Identification, Characterization, and Epitope Mapping of Human Monoclonal Antibody J19 That Specifically Recognizes Activated Integrin $\alpha_4\beta_7$ *

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Background: The activation-specific antibodies against integrins are powerful tools in integrin studies.

Results: An activation-specific antibody J19 against $\alpha_4\beta_7$ was identified and characterized.

Conclusion: J19 specifically binds to the activated $\alpha_4\beta_7$, by recognizing the epitope only exposed in extended conformation.

Significance: J19 is a potentially powerful tool for studying $\alpha_4\beta_7$ function and treatment of $\alpha_4\beta_7$-related inflammatory diseases.

Integrin $\alpha_4\beta_7$ is a lymphocyte homing receptor that mediates both rolling and firm adhesion of lymphocytes on vascular endothelium, two of the critical steps in lymphocyte migration and tissue-specific homing. The rolling and firm adhesions of lymphocytes rely on the dynamic shift between the inactive and active states of integrin $\alpha_4\beta_7$, which is associated with the conformational rearrangement of integrin molecules. Activation-specific antibodies, which specifically recognize the activated integrins, have been used as powerful tools in integrin studies, whereas there is no well characterized activation-specific antibody to integrin $\alpha_4\beta_7$. Here, we report the identification, characterization, and epitope mapping of an activation-specific human mAb J19 against integrin $\alpha_4\beta_7$. J19 was discovered by screening a human single-chain variable fragment phage library using an activated $\alpha_4\beta_7$ mutant as target. J19 IgG specifically bound to the high affinity $\alpha_4\beta_7$, induced by Mn2+, DT, ADP, or CXCL12, but not to the low affinity integrin. Moreover, J19 IgG did not interfere with $\alpha_4\beta_7$-MadCAM-1 interaction. The epitope of J19 IgG was mapped to Ser-331, Ala-332, and Ala-333 of $\beta_7$ I domain and a seven-residue segment from 184 to 190 of $\alpha_4$-propeller domain, which are buried in low affinity integrin with bent conformation and only exposed in the high affinity extended conformation. Taken together, J19 is a potentially powerful tool for both studies on $\alpha_4\beta_7$ activation mechanism and development of novel therapeutics targeting the activated lymphocyte expressing high affinity $\alpha_4\beta_7$.

Integrins are a family of $\alpha/\beta$ heterodimeric adhesion receptors that mediate cell-cell, cell-extracellular matrix, and cell-pathogen interactions and transmit signals bidirectionally across plasma membrane (1). Because of their unique function of integrating extracellular environment with cytoskeleton, integrins play important roles in adhesion-dependent cellular processes including cell migration, proliferation, survival, and differentiation (1–4). Integrin $\alpha_4\beta_7$ is a lymphocyte homing receptor, which can mediate both rolling and firm adhesion of lymphocytes, two of the critical steps in lymphocytes homing to the intestine and gut-associated lymphoid tissues (5, 6). Its ligand, mucosal addressin cell adhesion molecule-1 (MadCAM-1), is preferentially expressed on high endothelial venules of gut-associated lymphoid organs and on lamina propria venules, helping lymphocyte traffic to mucosal organs (7). The activation of integrin $\alpha_4\beta_7$ is a critical step in the progression of inflammatory bowel disease (8, 9). Thus, $\alpha_4\beta_7$ is a promising therapeutic target for the treatment of inflammatory bowel disease.

Different from most integrins supporting only firm adhesion of cells upon activation, integrin $\alpha_4\beta_7$ can mediate both rolling and firm adhesion of lymphocytes (10, 11). The resting integrin $\alpha_4\beta_7$ supports rolling adhesion of lymphocytes via its low affinity interaction with MadCAM-1. Upon activation, $\alpha_4\beta_7$ binds to MadCAM-1 in high affinity, which results in firm cell adhesion. The transition from rolling adhesion to firm adhesion is regulated by the shift of integrin from low affinity to high affinity state (11, 12). Affinity regulation is associated with the conformational rearrangement of the integrin molecule (13, 14). Previous studies have shown that integrin extracellular domains exist in at least three distinct global conformational states that differ in affinity for ligand: low affinity bent conformation with a closed headpiece, intermediate affinity extended conformation with a closed headpiece, and high affinity extended conformation with an open headpiece (15–19). The

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3 The abbreviations used are: MadCAM-1, mucosal addressin cell adhesion molecule-1; scFv, single-chain variable fragment; HBS, Hepes-buffered saline; SPL, splenic lymphocyte.
equilibrium among these different states is regulated by integrin inside-out signaling and extracellular stimuli, such as divalent cations (20, 21). Compared with the low affinity state in Ca\(^{2+}\) + Mg\(^{2+}\), removal of Ca\(^{2+}\) or addition of Mn\(^{2+}\) strikingly increases ligand binding affinity of almost all integrins (11). Electron microscopic studies of integrins \(\alpha_5\beta_3\) and \(\alpha_6\beta_4\) demonstrate that integrin activation is coupled with the switchblade-like extension of the extracellular domain and a change in angle between the \(\beta 1\) and hybrid domains (18, 19). Crystal structures of integrin \(\alpha_{I\beta}^{m}\beta_3\) headpiece in the high affinity conformation demonstrate that the C-terminal \(\alpha7\)-helix of the \(\beta 1\) domain moves axially toward the hybrid domain, causing the \(\beta\) hybrid domain to swing outward by 60°, away from the \(\alpha\) subunit (15, 22). The conformational rearrangement in the integrin headpiece destabilizes the bent conformation and induces integrin extension in which the headpiece extends and breaks free from an interface with the leg domains that connect it to the plasma membrane. This conversion from the low affinity to the high affinity conformation of integrin can be mimicked by the introduction of glycan wedges into the interface between the hybrid and the I domains of \(\beta_7\), \(\alpha_3\), and \(\beta_1\) integrins, which activates integrins by stabilizing the outward swing of the hybrid domain and the high affinity headpiece conformation (12, 23). This wedge mutant integrin therefore can be used as a target for screening activation-specific antibodies that exclusively recognize the activated integrin. Up to date, there is no well characterized activation-specific antibody against integrin \(\alpha_6\beta_3\). Thus, a well characterized activation-specific antibody to \(\alpha_4\beta_7\) will be extremely useful for both studies on \(\alpha_4\beta_7\) activation mechanism and development of drug delivery system targeting the activated lymphocyte for the treatment of inflammatory bowel disease.

In this study, we discovered the activation-specific human mAb J19 against integrin \(\alpha_4\beta_7\) by screening from the human scFv phage library using the activated wedge mutant \(\alpha_4\beta_7\). As a target, J19 IgG specifically bound to the \(\alpha_4\beta_7\) activated by Mn\(^{2+}\), DTT, ADP, or CXCL12 but not to the low affinity integrin. Moreover, J19 IgG did not interfere with \(\alpha_4\beta_7\)-MAdCAM-1 interaction, suggesting it was a mAb distinct from “ligand mimetic” group, and we demonstrate that J19 IgG recognizes an activation-dependent epitope on \(\alpha_4\beta_7\) consisting of residues from both \(\alpha_4\) and \(\beta_7\) subunits. This epitope is buried in the low affinity integrin with bent conformation and only exposed in the extended conformation induced by integrin activation. These data also provide strong supporting evidence for the conformational rearrangement during integrin \(\alpha_4\beta_7\) activation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and mAbs**—K562 cells stably expressing human \(\alpha_4\beta_7\) integrin were cultured in RPMI 1640 medium (Invitrogen) and 10% FBS (Biochrom AG) supplemented with 0.2 mg/ml hygromycin and 0.5 mg/ml G418 (all from Amresco). 293T stably cell lines expressing human integrin \(\alpha_4\beta_7\) WT or wedge mutant were cultured in DMEM (Invitrogen) with 10% FBS supplemented with hygromycin (0.1 mg/ml).

The following integrin antibodies were used in this study: mAb TS2/16 (Santa Cruz) to human \(\beta_7\), murine mAb Ber- ACT8 (Santa Cruz) to human \(\alpha_5\), murine mAb HP2/1 (Abcam) to human and rat \(\alpha_\alpha,\) and rat mAb FIB27 (BD biosciences) to human and mouse \(\beta_7\). The murine mAb 9F10 against human \(\alpha_4\) was prepared from hybridoma (Developmental Studies Hybridoma Bank, University of Iowa). The murine mAb Act-1 specific for human \(\alpha_6\beta_7\) was previously described (24, 25). Human IgG1 control was from Pierce.

**Plasmid Construction and Transient Transfection into 293T Cells**—J19 IgG expression constructs were built on the backbone of pIRE52-EGFP (Invitrogen). CDNAs encoding human IgG1 light chain and heavy chain constant regions were amplified by RT-PCR from human endothelial cell total mRNA.

cDNAs of human \(\alpha_4\), \(\alpha_6\), \(\beta_3\), and \(\beta_7\) subunits were inserted into vector pcDNA3.1/Hygro (Invitrogen).

**Protein Purification and Analytical Gel Filtration**—The J19 IgG was purified by protein A (Pierce) affinity chromatography. 293T cells were transiently transfected with J19 IgG expressing construct and cultured in DMEM supplemented with 10% ultralow IgG fetal bovine serum (Invitrogen).

The soluble integrin \(\alpha_4\beta_7\) with all ectodomains was purified as previously described (19, 22). Briefly, soluble integrin was purified from culture supernatant of 293T cells stably expressing soluble integrin \(\alpha_4\beta_7\) ectodomains with C-terminally fused His tag and Strep-tag II using nickel-nitrilotriacetic acid-agarose (Qiagen) followed by Strep-Tactin (IBA) affinity chromatography and gel filtration (Superdex 200; GE Healthcare).

Analytical gel filtrations were performed using precalibrated Superdex 200 on an AKTA purifier system running Unicorn 5.11 software at a flow rate of 0.5 ml/min at room temperature (29, 30). The elution profiles were monitored in-line by UV adsorption at 280 nm. Hepes-buffered saline (HBS) buffer (150 mM NaCl, 20 mM Hepes, pH 7.4) containing 1 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\) or 0.5 mM Mn\(^{2+}\) was used throughout. 5.2 μg of purified WT or wedge mutant integrin \(\alpha_4\beta_7\) was loaded.

**Selection of Integrin-binding Phages**—Human scFv phage library was purchased from Geneservice. Antibody screening was done according to the protocol provided by Geneservice with minor modifications. Soluble integrin \(\alpha_4\beta_7\) was biotinylated and immobilized to streptavidin-coated Dynabeads (Invitrogen). Phage displaying scFv binding to \(\alpha_4\beta_7\) was captured by integrin-labeled beads (so called “panning”). In each round of panning, the binders to WT \(\alpha_4\beta_7\) were depleted first followed by screening the specific scFv fragments against wedge mutant. After three rounds of panning, specific binders were identified by monoclonal phase ELISA.

**Flow Cytometry**—Immunofluorescence flow cytometry was done as described (31). Before staining with antibody, 2.5 × 10^5 cells were washed with HBS containing 5 mM EDTA and then
resuspended in either HBS containing 1 mM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\) or activating HBS containing 2 mM Mn\(^{2+}\). For activation of α\(_4β_7\) by other stimuli than divalent cations, 2.5 × 10\(^5\) cells were resuspended and incubated at 37 °C for 15 min in HBS (1 mM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\)) and blocked by 10% FBS. The cells were then stained with Alexa Fluor 488 goat anti-human IgG (H + L) and Cy3-conjugated goat anti-rat IgG (Invitrogen), respectively.

**Primary splenic lymphocytes (SPLs) were isolated as previously described (32) and suspended in HBS and stimulated by 2.5 μg/ml CXCL12 (R & D Systems) for 5 min at 37 °C. The cells were fixed with an equal volume of 2X formaldehyde (7.4%) before staining with 5 μg/ml J19 IgG.

**Fluorescence Microscopy**—Immunofluorescence staining assay was performed as reported (33). Cover glasses were coated with 10 μg/ml human MAdCAM-1 fused to the Fc1 and Fc2 regions of human IgG1 (huMAdCAM-1/Fc) in the presence or absence of 2 μg/ml CXCL12. The cells were incubated on the cover glass for 20 min at 37 °C, fixed with 3.7% paraformaldehyde and then stained with 5 μg/ml J19 IgG or FIB27, followed by Alexa Fluor 488 goat anti-human IgG (H + L) and Cy3-conjugated goat anti-rat IgG (Invitrogen), respectively.

**Flow Chamber Assay**—The flow chamber assay was performed as described (11, 12). A polystyrene Petri dish to be used as the lower wall of the chamber was coated with a 5-mm-diameter, 20-μl spot of 10 μg/ml purified huMAdCAM-1/Fc in coating buffer (phosphate-buffered saline, 10 mM NaHCO\(_3\), pH 9.0) for 1 h at 37 °C, followed by 2% BSA in coating buffer for 1 h at 37 °C to block nonspecific binding sites. The cells were washed twice with HBS containing 5 mM EDTA and 0.5% BSA, resuspended at 1 × 10\(^6\)/ml in buffer A (HBS, 0.5% BSA), and kept at room temperature. The cells were diluted to 1 × 10\(^6\)/ml in buffer A containing different divalent cations immediately before infusion in the flow chamber using a Harvard apparatus programmable syringe pump. Then shear stress was increased from 0.3 dyn/cm\(^2\) up to 16 dyn/cm\(^2\). The number of cells remaining bound at the end of 1 dyn/cm\(^2\) was determined.

**FRET**—For detecting the orientation of integrin ectodomain relative to cell membrane, FRET was measured as described (30, 35). Briefly, 293T cells stably expressing integrin α\(_4β_7\) treated with indicated cations were fixed by 3.7% paraformaldehyde and then stained with 20 μg/ml Alexa Fluor 488–Act-1 Fab, followed by staining with 10 μM FM4–64 FX (Invitrogen).

**RESULTS**

*Generation of High Affinity Integrin α\(_4β_7\) with Glycan Wedge Mutation*—Previous studies have shown that the introduction of N-glycan at the integrin β1/hybrid domain interface will activate integrins and stabilize the high affinity conformation (12, 23). To obtain the high affinity human integrin α\(_4β_7\), we introduced an N-glycosylation site at Asn-322 in the α\(_4\)-β\(_7\) loop of β\(_7\) 1 domain by mutating Gln-324 to Thr as previously described (12). The WT and wedge mutant (Q324T) human α\(_4β_7\) were transiently expressed in 293T cells, and the adhesive behavior in shear flow of those transfectants was characterized by allowing them to adhere to MAdCAM-1 in a parallel wall flow chamber. WT α\(_4β_7\) and 293T wedge mutant behaved as previously described for lymphoid cells expressing α\(_4β_7\) (36). In 1 mM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\), more than 75% of adherent cells expressing WT α\(_4β_7\) rolled on MAdCAM-1 substrates at the wall shear stress of 1 dyn/cm\(^2\) (Fig. 1A). In contrast, the cells were firmly adherent in 0.5 mM Mn\(^{2+}\) (Fig. 1A). Rolling and firm adhesions represent the low and high affinity interactions of integrin α\(_4β_7\) with MAdCAM-1, respectively. By contrast with the rolling adhesion of WT α\(_4β_7\), 293T wedge mutant expressing α\(_4β_7\) wedge mutation showed significantly increased firmly adherent cells in 1 mM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\), which is similar to the adhesion behavior of WT α\(_4β_7\) activated by 0.5 mM Mn\(^{2+}\) (Fig. 1A). α\(_4β_7\) wedge mutants treated with the α\(_4β_7\) blocking antibody Act-1 did not accumulate on MAdCAM-1 substrates. Thus, integrin α\(_4β_7\) is constitutively activated by the glycans introduced into the I/hybrid domain interface of β\(_7\) subunit.

Next, we introduced Q324T mutation into the human integrin α\(_4β_7\) soluble construct with all ectodomains. To eliminate the heterogeneity resulting from partial cleavage of α\(_4\) subunit, a previously described Arg-558 to Ala mutation was introduced into α\(_4\) subunit to remove the protease cleavage site (37). Both WT and wedge mutant α\(_4β_7\)-soluble proteins were expressed in
Activation-specific Antibody J19 against Integrin α4β7

To identify an activation-specific antibody against α4β7, we performed human single fold scFv phage display library (Tomlinson I + J) selection using the high affinity wedge mutant α4β7 as target. The Tomlinson I + J libraries purchased from Geneservice contain over 100 million different human scFv fragments. The library was first depleted by the low affinity WT α4β7 soluble protein and then selected against the high affinity wedge mutant α4β7. In this way, binders specific for high affinity α4β7 were enriched after each round of selection. After three rounds of selections, the remaining binders were validated by monoclonal phage ELISA using purified WT and wedge mutant protein as target, respectively. Among a number of isolates that showed higher binding signals to wedge mutant than to WT α4β7 (data not shown), phage clone J19 bound specifically to wedge mutant and high affinity WT α4β7, activated by 2 mM Mn2+, but not to the low affinity WT α4β7 in 1 mM Ca2+ + 1 mM Mg2+ (Fig. 2A).

To further characterize the J19 scFv phage isolate, the J19 scFv was expressed and purified. The binding of purified J19 scFv to integrin α4β7 was analyzed using flow cytometry with K562 cells stably expressing human α4β7 (Fig. 2B). Different from the specificity of J19 phage for high affinity α4β7, J19 scFv showed similar binding to the low affinity α4β7 in 1 mM Ca2+ + 1 mM Mg2+ and the high affinity α4β7 in 2 mM Mn2+. The binding of J19 scFv to α4β7 was comparable with that of α4β7 mAb Act-1 (Fig. 2B). Considering that scFv is of a smaller size than the phage particle, it is tempting to speculate that the binding sites of J19 in low affinity α4β7 can be accessed by smaller scFv but not the larger phage particle expressing the same scFv.

J19 IgG Specifically Binds to Activated Integrin α4β7—To further investigate the function of J19, we reformatted the J19 scFv to full-length human IgG1. The binding of J19 IgG to integrin α4β7 was determined using flow cytometric analysis with 293T cells stably expressing WT or wedge mutant α4β7 (Fig. 3A). Comparable with J19 phage, J19 IgG specifically bound to 293T cells expressing wedge mutant α4β7 in 1 mM Ca2+ + 1 mM Mg2+ and WT α4β7 293T transfectants in 2 mM Mn2+, but not WT α4β7 transfectants in 1 mM Ca2+ + 1 mM Mg2+, suggesting its specificity for the activated α4β7. As control, the binding activity of α4β7 mAb Act-1 was shown in parallel. In contrast to J19 IgG, Act-1 showed nonselective binding to both low and high affinity α4β7 (Fig. 3A). Both J19 IgG and Act-1 did not bind to mock 293T cells transfected with pcDNA3.1 vector alone (Fig. 3A). Similar results were obtained using K562 cells stably expressing α4β7, suggesting that the specificity of J19 IgG for activated integrin α4β7 is not cell type-dependent (Fig. 3B). Moreover, J19 IgG bound to Mn2+-activated α4β7 in a dose-dependent manner (Fig. 3C). From the best fit curve generated with GraphPad Prism 5.01 software, the EC50 of J19 IgG for binding to activated α4β7, is 29.96 nM.

In addition to the strong activation by Mn2+, integrin can also be activated by other stimuli like DTT and ADP (38–40). DTT has been shown to activate integrin in a number of systems (38, 39). ADP was reported to induce integrin activation through inside-out signaling by activating PI3K pathway (41). To further study the binding specificity of J19 IgG to α4β7, activated by different stimuli, K562 cells stably expressing α4β7...
Activation-specific Antibody J19 against Integrin \(\alpha_4\beta_7\)

**FIGURE 3.** J19 IgG specifically recognizes the activated integrin \(\alpha_4\beta_7\). A, 293T cells stably expressing WT or wedge mutant \(\alpha_4\beta_7\) were stained with human IgG1 control, Act-1, or J19 IgG at 5 \(\mu\)g/ml in the presence of 1 mM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\) or 2 mM Mn\(^{2+}\). Mock represents pcDNA3.1 vector transfectants. B, K562 cells stably expressing \(\alpha_4\beta_7\) were stained with 5 \(\mu\)g/ml indicated antibodies in the presence of 1 mM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\) or 2 mM Mn\(^{2+}\) and analyzed by flow cytometry. Mock represents K562 cells transfected with pcDNA3.1 vector. C, concentration-dependent binding of J19 IgG and human IgG1 control to K562 transfectants stably expressing WT \(\alpha_4\beta_7\) in the presence of indicated divalent cations. The cells were incubated with serially diluted J19 IgG or human IgG1 control. The error bars are \(\pm\) S.D. (\(n = 3\)). D, binding of J19 IgG to K562 cells stably expressing \(\alpha_4\beta_7\) in the presence of DTT (500 \(\mu\)M) or ADP (10 \(\mu\)M) were determined by flow cytometric analysis as described under “Experimental Procedures.” A representative experiment (of three) is shown as a histogram. The numbers within the panels show the specific mean fluorescence intensity of human IgG1, Act-1, or J19 IgG. The results are the means \(\pm\) S.D. of three independent experiments.

were treated with Mn\(^{2+}\), DTT, or ADP and then followed by staining with 5 \(\mu\)g/ml J19 IgG. As shown in Fig. 3D, J19 IgG specifically bound to K562 \(\alpha_4\beta_7\) cells treated with these stimuli. By contrast, J19 IgG did not bind to the same cells without any stimulation (Fig. 3D). These results suggested that epitope recognized by J19 IgG in integrin \(\alpha_4\beta_7\) was only expressed after integrin activation. Moreover, the binding of J19 IgG to K562 \(\alpha_4\beta_7\) cells treated with Mn\(^{2+}\) is higher in comparison with those stimulated by DTT or ADP. The different expression level of J19 epitope induced by the above stimuli could be due to the different activation states and conformations of integrin \(\alpha_4\beta_7\).

**J19 IgG Recognizes Integrin \(\alpha_4\beta_7\). Heterodimer from Human, Mouse, and Rat**—Because integrin \(\alpha_4\beta_7\) shares \(\alpha_4\) subunit with integrin \(\alpha_4\beta_1\) and \(\beta_7\) subunit with integrin \(\alpha_5\beta_7\), J19 IgG may also bind to \(\alpha_4\beta_1\) or \(\alpha_5\beta_7\), if it recognizes either subunit of \(\alpha_4\beta_7\) heterodimer. To evaluate the potential cross-reactivity of J19, its binding to human \(\alpha_4\beta_7\) and \(\alpha_5\beta_7\) was determined using 293T cells transiently expressing \(\alpha_4\beta_1\) or \(\alpha_5\beta_7\). (Fig. 4A). The expressions of \(\alpha_5\beta_7\) and \(\alpha_4\beta_7\) were confirmed by mAbs HP2/1 (\(\alpha_4\) mAb), TS2/16 (\(\beta_7\) mAb), Ber-ACT8 (\(\alpha_5\) mAb), and Fib27 (\(\beta_7\) mAb). The flow cytometric results showed no J19 IgG binding to \(\alpha_4\beta_1\) or \(\alpha_5\beta_7\) expressing 293T cells in both Ca\(^{2+}\) + Mg\(^{2+}\) and Mn\(^{2+}\), indicating that J19 IgG does not recognize \(\alpha_4\beta_1\) or \(\alpha_5\beta_7\), both pre- and postactivation (Fig. 4A). These results demonstrate that neither \(\alpha_4\) nor \(\beta_7\) subunit alone is sufficient to support J19 binding and that this antibody recognizes \(\alpha_4\beta_7\) heterodimeric complex.

We next test the cross-reactivity of J19 IgG with \(\alpha_4\beta_7\) from other species. The mouse and rat \(\alpha_4\beta_7\) were transiently expressed in 293T cells, respectively. The expression level of \(\alpha_4\beta_7\) was determined using mAb FIB27 against human and mouse \(\beta_7\), and mAb HP2/1 against rat \(\alpha_4\). J19 IgG showed comparable binding to the activated human, mouse, and rat \(\alpha_4\beta_7\) but not to inactive ones (Fig. 4B).

**J19 IgG Specifically Binds to Chemokine-activated Mouse SPLs**—Having shown that J19 IgG was specific for the activated \(\alpha_4\beta_7\) expressed on K562 and 293T cell lines, we next tested whether J19 IgG also bound to the high affinity form of \(\alpha_4\beta_7\) expressed on primary lymphocytes. The mouse SPLs that highly express \(\alpha_4\beta_7\) were isolated, and the activation of integrin \(\alpha_4\beta_7\) was determined using mAb HP2/1 against human and mouse \(\beta_7\), and mAb HP2/1 against rat \(\alpha_4\). J19 IgG showed comparable binding to the activated human, mouse, and rat \(\alpha_4\beta_7\) but not to inactive ones (Fig. 4B).

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IgG and β7, mAb FIB27 (Fig. 5B). J19 IgG signal was only observed at the surface of SPLs after 47 was activated by CXCL12 or Mn2+. J19 IgG signal was only observed at the surface of SPLs after 47 was activated by CXCL12 or Mn2+. J19 IgG signal was only observed at the surface of SPLs after 47 was activated by CXCL12 or Mn2+

**Effect of J19 IgG on Integrin 47-MAdCAM-1 Interaction**—To further characterize J19 IgG, we studied the effect of this antibody on cell adhesion mediated by 47-MAdCAM-1 interaction in shear flow using K562 cells stably expressing human 47. The cells were preincubated with J19 IgG or 47 blocking mAb Act-1 and then infused into a parallel wall flow chamber with MAdCAM-1 immobilized on the lower wall. 20 μg/ml Act-1 almost completely abolished the cell adhesion at the wall shear stress of 1 dyn/cm², whereas J19 IgG showed no effect on cell adhesion even at much higher concentration of 100 μg/ml (Fig. 6). Thus, J19 IgG does not affect the interaction between integrin 47 and MAdCAM-1, suggesting that it is distinct from the ligand-mimetic mAb.

**Epitope Mapping of J19 IgG**—Because of the lack of cross-reactivity with 47 by J19 IgG and the high homology between β7 subunits, we constructed a panel of 47/7 chimeras to locate the epitope of J19 IgG in 7 subunit.

![FIGURE 4. J19 IgG recognizes α4β7 heterodimer and cross-reacted with mouse and rat α4β7.](image-url)

**FIGURE 4.** J19 IgG recognizes α4β7 heterodimer and cross-reacted with mouse and rat α4β7. A, 293T cells were transfected with human α4β7 or α4β7, and the integrin expression level was determined by indicated antibodies, respectively. The binding of J19 IgG to α4β7 or α4β7 was analyzed by flow cytometry in the presence of 1 mM Ca2+ + 1 mM Mg2+ or 2 mM Mn2+. B, human, mouse, or rat α4β7 was transiently expressed in 293T cells, and the integrin expression level was determined by 5 μg/ml indicated antibodies, respectively. Reactivity of J19 IgG with α4β7, 293T transfectants was determined by flow cytometry. A representative experiment (of three) is shown as a histogram. The numbers within the panels show the specific mean fluorescence intensity of indicated mAbs. The results are the means ± S.D. of three independent experiments.
J19 IgG to H9251/4/H9252/7. All of the chimeras in which segment 331–348 was of H9252/7 origin failed to be stained with 5 μg/ml J19 IgG, whereas all other chimeras in which this segment was of H9252/7 origin were stained, as well as WT H9252/7 (Fig. 7A). Within region 331–348, seven amino acids differ between human H9252/1 and H9252/7. Thereafter, we substituted the seven β7 residues with the corresponding H9252/1 residues. Mutations of S331E, A332E, and A333F decreased 30–50% of J19 IgG binding in comparison with WT H9252/4. By contrast, mutations of the other four residues, L334Q, Q338K, S341K/K342N, almost had no effect on the recognition of H9252/4/H9252/7 by J19 IgG. Moreover, the H9252/7 triple mutation S331E/A332E/A333F completely abolished the recognition of H9252/4/H9252/7 by J19 IgG (Fig. 7B). These results strongly suggest that residues Ser-331, Ala-332, and Ala-333 in H9252/7 I domain represent a direct binding site for J19 IgG.

The J19 IgG binding site in human H9252/4 subunit was mapped by using H9252/4/E chimeras because H9252/4 and H9252/E share the same H9252/7 subunit. Considering H9252-domain in H9252/4 subunit is close to the above mapped J19 epitope in H9252/7 I domain, we first swapped H9252-domain of H9252/4 and H9252/E subunits, whereas the swap of H9252-domain of H9252/4 and H9252/E subunits resulted in no expression of both H9252/4/H9252/7 and H9252/E/H9252/7 chimeric integrins. The abnormal expression of H9252/4/E chimeras is possibly due to the difference in structure between H9252/I domain-less integrin H9252/4 and H9252/I domain-containing integrin H9252/E. Thus, based on J19 binding sites in H9252/7 subunit and the crystal structures of integrin H9252/IIb/H9252/3 and H9252/V/H9252/3, several segments in H9252/β-propeller domain close to the epitope in β7 subunit were substituted with corresponding H9252/E sequences, respectively. These chimeric cDNAs were all cloned into pcDNA 3.1 expression vectors and transiently co-expressed with human H9252/7 subunit in 293T cells, then followed by immunostaining with 5 μg/ml J19 IgG. The expression of these chimeras was confirmed by immunostaining with mAb FIB27 against β7. Swapping of α4 segments 211–216 and 240–246 with those of H9252/E had no effect on the binding of J19 IgG, whereas

**FIGURE 5. J19 IgG binds to activated α4β7 on primary lymphocytes.** A, binding of J19 IgG to mouse SPLs in the presence or absence of 0.2 μg/ml CXCL12 was analyzed by flow cytometry. A representative experiment (of three) is shown as a histogram. The numbers within the panels show the specific mean fluorescence intensity of J19 IgG. The results are the means ± S.D. of three independent experiments. Open histogram, isotype control IgG filled histogram, J19 IgG. B, mouse SPLs were plated on coverslips coated with 10 μg/ml MAdCAM-1 or 10 μg/ml MAdCAM-1 plus 2 μg/ml CXCL12 for 20 min and then stained with 5 μg/ml J19 IgG and FIB27 in the presence of 1 mM Ca2+ + 1 mM Mg2+ or 2 mM Mn2+ as indicated. Bar, 20 μm.

**FIGURE 6. J19 IgG has no effect on α4β7-MAdCAM-1 interaction.** K562 cells stably expressing human α4β7 were incubated with 100 μg/ml J19 IgG or 20 μg/ml mAb Act-1 in the presence of 1 mM Ca2+ + 1 mM Mg2+ or 2 mM Mn2+ for 15 min at 37°C, followed by flow chamber assay as described under “Experimental Procedures.” The number of rolling and firmly adherent cells was measured under the wall shear stress of 1 dyn/cm². The error bars are ± S.D. (n = 3).
swapping of the α4 184–190 segment completely abolished J19 IgG binding to chimera α4/E subunit in 293T cells. These results demonstrate that the epitope of J19 IgG in α4/β7 subunit locates in a seven-residue segment from 184 to 190 in β7-propeller domain. However, J19 did not bind to chimeric αE (αE,184α4,190α7) when swapping the 184–190 segment of α4 into αE subunit (Fig. 7C), which might be due to the interference of J19 binding by the α7 domain on the top of the β-propeller domain in αE subunit.

The Epitope Exposure of J19 IgG Is Coupled with Extension of Integrin α4β7 Ectodomain—The headpiece of integrin folds over its legs and faces down toward the membrane in the low affinity bend conformation and extends upward in a switchblade-like opening upon activation (2, 43). We next studied the relationship between J19 IgG epitope exposure and the conformational rearrangement of integrin α4β7 during its activation.

To assess the orientation of integrin α4β7 ectodomain relative to the cell membrane using a FRET system, α4β7 was labeled with Alexa Fluor 488-Act-1 Fab fragment as donor, which binds to the top of α4β7 β1 domain (44). The outer face of plasma membrane was labeled with FM4–64 FX (FM) as acceptor (30, 35). Compared with the inactive α4β7 in 1 mM Ca2+ + 1 mM Mg2+, the FRET efficiency of 293T transfectants bearing activated α4β7 in 2 mM Mn2+ was significantly decreased from 22 to 6%, indicating the extension of the α4β7 ectodomain (Fig. 8A). In parallel, immunostaining results showed that J19 IgG bound to activated α4β7 in 2 mM Mn2+ but not to inactive α4β7 in 1 mM Ca2+ + 1 mM Mg2+, suggesting that the epitope of J19 IgG was only exposed in the activated α4β7 with extended conformation in 2 mM Mn2+ (Fig. 8B). These data strongly indicate that the J19 IgG epitope is exposed when the integrin head domain moves away from the cell membrane in the extended conformation.

FIGURE 7. Epitope mapping of J19 IgG. A, mapping of J19 IgG epitope with β7 chimeras. mAb reactivity was determined with chimeric human β7 subunits co-expressed with human α4 in 293T cells in the presence of 2 mM Mn2+ by using flow cytometry. J19 IgG recognition was measured as specific mean fluorescence intensity and quantitated as a percentage of total α4β7 expression defined by staining with mAb 9F10 to α4. B, fine mapping of J19 IgG epitope in β7 subunit. Human WT β7 or mutant β7 containing multiple or single β7 to α4 amino acid substitutions was co-expressed with human α4 subunit in 293T cells. J19 IgG recognition was quantified as in A. C, mapping of J19 IgG epitope with α4 chimeras. The human α4/E α4 chimeras were co-expressed with human β7 in 293T cells. The transfectants were stained with J19 IgG or mAb FIB27 to β7, followed by flow cytometry in the presence of 2 mM Mn2+. J19 IgG recognition was quantified as in Fig. 7A. The error bars are ± S.D. (n = 3).
DISCUSSION

In this study, we screened and characterized an activation-specific human monoclonal antibody to integrin α4β7 by panning a scFv-displaying phagemid library using an active form of α4β7 (wedge mutant) as target. The subtractive selection was performed by depleting the library on the isolated, inactive WT α4β7 protein first and then panning against the constitutively activated α4β7 wedge mutant. The specific scFv clone J19 was isolated from the library and reformatted to full-length human IgG1. The J19 IgG specifically binds to integrin α4β7 activated by different stimuli, other than the inactive α4β7. The binding epitope of J19 IgG was mapped to two small segments located in α4 β-propeller domain and β7 I domain, respectively (supplemental Fig. S1). The seven-residue segment from 184 to 190 in α4 subunit locates between β-strands 2 and 3 in β-sheet 3 of the β-propeller domain. The other segment consists of residues Ser-331, Ala-332, and Ala-333 located in a turn between β-strand 5 and α-helix 5 at the top of βI domain. In the low affinity bend conformation, the integrin β-propeller domain and the βI domain sit on their leg domains and face the cell membrane, leading to the epitope buried in the inactive integrin. Upon activation, integrin converted from the bent to extended conformation with either closed headpiece (intermediate affinity state) or open headpiece (high affinity state), which led to the exposure of J19 epitope (supplemental Fig. S1). Thus, J19 IgG is an activation-dependent mAb, which recognizes an epitope expressed only in integrin α4β7 with extended conformation.

The high affinity wedge mutant α4β7 used for J19 selection contains a mutation-introduced N-glycan at the integrin β7 I/ hybrid domain interface and mimics the swing-out of hybrid domain in β7 subunit, which is predicted to activate integrins and stabilize the high affinity conformation. In our study, we showed that J19 bound both wedge mutant and WT integrin α4β7, activated by physiological agonist, such as CXCL12. Thus, the epitope recognized by J19 IgG is expressed in both high affinity wedge mutant and the physiologically activated WT integrin α4β7. These results strongly suggest that the hybrid domain swing-out is the key conformational rearrangement during integrin activation and can induce a high affinity conformation mimicking the physiological activation of integrin α4β7.

Different from the J19 phage and J19 IgG, which specifically recognize the activated integrin α4β7, J19 scFv showed similar binding to both inactive and activated α4β7. The loss of specificity to activated α4β7 could be due to the smaller size of scFv compared with phage particle and full-length IgG1. The epitope of J19 in the inactive α4β7 with bend conformation is not accessible to large size J19 phage or IgG, whereas the space is large enough for smaller scFv to access the epitope. This result also provides supporting evidence for the induction of J19 epitope expression by the conformational rearrangements of integrin.

In αI domain-containing integrins, the αI domain is inserted into a loop between β-sheets 2 and 3 at the top of the β-propeller domain (45, 46). As part of the J19 epitope, the seven-residue segment from 184 to 190 locates in β-sheet 3 of the β-propeller domain, which will be masked by the αI domain in the αε subunit (supplemental Fig. S2). Thus, J19 IgG cannot bind to integrin α4β7 even after replacement of the seven-residue segment in αε subunit with that from αI (Fig. 7C).

Interestingly, the binding of J19 IgG to α4β7 could be enhanced by a number of stimuli, including Mn2+, DTT, and ADP, but to different levels. The binding of J19 IgG showed stronger binding to α4β7 expressing cells treated with Mn2+ than the same cells stimulated with DTT or ADP, suggesting that integrin activated by different stimuli could have different conformations and expression of J19 epitope. The different expression levels of J19 epitope were consistent with the detection of increased affinity for ligand after those stimulations, as...
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measured by a sensitive flow chamber assay (34, 35, 47). Thus, J19 IgG could serve as a reporter for the activation extent of α₄β₇.

In addition to serving as a research tool for in vitro and in vivo studies of integrin activation, the J19 antibody also represents a therapeutic candidate for treatment of inflammatory bowel disease. J19 IgG selectively targets the activated lymphocytes and leaves the inactive ones intact, which may result in fewer potential side effects.

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