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Integrin $\alpha_v\beta_8$ Adopts a High Affinity State for Soluble Ligands Under Physiological Conditions

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ABSTRACT

It has been proposed that integrins adopt a low affinity conformation under physiological conditions. Integrin can either be activated through cytoplasm or by binding of cations such as Mn^{2+} to the head domain. The cytoplasmic activation pathway, that is, inside-out signaling has been regarded as the physiological pathway for integrin activation. Integrin β_8 is important for neuron vascular development. However, due to the highly divergent cytoplasmic domain, this integrin probably does not rely on inside-out signaling for affinity regulation. We therefore hypothesized that the β_8 integrin uniquely assumes a constitutively high affinity state under physiological conditions. We discovered that β_8 indeed exhibited high binding to soluble vitronectin in the presence of Ca^{2+} and the ligand binding could not be further enhanced by addition of Mn^{2+} . The lower ectodomain stalk of the integrin, which is comprised by the integrin epidermal growth factor-like (I-EGF) domains and β TD domain, is critical for this high affinity conformation. In addition, we found that unlike other integrins, Mg^{2+} at low concentration inhibited β_8 ligand binding. Mutagenesis studies indicated that β_8 integrin possesses a unique cation binding site which might contribute to the ligand binding affinity. Our study showed that both the β_8 lower ectodomain stalk and the head domain play an important role in its high affinity state under physiological conditions. *J. Cell. Biochem.* 118: 2044–2052, 2017. © 2016 Wiley Periodicals, Inc.

KEY WORDS: INTEGRIN; HIGH AFFINITY CONFORMATION; SIGNALING; METAL ION BINDING; SITE AFFINITY REGULATION

Integrins are α - β heterodimeric cell surface adhesive receptors that play important roles in many biological functions including cell migration, proliferation, differentiation, and survival [Hynes, 2002]. At least twenty-four integrins, which are composed of eighteen α and eight β subunits, have been identified in vertebrates [Humphries et al., 2006]. Different integrins have diverse functions due to distinct submembrane linkers to cytoskeleton and intracellular proteins as well as different ligand-binding specificities and affinities. Integrin $\alpha_v\beta_8$, is distinctive among the integrins. This integrin is almost exclusively expressed in the central and peripheral nervous systems and placenta. The β_8 integrin expression is correlated with central nervous system (CNS) development [Chernousov and Carey, 2003]. $\alpha_v\beta_8$ also regulates TGF- β 1 activation and uniquely aids in the repair of neural cells, especially Schwann cells, by binding to ECM ligands and inducing growth, differentiation, and healing. The integrin is essential for embryonic development as β_8 knock-out mice exhibited embryonic or perinatal lethality with abnormal vascular development [Zhu et al., 2002; Proctor et al., 2005]. Lack of interaction between latent TGF- β 1 and $\alpha_v\beta_8$, which is required for TGF- β 1 activation, has been proposed to be the cause of these defects [Mobley et al., 2009]. Despite its

interactions with vitronectin, the β_8 integrin was reported as a non-adhesive receptor [Nishimura et al., 1994]. Later studies found that the β_8 cytoplasmic domain is completely different from other α_v partners, including β_1 , β_3 , β_5 , and β_6 , which may explain why this integrin does not mediate cell adhesion like its siblings.

Integrins are considered as special adhesive receptors because their adhesiveness can be dynamically regulated through a process termed inside-out signaling. On the cell surface, integrins normally adopt a low affinity state, which can be switched into high affinity state through inside-out signaling. Binding of the integrin extracellular ligands in turn transmits signals inward, a process called outside-in signaling, which exerts significant influences on cell mobility, proliferation, and differentiation, etc. [Ginsberg et al., 2005]. It has been shown that many integrins, including the β_3 integrin, can transmit these bidirectional signals across the plasma membrane. However, the β_8 cytoplasmic domain lacks all the protein-protein interaction sites that have been identified on the β_3 cytoplasmic domain and shows little homology to other α_v pairing partners. This fact implicates that either the β_8 integrin does not rely on inside-out signaling as other integrins or it uses a completely different set of machinery for cytosolic activation.

Conflict of interest: The authors declare that they have no conflict of interest with the contents of this article.

Authors' contributions: PH designed, performed, and analyzed the experiments and wrote the manuscript. BHL conceived the study, designed, and analyzed the experiments, and wrote the manuscript.

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Likewise, the integrin probably mediates a distinct outside-in signaling.

It has been proposed that integrin affinity is regulated through global conformational changes of the integrin extracellular domains [Luo and Springer, 2006; Luo et al., 2007]. Many studies suggest that integrins exist on the cell surface as three different conformational states: bent, extended-closed, and extended-open. In the crystal structures of the full length ectodomains of $\alpha_v\beta_3$ [Xiong et al., 2001, 2002, 2009; Dong et al., 2012], $\alpha_{IIb}\beta_3$ [Zhu et al., 2008], and $\alpha_X\beta_2$ [Xie et al., 2010], integrins are all in the bent conformation with the α - and β -subunit C-termini only a few angstroms apart, consistent with association of the α - and β -subunit transmembrane (TM) domains. It was hypothesized that this bent conformation represents a resting, low affinity state, and inside-out activating signals induce separation of the integrin TM and cytoplasmic domains followed by separation of two ectodomain lower stalks and extension of these two legs [Wang and Luo, 2010; Ye et al., 2011; Hu and Luo, 2013]. Integrin extension then results in swing-out of the β -subunit hybrid domain, which causes the downward movement of the $\alpha 7$ helix of the β -I domain, leading to exposure of the ligands binding site and high affinity for soluble ligand binding [Luo et al., 2007]. Even though the activating model has been supported by numerous experimental data, it is still unclear whether every integrin assumes a bent conformation with low affinity for ligands under physiological conditions and goes through activation during integrin signaling. Based on previous studies [Mu et al., 2002], we proposed that the β_8 integrin might be an exception, which could adopt a conformation with high affinity for soluble ligands under physiological conditions.

In addition to global conformational changes, cations also play a critical role in regulating integrin affinity by interacting with the three ion binding sites [Luo et al., 2007; Campbell and Humphries, 2011]. The metal ion dependent adhesion site (MIDAS) sits in the middle and is flanked by adjacent to MIDAS (ADMIDAS) and synergistic metal ion binding site (SyMBS) [Campbell and Humphries, 2011]. The MIDAS functions as a key interacting site for integrin ligands as the cation bound in the site coordinates binding of the ligands to integrin through interacting the carboxyl group of the ligands. In contrast, ADMIDAS has been reported as a negative regulatory site for ligand-integrin interaction. The inhibitory effect of Ca^{2+} occupancy in ADMIDAS has been observed in various integrins including β_7 [Chen et al., 2003], β_1 [Mould et al., 2003; Valdramidou et al., 2008], and β_3 [Pesho et al., 2006]. ADMIDAS mutations increased affinity in ligand binding for rolling-mediating integrins and inhibited ligand binding in integrins that only mediate firm adhesion [Raborn et al., 2011; Zhang and Chen, 2012]. These conflicting results could be caused by a single residue difference located in MIDAS, implicating a critical role of the ion binding sites in diverse functions of integrins [Raborn and Luo, 2012; Raborn et al., 2013]. Among the ions that interact with the β I domain, Mg^{2+} is known to bind to MIDAS [Zhu et al., 2008] whereas Ca^{2+} can bind to all the three sites but with higher affinity toward SyMBS and ADMIDAS [Chen et al., 2003; Pesho et al., 2006].

In this study, we expressed the $\alpha_v\beta_8$ integrin in HEK293T cells, and showed that this integrin bound soluble ligands much better than the β_3 integrin under physiological conditions. Follow up studies indicated that the lower leg of β_8 integrin is crucial in this

constitutively high ligand binding. Furthermore, we discovered that unlike most other integrins, Mg^{2+} in the low concentration inhibited binding of the integrin to its natural ligand vitronectin. Alignment and mutagenesis study unveiled a distinct ADMIDAS on β_8 integrin, which contributes to the inherent high affinity for soluble ligands of the integrin. Our study suggested that both the lower stalk and cation binding sites on the integrin head are important for the high affinity conformation of the β_8 integrin.

EXPERIMENTAL PROCEDURES

CELLS AND PLASMIDS

HEK293T cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 1% Non-Essential Amino Acids, 1% L-Glutamine, 1% Sodium Pyruvate, and 1% Penicillin-Streptomycin (all from Life Technologies). Plasmids containing wild-type human integrin α_v and β_3 were described previously [Takagi et al., 2002]. Wild-type β_8 integrin was generously gifted by Dr. Nishimura [Nishimura et al., 1994]. All of the chimera constructs were created via overlap PCR and the mutants were created by QuikChange kit (Stratagene, La Jolla, CA).

CHIMERIC INTEGRIN CONSTRUCTION

The integrin chimeras were generated by replacing different regions of β_3 with the corresponding regions on β_8 . The chimera $\beta_3\beta_8\text{Leg}$ was constructed by replacing the β_3 residues 461–788 with β_8 468–769 (whole lower leg swapping). Briefly, the DNA fragment coding the β_3 integrin head domain (PSI, β I, and hybrid domains) was produced by using a forward primer: AGACTAGTACCATGC-GAGCGCGGCCGCG and an overlapping reverse primer which contains part of the β_8 EGF1 domain: GTCCTCTGTGTCCTCA-CACTGACAGTCACAATCAAAGGTGACCTGGACG. Likewise, the leg domain fragment of β_8 was created using an overlapping forward primer which contains part of the β_3 hybrid domain: CGTCCAGGT-CACCTTTGATTGTGACTGTGAGTGTGAGGACAACAGAGGAC and a reverse primer of β_8 : GGATATCTTAGAAGTTGCACCTGAAAGTTTC. The PCR reaction was performed by using Phusion[®] high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA). The two fragments were then fused together and amplified by adding both the forward primer of β_3 and the reverse primer of β_8 . The chimeric cDNA was then purified and subcloned into pCDNA3.1 vector. Similarly, the $\beta_3\beta_8$ TM-Cyt chimera was created by replacing β_3 717–788 with β_8 680–769 using following primers: TGATGAAAA-TATTCTCAAGTAGCTAGGGCCCTTGGGACACTCTGGCTCT and AGA GCCAGAGTGTCCCAAGGGCCCTAGCTACTTGAGAATATTTTCAT CA. The $\beta_3\beta_8\text{EGF-}\beta\text{Td}$ chimera was created by replacing β_3 461–717 with β_8 468–680 and following primers were used: CTGAGAGCAGGACCAGGATGTCTGGGCTGGAGAAACATTCT-GAAGTT and AACTTCAGAATGTTTCTCAGCCCAGACATCCTGGTG GTCCTGCTCTCAG.

TRANSIENT TRANSFECTION

Constructs were transiently transfected into HEK293T cells using X-tremegene 9 transfection kit (Roche Diagnostics, Indianapolis, IN).

The expression levels of the wild-type integrins, the chimeras, and the mutants were determined by flow cytometry staining with monoclonal antibody AP3 (anti- β_3 mAb, American Type Culture Collection), MAb1980 (anti- α_v mAb, EMD Millipore Corporation, Billerica, MA), and 37E1 (anti- β_8 mAb, a kind gift from Dr. Nishimura [Nishimura et al., 1994]).

SOLUBLE LIGAND BINDING ASSAY

Soluble ligand binding of Alexa Fluor 488-labeled human vitronectin and fibronectin (both from Sigma-Aldrich, St. Louis, MO) was determined as previously described [Wang et al., 2010]. Briefly, transfected cells were suspended in 20 mM HEPES-buffered saline (pH 7.4) (HBS) supplemented with 5.5 mM glucose and 1% BSA were pre-blocked with 1 μ g anti- β_1 antibody (EMD Millipore Corporation) on ice for 30 min. Cells were then incubated with 20 μ g/mL labeled vitronectin or fibronectin at room temperature in the presence of different ion conditions. For vitronectin binding, cells were then stained with MAb1980 on ice for 30 min and labeled with dylight 633 conjugated anti-mouse IgG antibody (Thermo Scientific Pierce, Rockford, IL). For fibronectin binding, cells were incubated with Alexa Fluor 543-labeled AP3 on ice for 30 min. Binding activity was presented as the percentage of the mean fluorescence intensity (MFI) of ligands staining relative to the MFI of MAb1980 or AP3 staining.

RESULTS

EXPRESSION AND SOLUBLE VITRONECTIN BINDING OF INTEGRIN $\alpha_v\beta_8$ ON THE CELL SURFACE

It has been proposed that integrins normally assume a bent conformation with low affinity for ligands on the cell surface whereas extracellular stimuli activate integrin from cytosol and induce extension with high affinity toward soluble ligands [Luo et al., 2007; Shattil et al., 2010; Wang and Luo, 2010; Kim et al., 2011; Hu and Luo, 2013]. However, based on the fact that there is no binding site on the β_8 cytoplasmic domain for the inside-out activators, we proposed that $\alpha_v\beta_8$ integrin may adopt a different conformation with high affinity to its ligands under physiological conditions. To verify our hypothesis, we first expressed integrins $\alpha_v\beta_3$ and $\alpha_v\beta_8$ in the 293T cells, and used antibody staining to confirm their expression on the cell surface. As shown in Figure 1A, α_v -specific MAb1980 bound both transfectants well. The anti- β_3 mAb AP3 bound to 293T cells expressing $\alpha_v\beta_3$ only, but not $\alpha_v\beta_8$ and control cells, confirming previous observation that there was no endogenous $\alpha_v\beta_3$ expression in 293T cells. By contrast, the anti- β_8 mAb 37E1 bound cells transfected with $\alpha_v\beta_8$, indicating efficient expression and proper folding of the integrin on the cell surface (Fig. 1A).

To determine the ligand binding capacity, soluble ligand binding assay was performed using Alexa-488 labeled recombinant human vitronectin (Fig. 1B). As expected, $\alpha_v\beta_3$ bound little soluble vitronectin in the presence of Ca^{2+} , whereas the presence of Mn^{2+} increased the binding significantly. By contrast, integrin $\alpha_v\beta_8$ bound soluble vitronectin in the presence of Ca^{2+} at maximal level, even better than that of $\alpha_v\beta_3$ in Mn^{2+} . Addition of Mn^{2+} did not further increase binding ability, suggesting that most population of this

integrin has already adopted a high affinity state under physiological conditions.

We noticed that in a recent publication, a purified disulfide-linked extracellular fragment of $\alpha_v\beta_8$ integrin expressed marginal affinity for vitronectin [Ozawa et al., 2016] which is inconsistent with our observation and the previous report [Nishimura et al., 1994]. We believe that this contradictory may be caused by the artificially introduced disulfide-bond which restricted separation of the β_8 leg from the α_v subunit, leading to a structural alternation on the ligand binding head domain.

THE ECTODOMAIN LOWER STALK OF β_8 IS IMPORTANT FOR THE HIGH AFFINITY STATE

This high affinity of β_8 integrin could be caused by the ligand binding head domain or the lower leg or by both. We therefore created a chimera swapping the β_8 lower leg with that of the β_3 to determine whether this region plays important role in the constitutively high affinity state. The chimera showed about 30% lower binding in comparison to the wild-type β_8 , indicating this region's critical role in maintaining high affinity conformation (Fig. 1C).

The lower leg of β integrin is composed by four EGF-like domains (I-EGF1-4), a β TD domain, a TM domain, and a short cytoplasmic tail. We created $\beta_3\beta_8$ chimeras in which the whole β_3 lower leg or only the TM-cytoplasmic domains were swapped with the corresponding β_8 domains (Fig. 2A). The inherent low affinity of β_3 integrin at resting state can provide a better sensitivity in studying the roles of β_8 lower leg domains. Soluble ligand binding assay was performed to test ligand binding ability of the chimeras (Fig. 2B). We used human fibronectin labeled with Alexa-488 instead of vitronectin because fibronectin is more affordable, well studied, and more importantly, unlike vitronectin as shown in Figure 1, the basal binding of integrin $\alpha_v\beta_3$ to soluble fibronectin is very low under the physiological Ca^{2+} condition. Therefore, fibronectin is more sensitive to identify potential change of the ligand binding. As expected, the wild-type $\alpha_v\beta_3$ bound soluble fibronectin poorly in the presence of Ca^{2+} , whereas the addition of Mn^{2+} significantly increased the binding capability. By contrast, the chimera containing the whole β_8 lower leg ($\beta_3\beta_8\text{Leg}$) bound soluble fibronectin with much higher capability in the presence of Ca^{2+} , comparable to the wild-type $\alpha_v\beta_3$ in Mn^{2+} (Fig. 2B), implicating a potent activating effect of the region. In contrast, the chimera with the β_8 TM and cytoplasmic domains ($\beta_3\beta_8\text{TM-Cyt}$) showed only moderately increased binding to soluble fibronectin under Ca^{2+} condition. The results suggested a critical role of the β_8 lower ectodomain stalk, which consists of four EGF domains, in high ligand binding affinity. Indeed, swapping in only the lower stalk ($\beta_3\beta_8\text{EGF-}\beta\text{TD}$) was sufficient to promote ligand binding to a similar level as the whole leg swapping chimera (Fig. 2C). We also noticed that although both $\beta_3\beta_8\text{Leg}$ and $\beta_3\beta_8\text{EGF-}\beta\text{TD}$ expressed high affinity for soluble ligands, the binding could be further enhanced by addition of Mn^{2+} . This suggests that swapping in the β_8 lower stalk may shift the conformation equilibrium toward high affinity state, whereas Mn^{2+} activated the remaining integrin population that resides in low affinity state. Taking together, these discoveries strongly indicated that the β_8

Fig. 1. Expression and soluble ligand binding of integrin β_8 and $\beta_8\beta_3$ chimera. A. HEK293T cells were transfected with either $\alpha_v\beta_3$ or $\alpha_v\beta_8$ and stained with MAb1980 (anti- α_v antibody), AP3 (anti- β_3 antibody), and 37E1 (anti- β_8 antibody), respectively. Bold-solid line: untransfected HEK293T cells; thin-dashed line: transfected cells. B. Binding of Alexa A488 labeled vitronectin to HEK293T cells transiently transfected with either $\alpha_v\beta_3$ or $\alpha_v\beta_8$ in the presence of EDTA (5 mM), Ca^{2+} (5 mM), or Mn^{2+} (1 mM) as indicated. C. HEK293T cells were transiently transfected with the chimera containing a β_8 ligand binding head domain and a full length β_3 lower leg ($\beta_8\beta_3\text{Leg}$) and tested for Alexa A488 conjugated vitronectin binding together with wild-type β_3 and β_8 in the presence of EDTA (5 mM), Ca^{2+} (5 mM), or Mn^{2+} (1 mM) as indicated. Error bars represent standard deviation (S.D.) from at least three independent assays.

Fig. 2. The $\beta_3\beta_8$ chimera carrying the β_8 ectodomain lower stalk showed high ligand binding. **A.** The $\beta_3\beta_8$ chimeras were created by swapping different β_3 domains with the corresponding β_8 domains. $\beta_3\beta_8$ Leg: the construct contains the β_3 head domain with the β_8 ectodomain lower stalk (I-EGF domains and β TD domain) and the β_8 TM-Cytoplasmic domain. $\beta_3\beta_8$ EGF- β TD: the construct contains the chimeric β_3 integrin which carries the β_8 ectodomain lower stalk. $\beta_3\beta_8$ TM-Cyt: the construct contains the β_3 integrin ectodomain with the β_8 TM-Cytoplasmic domain. **B.** Binding of Alexa A488 labeled fibronectin to HEK293T cells that were transiently transfected with the wild-type β_3 or different $\beta_3\beta_8$ chimeras in the presence of EDTA (5 mM), Ca^{2+} (5 mM), or Mn^{2+} (1 mM) as indicated. The $\beta_3\beta_8$ Leg chimera showed much higher ligand binding ability by comparing to the wild-type and $\beta_3\beta_8$ TM-Cyt. **C.** Binding of Alexa A488 labeled fibronectin to HEK293T cells that were transiently transfected with different $\beta_3\beta_8$ chimeras in the presence of EDTA (5 mM), Ca^{2+} (5 mM), or Mn^{2+} (1 mM) as indicated. The chimera containing β_8 exofacial lower stalk but not TM and cytoplasmic domains ($\beta_3\beta_8$ EGF- β TD) expressed similar affinity as the $\beta_3\beta_8$ Leg chimera. Error bars represent S.D. from at least three independent assays.

Fig. 3. Effect of different ionic conditions on integrin mediated vitronectin binding. **A.** Binding of Alexa A488 labeled vitronectin to HEK293T cells transiently transfected with either $\alpha_v\beta_3$ or $\alpha_v\beta_8$ in the presence of different ion conditions as shown on the Figure. **B.** Binding of Alexa A488 labeled vitronectin to HEK293T cells transiently transfected with either $\alpha_v\beta_3$ or $\alpha_v\beta_8$ under gradually increased Mg^{2+} concentrations. Error bars represent standard deviation (S.D.) from at least three independent assays.

lower ectodomain stalk is important for the overall high affinity conformation.

Ca^{2+} IS ESSENTIAL FOR INTEGRIN $\alpha_v\beta_8$ MEDIATED LIGAND BINDING, WHEREAS Mg^{2+} INHIBITS LIGAND BINDING AT LOW CONCENTRATIONS BUT ENHANCES IT AT HIGHER CONCENTRATIONS

Previous studies indicated that Mn^{2+} and Mg^{2+} are positive regulators of integrin ligand binding for most integrins [Mould, 1996], by the interaction of the cations with MIDAS on the α I or β I domain and by competition of Mn^{2+} against Ca^{2+} at ADMIDAS, a negatively regulatory site when occupied by Ca^{2+} , respectively [Zhang and Chen, 2012]. However, these studies have all been carried out in integrins expressing low affinity under physiological conditions. We therefore tested affinity states of integrin $\alpha_v\beta_8$ with Alexa 488 labeled vitronectin under various ion conditions and used $\alpha_v\beta_3$ as a comparison (Fig. 3a). The results clearly suggested a dramatically different role of Mg^{2+} on $\alpha_v\beta_8$ regulation. Addition of Mg^{2+} at 1 mM concentration reduced ligand binding by about 30% from the level of Ca^{2+} only condition for $\alpha_v\beta_8$. In contrast, the ligand binding of $\alpha_v\beta_3$ did not change after addition of Mg^{2+} . Furthermore, chelating Ca^{2+} with 1 mM EGTA in the presence of Mg^{2+} completely ablated ligand binding of the $\alpha_v\beta_8$ integrin. We further studied the effects of Mg^{2+} in a concentration dependent manner (Fig. 3b). The results indicated that low concentration of Mg^{2+} could reduce ligand binding in $\alpha_v\beta_8$. However, appearance of Mg^{2+} at high

Fig. 4. The unique ADMIDAS plays a critical role in β_8 integrin mediated ligand binding. **A.** Sequence alignment of MIDAS (Bold), ADMIDAS (Bold and underlined), and SyMBS (Underlined) between all β integrins. Note that the β_8 ADMIDAS is distinct from others. **B.** Binding of Alexa 488 labeled vitronectin to HEK293T cells transiently transfected with different $\alpha_v\beta_3$ or $\alpha_v\beta_8$ constructs in the presence of different ions as indicated. Error bars represent standard deviation (S.D.) from at least three independent assays.

concentration (>5 mM) restored the $\alpha_v\beta_8$ ligand binding to a level similar to the Ca^{2+} only condition. Our results suggested that Mg^{2+} at low concentration inhibits interaction between $\alpha_v\beta_8$ and its ligand, but this inhibitory effect could be reversed by high concentration of Mg^{2+} . Therefore, we propose that there is another Mg^{2+} binding site other than MIDAS in the β_8 integrin. The site has a lower affinity for Mg^{2+} and can only be occupied at high ion concentration, and the occupancy of Mg^{2+} at this site could promote affinity of the β_8 integrin toward its ligand. More study is needed for a better understanding of this potential ion binding site.

THE UNIQUE ADMIDAS CONTRIBUTES TO HIGH AFFINITY FOR SOLUBLE LIGANDS OF β_8 INTEGRIN

Due to the importance of the cation regulation of integrins, it is possible that the high affinity of $\alpha_v\beta_8$ is also caused by the ion coordinating sites. By comparing the primary sequences of beta integrin subunits, we found that the ADMIDAS domain on β_8 is

unique among all the beta integrins (Fig. 4A). The two Asp residues which are universal at ADMIDAS in all other integrins are replaced with two Asn (Asn¹²⁰ and Asn¹²¹). Since Asn is polar instead of negatively charged under physiological conditions, this unique ADMIDAS likely plays a distinct role in integrin affinity regulation. We therefore designed two mutants: $\beta_3\text{Asn}^{126-127}\text{Asn}$ ($\beta_3\text{N}$) and $\beta_8\text{Asn}^{120-121}\text{Asp}$ ($\beta_8\text{D}$), to study the effect of this specific residue replacement (Fig. 4B). The results indicated that mutating β_3 ADMIDAS did not cause significant change, and Mn^{2+} could increase ligand binding of the $\beta_3\text{N}$ mutant similar to the wild type. By contrast, replacing the two Asn with Asp at β_8 ADMIDAS reduced ligand binding and this negative effect was more prominent in the presence of Mg^{2+} . Our result suggests that the lower negativity of the β_8 ADMIDAS may play an important role on stabilizing the integrin in high affinity conformation.

We further tested the effects of Mg^{2+} in β_8 mediated ligand binding with several chimeric/mutant β_8 construct. The $\beta_8\beta_3\text{Leg}$ is

Fig. 5. β_8 ADMIDAS is a potential regulatory site of Mg^{2+} . Binding of Alexa A488 labeled vitronectin to HEK293T cells transiently transfected with different $\alpha_v\beta_8$ constructs under gradually increasing Mg^{2+} concentrations as indicated. Note that the ADMIDAS mutation greatly jeopardized the ability of high concentration Mg^{2+} to promote ligand binding. Error bars represent standard deviation (S.D.) from at least three independent assays.

comprised by a complete β_8 head domain and a β_3 lower leg, whereas $\beta_8\beta_3$ LegD is an ADMIDAS mutant of $\beta_8\beta_3$ Leg, carrying the Asn¹²⁰⁻¹²¹ Asp mutation. We already observed reduced ligand binding in $\beta_8\beta_3$ Leg (Fig. 1C). However, with the ADMIDAS mutation incorporated into the chimera, we managed to found further decreased binding of soluble vitronectin, suggesting that both the β_8 ADMIDAS and its leg independently contribute to the high affinity state (Fig. 5). Moreover, since $\beta_8\beta_3$ Leg showed similar susceptibility to high Mg^{2+} concentration with the wild type, our result suggests that the Mg^{2+} regulatory site is unlikely located on the lower leg of the β_8 integrin. We further discovered that on both ADMIDAS mutants, high Mg^{2+} concentration can only moderately promote ligand binding by comparing with the wild-type and the leg swapping chimera. This suggested that the ADMIDAS mutation reduced the capacity of high concentration Mg^{2+} to promote ligand binding, making the β_8 ADMIDAS a very plausible candidate of the hypothesized Mg^{2+} binding site.

DISCUSSION

Two β_3 integrins ($\alpha_{IIB}\beta_3$ and $\alpha_v\beta_3$) have been widely studied as models for integrin bidirectional signaling. On the contrary, little is known about the β_8 , which also associates with α_v integrin and belongs to arginine-glycine-aspartic acid receptor family. Its affinity and conformational states still remain elusive. In this study, we identified integrin β_8 as the first integrin to express high affinity for soluble ligands under physiological conditions. We also discovered that both the head and the leg of the integrin contribute to this high affinity state. By using the chimeras that combined the ligand binding head domain of the β_3 integrin with different domains of the β_8 integrin leg, we revealed an important role of β_8 ectodomain lower stalk in the high affinity conformation. We also

discovered that the ADMIDAS on β_8 integrin is strikingly different from all other β integrins, as the two highly conservative, negatively charged Asp are replaced with Asn at β_8 ADMIDAS. Our mutagenesis study suggested that this difference may contribute to the high affinity conformation of $\alpha_v\beta_8$.

It has been shown that many integrins adopt a bent conformation with low affinity for ligands in resting state and can be converted into extended, high affinity conformation upon inside-out activation [Luo and Springer, 2006; Kim et al., 2011]. Our study indicated that the β_8 integrin is an exception. This integrin lacks all the identified binding sites of the integrin cytosolic activators, indicating that the integrin is unable to be activated from the cytosol. In this study, our experiments showed that β_8 integrin expressed high affinity for soluble ligands even under physiological conditions. Therefore, this integrin may not necessarily rely on intracellular signal-induced conformational change to switch into a high affinity state and become functional. This is the first integrin that has been shown to exist in an active state under physiological conditions. The discovery has many important implications for the mechanism of the integrin conformational regulation as well as the relationship between the integrin signal transduction and diverse biological functions.

The β integrin lower leg is comprised by several domains. Previous studies have shown that the disruption of the cytoplasmic domain association and afterward dissociation of the TM domain are essential in integrin activation, making the TM/cytoplasmic domains the most important regulatory sites during integrin activation [Hughes et al., 1996; Zhu et al., 2007; Hu and Luo, 2015]. However, our results suggested that in β_8 integrin, the TM domain and cytoplasmic tail do not play a key role in maintaining high affinity conformation. In contrast, the ectodomain lower stalk, which is comprised by the EGF domains and β TD, is sufficient to transform the $\beta_3\beta_8$ chimera into high affinity state. On the plasma membrane, integrins are proposed to dynamically adopt three conformational states: bent-closed, extended-closed, and extended-open [Luo and Springer, 2006]. These three states are believed to coexist in a dynamic equilibrium whereas ligand interaction or inside-out activation alters the equilibrium toward extended-open conformation [Chen et al., 2010; Springer and Dustin, 2012]. It is plausible that the lower leg of β_8 integrin shifts the equilibrium toward extended-open state. The lower β leg is believed to bend sharply between the EGF1 and EGF2 domains under physiological conditions, forming a β knee which is extended and straightened during integrin activation [Zhu et al., 2008]. Since the EGF domains are crucial in integrin conformational change, it is possible that the β_8 EGF domains, especially the EGF 1–2, are important in stabilizing the high affinity conformation.

Metal ions have been shown to play a critical role in integrin affinity regulation [Luo et al., 2007; Zhang and Chen, 2012]. Three ion binding sites have been identified on beta integrin head: MIDAS, ADMIDAS, and SyMBS. Cation occupancy in MIDAS is known to direct ligands binding through coordinating negatively charged carboxyl group of the ligands. In contrast, it has been reported that ADMIDAS can only be bound by Ca^{2+} and Mn^{2+} , and the ligand binding ability can be inhibited by Ca^{2+} occupancy or promoted by Mn^{2+} occupancy [Chen et al., 2003; Mould et al., 2003; Pesho et al., 2006]. However, the previous studies had been carried out in integrins

adopting low affinity conformation at resting state. We have shown that β_8 integrin adopts a high affinity state in the presence of Ca^{2+} and therefore, the metal ions may regulate the integrin in a different manner. Indeed, our study showed that the β_8 ADMIDAS is critical for high affinity conformation of the integrin. We also found that low concentration of Mg^{2+} inhibited β_8 integrin for ligand binding, whereas high concentration of Mg^{2+} promoted ligand binding. The discovery suggests the existence of another Mg^{2+} binding site rather than MIDAS on the β_8 head domain. The unique β_8 ADMIDAS might be occupied by Mg^{2+} at high concentration and this occupancy can promote β_8 mediated ligand binding. The extracellular calcium with mM concentration plays a vital role in nervous system [Simons, 1988; Burgoyne and Haynes, 2012]. Peripheral nerve is exposed to extracellular fluid which contains about 1–2 mM Ca^{2+} ; whereas central neurons are bathed in an interstitial fluid with about 1 mM Ca^{2+} . Changes in the calcium concentration have a profound effect on neuronal functions. On the other hand, magnesium may have more complex effects on neuronal functions [Lee et al., 2010]. It is possible that the β_8 ADMIDAS might have an essential role in sensing a fluctuating environmental ion condition as this integrin lacks a cytosolic “on/off” switch.

In conclusion, our research revealed for the first time that the β_8 integrin adopts a constitutively high affinity state toward its soluble ligands under physiological conditions. Our study showed that both the β_8 ectodomain lower stalk and its unique ADMIDAS are important for this high affinity conformation. One of the most important biological roles of the β_8 integrin is mediating maturation and activation of TGF- β 1. This is achieved through binding to latent TGF- β 1 and introducing membrane type 1 metalloprotease dependent active TGF- β 1 release [Mu et al., 2002]. Since the β_8 cytoplasmic tail lacks binding sites for all the known integrin cytosol activators, it is most likely that the only way for this integrin to efficiently bind latent TGF- β 1 is to maintain high affinity state under physiological conditions. In fact, a recent study showed that recombinant integrin $\alpha_v\beta_8$ extracellular domain indeed strongly interacts with latent TGF- β 1 [Ozawa et al., 2016]. However, more research is required to confirm if the high affinity conformation is indispensable for β_8 to function properly.

REFERENCES

Burgoyne RD, Haynes LP. 2012. Understanding the physiological roles of the neuronal calcium sensor proteins. *Mol Brain* 5:2.

Campbell ID, Humphries MJ. 2011. Integrin structure, activation, and interactions. *Cold Spring Harb Perspect Biol* 3(3).

Chen JF, Salas A, Springer TA. 2003. Bistable regulation of integrin adhesiveness by a bipolar metal ion cluster. *Nat Struct Biol* 10:995–1001.

Chen X, Xie C, Nishida N, Li Z, Walz T, Springer TA. 2010. Requirement of open headpiece conformation for activation of leukocyte integrin $\alpha\text{X}\beta\text{2}$. *Proc Natl Acad Sci USA* 107:14727–14732.

Chernousov MA, Carey DJ. 2003. $\alpha\text{V}\beta\text{8}$ integrin is a Schwann cell receptor for fibrin. *Exp Cell Res* 291:514–524.

Dong X, Mi LZ, Zhu J, Wang W, Hu P, Luo BH, Springer TA. 2012. $\alpha\text{V}\beta\text{3}$ integrin crystal structures and their functional implications. *Biochemistry* 51:8814–8828.

Ginsberg MH, Partridge A, Shattil SJ. 2005. Integrin regulation. *Curr Opin Cell Biol* 17:509–516.

Hu P, Luo BH. 2013. Integrin bi-directional signaling across the plasma membrane. *J Cell Physiol* 228:306–312.

Hu P, Luo BH. 2015. Integrin $\alpha\text{IIb}\beta\text{3}$ transmembrane domain separation mediates bi-directional signaling across the plasma membrane. *PLoS ONE* 10:e0116208.

Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, Shattil SJ, Ginsberg MH. 1996. Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. *J Biol Chem* 271:6571–6574.

Humphries JD, Byron A, Humphries MJ. 2006. Integrin ligands at a glance. *J Cell Sci* 119:3901–3903.

Hynes RO. 2002. Integrins: Bidirectional, allosteric signaling machines. *Cell* 110:673–687.

Kim C, Ye F, Ginsberg MH. 2011. Regulation of integrin activation. *Annu Rev Cell Dev Biol* 27:321–345.

Lee MH, Lin SR, Chang JY, Schultz L, Heath J, Hsu LJ, Kuo YM, Hong Q, Chiang MF, Gong CX, Sze CI, Chang NS. 2010. TGF- β induces TIAF1 self-aggregation via type II receptor-independent signaling that leads to generation of amyloid beta plaques in Alzheimer's disease. *Cell Death Dis* 1:e110.

Luo BH, Carman CV, Springer TA. 2007. Structural basis of integrin regulation and signaling. *Annu Rev Immunol* 25:619–647.

Luo BH, Springer TA. 2006. Integrin structures and conformational signaling. *Curr Opin Cell Biol* 18:579–586.

Mobley AK, Tchaicha JH, Shin J, Hossain MG, McCarty JH. 2009. β8 integrin regulates neurogenesis and neurovascular homeostasis in the adult brain. *J Cell Sci* 122:1842–1851.

Mould AP. 1996. Getting integrins into shape: Recent insights into how integrin activity is regulated by conformational changes. *J Cell Sci* 109(Pt 1):2613–2618.

Mould AP, Barton SJ, Askari JA, Craig SE, Humphries MJ. 2003. Role of ADMIDAS cation-binding site in ligand recognition by integrin $\alpha\text{5}\beta\text{1}$. *J Biol Chem* 278:51622–51629.

Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, Sheppard D, Broaddus VC, Nishimura SL. 2002. The integrin $\alpha\text{v}\beta\text{8}$ mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF- β1 . *J Cell Biol* 157:493–507.

Nishimura SL, Sheppard D, Pytela R. 1994. Integrin $\alpha\text{v}\beta\text{8}$. Interaction with vitronectin and functional divergence of the β8 cytoplasmic domain. *J Biol Chem* 269:28708–28715.

Ozawa A, Sato Y, Imabayashi T, Uemura T, Takagi J, Sekiguchi K. 2016. Molecular basis of the ligand-binding specificity of $\alpha\text{v}\beta\text{8}$ integrin. *J Biol Chem* 291:11551–11565.

Pesho MM, Bledzka K, Michalec L, Cierniewski CS, Plow EF. 2006. The specificity and function of the metal-binding sites in the integrin β3 A-domain. *J Biol Chem* 281:23034–23041.

Proctor JM, Zang K, Wang D, Wang R, Reichardt LF. 2005. Vascular development of the brain requires β8 integrin expression in the neuroepithelium. *J Neurosci* 25:9940–9948.

Raborn J, Fu T, Wu X, Xiu Z, Li G, Luo BH. 2013. Variation in one residue associated with the metal ion-dependent adhesion site regulates $\alpha\text{IIb}\beta\text{3}$ integrin ligand binding affinity. *PLoS ONE* 8:e76793.

Raborn J, Luo BH. 2012. Mutagenesis studies of the β1 domain metal ion binding sites on integrin $\alpha\text{V}\beta\text{3}$ ligand binding affinity. *J Cell Biochem* 113:1190–1197.

Raborn J, Wang W, Luo B. 2011. Regulation of integrin $\alpha\text{IIb}\beta\text{3}$ ligand binding and signaling by the metal ion binding sites in the β1 domain. *Biochemistry* 50:2084–2091.

Shattil SJ, Kim C, Ginsberg MH. 2010. The final steps of integrin activation: The end game. *Nat Rev Mol Cell Biol* 11:288–300.

Simons TJ. 1988. Calcium and neuronal function. *Neurosurg Rev* 11:119–129.

- Springer TA, Dustin ML. 2012. Integrin inside-out signaling and the immunological synapse. *Curr Opin Cell Biol* 24:107–115.
- Takagi J, Petre BM, Walz T, Springer TA. 2002. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* 110:599–611.
- Valdramidou D, Humphries MJ, Mould AP. 2008. Distinct roles of beta1 metal ion-dependent adhesion site (MIDAS), adjacent to MIDAS (ADMIDAS), and ligand-associated metal-binding site (LIMBS) cation-binding sites in ligand recognition by integrin alpha2beta1. *J Biol Chem* 283:32704–32714.
- Wang W, Fu G, Luo BH. 2010. Dissociation of the alpha-subunit Calf-2 domain and the beta-subunit I-EGF4 domain in integrin activation and signaling. *Biochemistry* 49:10158–10165.
- Wang W, Luo BH. 2010. Structural basis of integrin transmembrane activation. *J Cell Biochem* 109:447–452.
- Xie C, Zhu J, Chen X, Mi L, Nishida N, Springer TA. 2010. Structure of an integrin with an alphaI domain, complement receptor type 4. *EMBO J* 29:666–679.
- Xiong JP, Mahalingham B, Alonso JL, Borrelli LA, Rui X, Anand S, Hyman BT, Rysiok T, Muller-Pompalla D, Goodman SL, Arnaout MA. 2009. Crystal structure of the complete integrin alphaVbeta3 ectodomain plus an alpha/beta transmembrane fragment. *J Cell Biol* 186:589–600.
- Xiong JP, Stehle T, Diefenbach B, Zhang R, Dunker R, Scott DL, Joachimiak A, Goodman SL, Arnaout MA. 2001. Crystal structure of the extracellular segment of integrin alpha Vbeta3. *Science* 294:339–345.
- Xiong JP, Stehle T, Zhang R, Joachimiak A, Frech M, Goodman SL, Arnaout MA. 2002. Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an Arg-Gly-Asp ligand. *Science* 296:151–155.
- Ye F, Kim C, Ginsberg MH. 2011. Molecular mechanism of inside-out integrin regulation. *J Thromb Haemost* 9(Suppl1):20–25.
- Zhang K, Chen J. 2012. The regulation of integrin function by divalent cations. *Cell Adh Migr* 6:20–29.
- Zhu J, Carman CV, Kim M, Shimaoka M, Springer TA, Luo BH. 2007. Requirement of α and β subunit transmembrane helix separation for integrin outside-in signaling. *Blood* 110:2475–2483.
- Zhu J, Luo BH, Xiao T, Zhang C, Nishida N, Springer TA. 2008. Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. *Molecular Cell* 32:849–861.
- Zhu J, Motejlek K, Wang D, Zang K, Schmidt A, Reichardt LF. 2002. Beta8 integrins are required for vascular morphogenesis in mouse embryos. *Development* 129:2891–2903.