Figure 5 (0 mM Mn$^{2+}$, 2 mM Mg$^{2+}$), however, seems not to fall within this argumentation. To investigate this further, we measured the spectrum at identical ion concentrations, but at a much higher invasin concentration of 2 μM (Figure 6). This measurement resulted in higher yield forces and a higher binding frequency than the values obtained from a parallel measurement at ion concentrations favorable for integrin activity (5 mM Mn$^{2+}$; 2 mM Mg$^{2+}$). In view of the fact that divalent ions are mandatory for correct integrin function, we explain these findings as follows: At high concentrations of divalent ions, especially Mn$^{2+}$, the integrin assumes a conformation that is favorable for binding. Therefore invasin present in a concentration of 2 μM will efficiently bind to integrin and saturate practically all available binding sites on the test bead. In effect, this leads to low binding frequencies and low

![Figure 6](image-url). Yield force distributions measured for the α$\beta_1$ integrin–invasin interaction under full blocking conditions (2 μM free buffer invasin) and a fixed Mg$^{2+}$ concentration of 2 mM. The series correspond to two different concentrations of Mn$^{2+}$, 0 and 5 mM, and comprise 251 and 103 rupture events, respectively. (a): Histogram representation. (b): Cumulative distributions.
yield forces when the system is then explored by a bond rupture experiment; the observations are representing the non-specific background of bead–bead interactions. However, in the complete absence or at low concentrations of divalent ions, the integrin is likely to assume an altered conformation with much less affinity for invasin. If this is indeed the case, only few free invasin molecules from the buffer will bind to the integrin covered beads at low ion concentrations. The resulting increase in binding frequency and rupture force is likely caused by a residual low-affinity interaction between integrin and invasin. For this proposed low affinity bond, 2 μM invasin might not be sufficient to block all binding sites. Thus, upon bead encounter, weak bonds can be formed resulting in frequent attachments of moderate mechanical strength.

As the initial and essential part of pathogenesis, invasin-bearing enteropathogenic *Yersinia* bacteria attach to the integrins of eukaryotic host cell. Consequently, the bacterium grasps more and more integrins that are located in the plasma membrane of the host cells. This leads eventually to engulfment into vesicles. The wrapping of the pathogens with the host cell membrane requires the deformation of the cell membrane and the formation of lamellipodia in an array of a phagocytic cup, which requires mechanical forces to overcome membrane bending and cell surface tension (Nambari *et al*., 2010). These forces are mediated by the invasin–integrin interactions. In fact, bacteria with mutations of invasin, which decrease the affinities to integrins, are much less likely to be taken up by eukaryotic host cells, despite their extracellular attachment to the target cells (Isberg and Barnes, 2001 and references therein). The exertion of mechanical forces between the bacteria and the host cell membrane mediated by invasin and integrins is an essential step of the pathogenesis of enteropathogenic *Yersinia* bacteria and therefore a valid target to prevent infection. Our studies, conducted at the molecular level of the integrin–invasin interaction, lay the foundation to analyze the force exertion at the cellular and biological level. Moreover, they establish an assay system to test for compounds which interfere in this pathologically relevant force exertion process.

**CONCLUSIONS**

Taken together our data clearly demonstrate that divalent ions enhance the binding affinity of α3β1 integrin–invasin bonds and that manganese and magnesium ions act in a synergistic way. These effects have been well studied on whole cells. Our results show that their origins are directly related to the integrin molecule and the modulation of its bond to the interaction partner by the presence of bivalent cations. We also observe clear indications of a low affinity bond at very low concentrations of divalent ions. Our values for the interaction of an integrin with its ligand are in the force range of other integrin–ligand interactions (reviewed and listed in Weisel *et al*., 2003) and highlight the distinct roles of Mn2+ and Mg2+ for the mechanical strength of integrin–invasin bonds.

**Acknowledgements**

We thank A. Fischbach, W. Hürttlen, N. Kirchgessner and S. Houben for programming the bead tracking software, U. Linke and S. Dieluweit for gold sputtering and scanning electron microscopy of microneedles and W. Rubner for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft via the grant ME1458/3 (R.M.) and the grant SFB815 A6 (J.A.E.). K.B. benefited from grants via the Graduate School of Molecular Biophysics (Danish National Research Foundation), BioNET (Vilum Kann Rasmussen Foundation) and the Faculty of Sciences at SDU. The SDU laboratory is sponsored by a grant to MEMPHYS – Center for Biomembrane Physics (Danish National Research Foundation).

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