Two Functional Active Conformations of the Integrin α2β1, Depending on Activation Condition and Cell Type*

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For several integrins, the existence of multiple conformational states has been studied intensively. For the integrin α2β1, a major collagen receptor on platelets and other cell types, however, no such experimental data were available thus far. Recently, our group has developed a monoclonal antibody IAC-1 sensitive to the molecular conformation of α2β1 because it only binds to the activated state of α2β1 on platelets, induced upon inside-out signaling. By investigating IAC-1 binding in combination with our group has developed a monoclonal antibody IAC-1 sensitive major collagen receptor on platelets and other cell types, how-

... depending on the activation condition and cell type. Significant progress is currently made in demonstrating that integrins contain a number of flexible joints and connections, leading to a broad spectrum of possible conformational states with distinct effects on the overall ligand binding affinity of the receptor. For the I domain lacking integrins αVβ3 and αIIbβ3, three different conformational states have been demonstrated by electron microscopy and crystal structures as follows: (i) a closed conformation (resting state), not able to bind its natural ligand; (ii) an extended conformation with closed headpiece (low affinity state); and (iii) an extended conformation with open headpiece (high affinity state) (10–13). These two extended, active conformations are both favored by outside-in and inside-out signaling and can bind their natural ligand. The integrins α4β1 and α5β1 also exhibit multiple affinity states on the cell surface depending on the activation condition, similar to that described for αVβ3 and αIIbβ3 (14, 15). For the I domain containing integrins αLβ2 and αMβ2, the existence of such conformational states has been demonstrated as well. In the study by Jin et al. (16), molecular dynamic simulations, with pulling forces applied to the α7 helix of the I domain, were used to reproduce intermediate and open conformations of the integrin αL subunit, in agreement with the crystal structures described previously (17).

The integrin α2β1 (also designated VLA-2, GPⅡa/Ⅲa, or CD49b/CD29) is an I domain-containing integrin, expressed on a variety of cell types including platelets, white blood cells, endothelial cells, and fibroblasts (18–20). This integrin acts mainly as a collagen receptor on platelets (21), and on other cell types it can serve both as a collagen and laminin receptor (22). On platelets, α2β1 plays an important role in primary hemostasis by binding to collagen in the subendothelium exposed after vessel injury (23). On white blood cells, α2β1 is thought to be involved in tissue injury and inflammation situations (18, 19). More recently, α2β1 on activated T lymphocytes and Jurkat cells was found to influence Fas-induced apoptosis (24).

For α2β1, studies on the existence of different conformational states have been hampered, mainly because until recently no antibodies were available that specifically recognize the activated or ligand-bound forms...
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MATERIALS AND METHODS

Antibodies and Proteins

The anti-α2I domain mAb IAC-1 recognizes the activated state of α2β1 and was described previously (25). The anti-α2 mAb AK7 was from BD Biosciences. The anti-α2 mAbs Gi9 (both unlabeled and FITC-labeled) and 15D7 were from Immunotech (Marseille, France) or made in-house, respectively, and both inhibit collagen-induced platelet aggregation. Other monoclonal antibodies used are as follows: 7E3, a mouse anti-human αIIbβ3 that also recognizes the activated state of Mac-1 (a kind gift of Dr. T. Edgington, Scripps Institute, La Jolla, CA) (26); TS2/16, an activating mouse anti-human β1 (a kind gift of Dr. M. Kahn, University of Pennsylvania); MOPC-21-FITC, an unrelated mouse anti-human IgG1 (Sigma); and CD62P-PE, mouse anti-human P-selectin (BD Biosciences). Polyclonal rabbit anti-mouse IgG-FITC was from Dako (Glostrup, Denmark).

Monoclonal antibodies and soluble human collagen type I (type VIII, Sigma) were labeled with FITC according to the manufacturer's instructions (Pierce). The FITC labeling of collagen was performed in 50 mM NaHCO3; 0.2 mM KCl; 0.1 mM MgCl2; 0.03 mM NaHPO4; 1.2 mM NaHCO3; 0.5 mM Hapes; 0.01% (w/v) glucose; pH 7.4) containing 0.3% BSA. For the isolation of PBMC, blood was taken on low molecular weight heparin (250 units/ml final, Clexane, Aventis Pharma, Brussel, Belgium) and separated on Ficoll-Paque (Amersham Biosciences) following the manufacturer’s instructions. PBMC were finally resuspended at a concentration of 5 × 105 cells/ml in MEM (Invitrogen).

For the activation experiments, α2β1-expressing CHO cells and Jurkat cells were harvested, washed with XL buffer (13.7 mM NaCl; 0.5 mM KCl; 0.1 mM MgCl2; 0.5 mM Hapes; 0.01% (w/v) glucose; pH 7.4), and resuspended at 5 × 105 cells/ml, except where indicated otherwise. Platelets and PBMC were resuspended in their respective buffer or medium as described above. Reaction mixtures of cells (50 μl in a total of 100 μl) and an agonist were incubated for 30 min at room temperature. The agonists Mn2+ and Co2+ were used at a final concentration of 1 mM DTT at a final concentration of 10 μM. The peptide Ac-KRFFYVMWKK-NH2 and the cell-permeable peptides were used at a final concentration of 50 and 200 μM, respectively. Where indicated, platelets were also incubated with the antagonist PGE1, at 5 μM final concentration. After washing twice with an excess of their respective buffer, cells were used for further experimentation.

Peptide Design

All peptides (see TABLE ONE) were synthesized by a manual solid phase technique on 4-methylbenzhydrylamine resin using the standard t-butoxycarbonyl/benzyl protocol (Pierce) (28). Some peptides were N-terminally acetylated. Purification was performed by reverse phase-

| Peptide Sequence | Molecular mass monoisotopic (Da) | Molecular mass measured [MH+] (Da) | HPLC retention time (min) |
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| Ac-KRFFYVMWKK-NH2 | 1424.8                          | 1425.3                           | 25.6*                    |

The abbreviations used are: mAb, monoclonal antibody; PBS, phosphate-buffered saline; Mn2+, manganese(II) chloride tetrahydrate; Co2+, cobalt chloride; DTT, dithiothreitol; PGE1, prostaglandin E1; CHO, Chinese hamster ovary; PBMC, peripheral blood mononuclear cells; RP-HPLC, reverse phase high performance liquid chromatography; MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; MEM, minimum Eagle’s medium.

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of α2β1. Our group developed a monoclonal antibody (mAb),3 IAC-1, that recognizes an epitope within the α2I domain on platelets, hidden in the resting state but accessible when platelets are stimulated with agonists such as thrombin, ADP, or the snake venom convulxin (25). Moreover, because this antibody does not interfere with platelet collagen binding, it defines a new class of antibodies that is distinct from those belonging to the “ligand-induced binding sites” and the “ligand mimetic” groups.

The objective of this study was to use IAC-1 to provide further insight into the conformational states of α2β1 after inside-out and outside manipulations and to do this on four different cell types (platelets, α2β1-expressing CHO cells, PBMC, and Jurkat cells). Moreover, the functional relevance of these different conformational states of α2β1 was evaluated.

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high performance liquid chromatography (RP-HPLC; Jasco Co.), and all peptides were characterized by electrospray ionization-mass spectrometry (Bruker Daltonics Esquire 3000 plus) and amino acid analysis. Analytical data of peptides showed more than 95% purity.

The agonist peptide Ac-KRFYYVWMK-NH2, based on the binding domain of thrombospondin-1 to the CD47 receptor, was used to stimulate α2β1 on different cell types via inside-out signaling (29, 30). Cell-permeable chimeric peptides were designed by placing the hydrophobic delivery sequence VTVLALGALAGVGV to the human α2 cytoplasmic tail sequence KLGGFFKR, as described by Wang et al. (31). The control peptide, consisting of the non-sense sequence TKSKYNE, was used as a negative control (31).

Flow Cytometry

Cells were incubated in the presence or absence of an activating agent for 30 min as described above. After washing, cells were incubated with either 10 μg/ml IAC-1-FITC or 40 μg/ml collagen-FITC (final concentration) for 30 min at room temperature. Afterward, cells were washed in their respective buffer or medium and finally resuspended in 400 μl of PBS, supplemented with 7-aminoactinomycin (Molecular Probes, Eugene, OR) diluted 1:400. When an indirect staining was used, cells were incubated with 10 μg/ml of primary antibody for 30 min at room temperature, followed by incubation with a polyclonal rabbit-antimouse IgG-FITC (diluted 1:100) for 30 min at room temperature. Cells were immediately analyzed by flow cytometry (Beckman Coulter, Miami, FL), and the mean fluorescence intensity (MFI) was determined (10,000 cells for each condition).

Cell Adhesion Assays

**Cell Adhesion to Collagen Under Static Conditions**—Wells of a microtitrater plate (Greiner Bio-One GmbH, Frickenhauer, Germany) were coated with human collagen type I (25 μg/ml in PBS) overnight at 4°C. Wells were blocked with 3% milk powder in PBS for 2 h at room temperature. α2β1-Expressing CHO cells or Jurkat cells were preincubated for 30 min in the presence or the absence of an activating agent. After washing with XL buffer, a dilution series of activated and nonactivated cells was added to the cells and incubated for 0, 5, 10, 20, 30, 60, and 120 min at room temperature. Cell adhesion was determined by measuring endogenous phosphatase activity colorimetrically at 405 nm, after lysis of the cells with Triton X-100, using p-nitrophenyl phosphate (Merck) as a substrate (32). Wells were washed six times after coating and blocking and three times elsewhere. All assays were performed in duplicate and repeated at least three times.

**Cell Attachment and Spreading Assay**—Cell attachment and spreading was studied as described previously with modifications (33). Coverslips (18 × 18 mm, Menzel-Glaser, Braunschweig, Germany) were coated with human collagen type I (25 μg/ml in PBS) overnight at 4°C. Coverslips were blocked with 1% BSA and 0.1% glucose in Hepes/Tyrode buffer for 30 min at room temperature. Here the α2β1-expressing CHO cells and Jurkat cells were used at a concentration of 6 × 10^5 cells/ml in Iscove’s medium and were preincubated for 30 min in the presence or the absence of an activating agent. Coverslips were washed twice with Hepes/Tyrode buffer before the addition of 300 μl of cell suspension and incubated for either 10 or 60 min at room temperature. Coverslips were thoroughly washed to remove unbound cells and then fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. After washing, coverslips were stained with Mayer’s hematoxylin (2 min), followed by Fuchsin Ponceau (5 min), and mounted with DPX Mountant (Canemenco and Marivac Inc., Quebec, Canada). Adhesion to BSA-coated coverslips and adhesion of CHO-dhfr<sup>+</sup> cells to collagen-coated coverslips, used as negative controls, never exceeded 0.2–1.0% of the cell input.

**Double Immunofluorescence Labeling of Actin and α2β1**—Four-well LabTek cover glasses (Nunc A/S, Roskilde, Denmark) were coated with human collagen type I and blocked as described above. The α2β1-expressing CHO cells, at 6 × 10^5 cells/ml in Iscove’s medium, were preincubated for 30 min in the presence or absence of an activating agent, added to the cover glasses, and incubated for 60 min at room temperature. After washing, cells were fixed with 2% paraformaldehyde for 20 min at 37°C, followed by permeabilization in a 0.1% solution of Triton X-100 for 5 min at room temperature. Cells were incubated with 200 nM phalloidin/Texas Red to stain actin and 10 μg/ml IAC-1-FITC to stain activated α2β1 for 1 h at 37°C. As a positive control, cells were first incubated with 5 μg/ml AK7 for 1 h, followed by polyclonal rabbit anti-mouse IgG-FITC (diluted 1:50) for 1 h. As a negative control, 5 μg/ml MOPC-21-FITC was used. After washing, cells were mounted with Prolong Gold antifade with 4,6-diamidino-2-phenylindole (Molecular Probes).

Analysis was performed with an inverted Nikon Eclipse TE200 microscope connected to an Image Analyzer (Lucia, Laboratory Imaging Ltd., Analis, Namur, Belgium). Quantitative results were obtained by determining the number of adherent cells by light microscopy, and fluorescence microscopy was used to visualize actin and α2β1, using standard emission and excitation filters.

The number of adherent cells per coverslip was calculated as follows: (number of adherent cells at time point x/number of cells added per coverslip) × 100. Adherent cells are defined as cells that are immobilized to the substrate, independently of cell morphology. Cell morphol-
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INSIDE-OUT SIGNALING AND OUTSIDE MANIPULATION RESULT IN DIFFERENT CONFORMATIONAL STATES OF α2β1—We demonstrated previously that inside-out activation of human platelets by convulxin, for example, resulted in exposure of both the collagen-binding sites in inside-out activation of human platelets by convulxin, for example, Conformational States of α2β1. Here we extended this observation by manipulating the inside of the cell by using a cell-permeable peptide containing the KLGFFKR sequence of the neo-epitope of the receptor, recognized by the mAb IAC-1 (25). Here we extend this observation by manipulating the inside of the cell by using a cell-permeable peptide containing the KLGFFKR sequence of the neo-epitope of the receptor, recognized by the mAb IAC-1 (25). Here we extended this observation by manipulating the inside of the cell by using a cell-permeable peptide containing the KLGFFKR sequence of the neo-epitope of the receptor, recognized by the mAb IAC-1 (25).

**RESULTS**

Inside-out Signaling and Outside Manipulation Result in Different Conformational States of α2β1—We demonstrated previously that inside-out activation of human platelets by convulxin, for example, resulted in exposure of both the collagen-binding sites in α2β1 and a neo-epitope of the receptor, recognized by the mAb IAC-1 (25). Here we extended this observation by manipulating the inside of the cell by using a cell-permeable peptide containing the KLGFFKR sequence of the neo-epitope of the receptor, recognized by the mAb IAC-1 (25). Here we extended this observation by manipulating the inside of the cell by using a cell-permeable peptide containing the KLGFFKR sequence of the neo-epitope of the receptor, recognized by the mAb IAC-1 (25).

Statistical Analysis—Student’s t test for paired data were used to test statistically significant differences. Data given are the mean ± S.E. of the MFI of triplicate assays (B).

**FIGURE 2.** Effect of PGE1 on anti-P-selectin, collagen, and IAC-1 binding by platelets stimulated with the KLGFFKR peptide. Washed human platelets were incubated with the activating peptide H-VTVLALGALAGVGVGKLGFFKR-NH2 (200 μM) in the absence (black line or white bar) or presence (gray shaded or gray bar) of 5 μM PGE1 for 30 min at room temperature. Next, cells were incubated with either anti-P-selectin-PE (10 μg/ml), collagen-FITC (40 μg/ml), or IAC-1-FITC (10 μg/ml) for 30 min at room temperature. A representative histogram is shown for each cell type (A). Data are presented as the mean ± S.E. of the MFI of triplicate assays (B).

Up-regulation of α2β1, because no increase in MFI was observed when staining with the control mAbs Gi9 and AK-7, nor was a significant binding of the unrelated mouse anti-human IgG1 MOPC-21 observed (data not shown). Besides, addition of this peptide resulted in an overall up-regulation of platelets as determined by P-selectin exposure (Fig. 2). However, addition of PGE1 before addition of the peptide still induced collagen and IAC-1 binding without the exposure of P-selectin (Fig. 2), demonstrating that addition of the peptide per se induces conformational changes of α2β1, independent of general cell activation.

Most interestingly, by using this cell-permeable peptide we could for the first time induce inside-out activation of α2β1 on CHO cells. Indeed, the activated receptor on CHO cells bound both collagen and IAC-1 (Fig. 1). By using this heterologous cell line, we could therefore further prove the specificity of IAC-1 for activated α2β1.

Next, we investigated the binding of collagen and IAC-1 to platelets and α2β1-expressing CHO cells after outside manipulation with divalent cations (Mn2+ and Co2+), the reducing agent DTT or TS2/16, an activating anti-β1 antibody, and found that all resulted in a significant increase in collagen binding, indicating that α2β1 was activated (p < 0.05). Most interestingly, this outside manipulation was not accompanied by an increase in IAC-1 binding (Fig. 3). The different outside manipulations did not result in an up-regulation of α2β1 on the cells, as determined by Gi9 or AK-7 binding, nor was P-selectin exposed (data not shown). Hence, because these results demonstrate that the neo-epitope of IAC-1 is not exposed after outside manipulation in contrast to inside-out signaling, we presumed that at least two different active conformations of α2β1 exist.

The Different Conformational States of α2β1 Regulate Adhesion and Spreading to Collagen—To evaluate whether these two different active conformations have a functional role in cell adhesion, the adhesion of α2β1-expressing CHO cells to coated collagen type I was studied under static conditions.
First, the time course of cell adhesion to collagen type I was determined. Adhesion of nonactivated α2β1-expressing CHO cells to collagen increased with time and reached a maximum at around 60 min (Fig. 4A). When the cells were stimulated with either Mn2+ (outside) or the cell-permeable α2-cytosolic peptide H-VTVLALGALAGVGKVGLFFKR-NH2 (inside-out), cell adherence was significantly increased \((p < 0.05; \text{Fig. } 4A)\). Most interestingly, no significant difference in adhesion of α2β1-expressing CHO cells was observed between outside and inside-out-stimulated cells, indicating that adhesion of the two active conformations of α2β1 to coated collagen occurs with the same efficacy (Fig. 4A).

Next, the effect of outside and inside-out stimulation on cell spreading was evaluated as described before (33). Therefore, α2β1-expressing CHO cells, stimulated or not, were allowed to adhere to collagen-coated coverslips for 10 or 60 min, after which the cells were fixed and stained. Both outside and inside-out manipulation gave similar adhesion results as observed above (data not shown). Moreover, the adhesion to collagen was α2β1-specific because (i) CHO-dhfr+ cells did not adhere to collagen, and (ii) preincubation with the inhibitory mAb 15D7 almost completely abolished adhesion (data not shown). When the adherent cells were analyzed in more detail, an interesting observation was made. At 10 min, almost all adherent cells were simply attached to collagen, independent of activation, with 94 ± 1.3% of attached nonactivated cells and 98 ± 2.1% attached cells after outside and 96 ± 1.9% after inside-out stimulation, respectively. At 60 min, however, a significant proportion of the adherent α2β1-expressing CHO cells stimulated with Mn2+ (outside) obtained a spread morphology in contrast to nonactivated and inside-out stimulated α2β1-expressing CHO cells \((p < 0.05; \text{Fig. } 4B)\). These results indicate that the different active conformational states, induced after inside-out and outside manipulation, have a functional relevance in adhesion and spreading. Finally, we could demonstrate by fluorescent images that spread α2β1-expressing CHO cells, obtained after outside manipulation, did not stained positive for IAC-1 in contrast to attached α2β1-expressing CHO cells, obtained after inside-out manipulation (Fig. 5). Staining of α2β1-expressing CHO cells with the unrelated mouse anti-human Igg MOPC-21 did not result in a fluorescence signal (data not shown), and staining with the anti-α2 I domain mAb AK7 always resulted in a positive FITC signal, independent of stimulation (Fig. 5).

The Different Conformational States of α2β1 Are Cell Type-Dependent—Because the collagen receptor α2β1 is also present on other cell types, we included both PBMC and Jurkat cells to substantiate our discovery of several active conformational states of α2β1. We started by inducing inside-out signaling on these cell types with the synthetic peptide Ac-KRFYVVMWK-NH2, known to activate indirectly α2β1 on platelets and smooth muscle cells and αVβ3 on white blood cells by binding to the thrombospondin-1 receptor CD47 (29, 30, 35). As expected, incubation with Ac-KRFYVVMWK-NH2 resulted in a significant increase of both collagen and IAC-1 binding on platelets from 1.8 ± 0.8 to 29.7 ± 1.9% and from 1.5 ± 0.9 to 24.5 ± 1.5%, respectively \((p < 0.05; \text{Fig. } 6A)\). Moreover, addition of Ac-KRFYVVMWK-NH2 to PBMC was also able to induce both collagen and IAC-1 binding (Fig. 6), demonstrating that CD47 on white blood cells cannot only modulate β3 integrins but also the β1 integrin α2β1. This peptide, however, was not able to activate α2β1 on Jurkat cells because no increase in collagen nor IAC-1 binding was observed (Fig. 6). This is
consistent with previous reports where it has been suggested that the function of CD47, although highly expressed on Jurkat cells (36), appears to be integrin-independent (37, 38).

Next, we also used the cell-permeable α2-cytosolic peptide H-VTV-LALGALAGVGVGKLGFFKR-NH₂ to activate α2β1 on PBMC and Jurkat cells via inside-out signaling. For both cell types, this activation resulted in a significant increase in collagen and IAC-1 binding (p < 0.05; Fig. 7), similar to that described for platelets and α2β1-expressing CHO cells. Furthermore, the inside-out stimulation by peptides resulted in MAC-1 activation on PBMC, similar to the P-selectin exposure described for platelets (data not shown).

Just as for platelets and α2β1-expressing CHO cells, we stimulated PBMC and Jurkat cells via outside manipulation. Here we only used divalent cations for activation because TS2/16 had no effect on these cell types, and addition of DTT resulted in a marked down-regulation of α2β1, as determined by AK-7 binding (data not shown). The latter is consistent with previous reports describing that DTT can affect integrin expression on lymphocytes (39). Activation of α2β1 on PBMC and Jurkat cells by the divalent cations Mn²⁺ and Co²⁺ resulted in a significant increase in collagen binding, indicating that α2β1 was activated (p < 0.05; Fig. 8). Most surprisingly, and in striking contrast to platelets and α2β1-expressing CHO cells, this outside activation of α2β1 was accompanied by a significant increase in IAC-1 binding (p < 0.05; Fig. 8). Next, in line with α2β1-expressing CHO cells, a time course of adhesion to coated collagen type I was determined for the Jurkat cells. Also, a significantly increased cell adhesion after stimulation was observed, with no difference between outside and inside-out manipulation (Fig. 9A). Evaluation of the cell morphology of the adherent Jurkat cells revealed at every time point and after every stimulation merely attached cells (Fig. 9B). Taken together, these results demonstrated that the different conformational states of α2β1 are cell type-dependent and confirmed their functional relevance in adhesion and spreading.

**DISCUSSION**

During the last years, much progress has been made in unraveling the complexity of the broad spectrum of possible conformational states in integrins (9, 40, 41). Collective observations from electron microscopy, crystal structures, mutational introduction of disulfides, and other functional studies suggest that integrins may assume at least three different activation states, represented by different quaternary conformations as follows: (i) a closed conformation (resting state), not able to bind its natural ligand; (ii) an extended conformation with closed headpiece (low affinity state); and (iii) an extended conformation with open headpiece (high affinity state) (10, 15–17, 42). These two extended, active conformations are both favored by outside-in and inside-out signaling and can bind their natural ligand.

For the integrin α2β1, a major collagen receptor on platelets, no such experimental data were reported thus far, mainly because until recently,
no antibodies were available that specifically recognize activated or ligand-bound forms. We have developed mAb IAC-1, sensitive to the molecular conformation of \( \beta_2 \) because it only binds to the activated form of \( \alpha_2 \beta_1 \) on platelets, induced via inside-out signaling (25). The aim of the this study was to use this mAb IAC-1 to evaluate the effects of different inside-out and outside manipulations on the conformational state of \( \alpha_2 \beta_1 \).

First, the effect of inside-out and outside manipulations of \( \alpha_2 \beta_1 \) on platelets and CHO cells was investigated. For inside-out signaling, we designed a cell-permeable peptide containing the \( \beta_2 \)-cytoplasmic tail sequence KLGFFKR, known to be important for regulating inside-out activation of several integrins (6, 31, 43). Addition of this peptide resulted in activation of \( \alpha_2 \beta_1 \), as observed by a significant increase in collagen binding, and also exposed the neo-epitope of IAC-1. For outside manipulation, three different agents were used known to activate integrins as follows: the divalent cations Mn\(^{2+}\) and Co\(^{2+}\) (44–46); the reducing agent DTT, which breaks two disulfide bonds within the cysteine-rich domain of the \( \beta_2 \)-subunit (47, 48); and the activating anti-\( \beta_1 \) antibody TS2/16 (45). Manipulation of platelets and \( \alpha_2 \beta_1 \)-expressing CHO cells with those three activating agents resulted in a significant collagen binding, indicating that \( \alpha_2 \beta_1 \) on these cells became activated. Most interestingly, this collagen binding was not accompanied by IAC-1 binding, implying that the neo-epitope of IAC-1 is not exposed after outside manipulation. Hence, based on these data it can be suggested that \( \alpha_2 \beta_1 \) can be present in three different conformational states as follows: (i) the nonactivated, resting state with no collagen nor IAC-1 binding; (ii) an intermediate state, induced after outside manipulation, with collagen but no IAC-1 binding; and (iii) a fully activated state, induced after inside-out stimulation, with both collagen and IAC-1 binding. In addition, we hypothesize that these conformational states of \( \alpha_2 \beta_1 \) resemble the closed conformation, the extended conformation with closed headpiece, and the extended conformation with open headpiece, as already described for several integrins. However, more detailed research using electron microscopy and crystal structures is necessary to confirm this hypothesis.

For \( \alpha_2 \beta_2 \), the mAb MEM148 has been described that recognizes an epitope not expressed in the resting integrin state but exposed upon Mg\(^{2+}\)/EGTA treatment (49). Just recently, the epitope of this mAb has been mapped to a critical proline residue located on the inner face of the \( \beta_2 \) hybrid domain (50). In this study, \( \alpha_2 \beta_2 \)-expressing MOLT-4 cells could bind MEM148 after Mg\(^{2+}\)/EGTA treatment and were able to bind the ligand ICAM-1 as expected, but most interestingly, treatment with the activator phorbol ester 12-myristate 13-acetate (PMA) was not able to induce detectable expression of the MEM148 epitope on these
cells. This was rather unexpected because PMA is known to promote $\alpha_2\beta_1$ ligand binding (51). So it appears that the mAb MEM148 recognizes an epitope that becomes accessible after outside manipulation (by the divalent cation magnesium) but not after inside-out stimulation (by PMA). The specificity of this mAb for different conformational changes in the integrin is in line with our present data; however, we observed no binding of IAC-1 after outside manipulation but only after inside-out stimulation. Still, we could demonstrate also that our mAb IAC-1 can distinguish between conformational states of $\alpha_2\beta_1$ induced after different agonistic stimulations.

Next, we tried to determine whether these different conformational states would influence the function of $\alpha_2\beta_1$ and therefore allow $\alpha_2\beta_1$-expressing CHO cells, stimulated with an inside-out or outside agonist, to adhere to coated collagen for various periods of time. Although both activation ways clearly enhanced the adhesion of CHO cells to coated collagen as compared with resting cells, no significant difference in adhesion was observed between cells treated either way. More detailed investigation of this adhesion, however, revealed some interesting observations. First, $\alpha_2\beta_1$-expressing CHO cells manipulated with the outside stimulating agent Mn$^{2+}$ spread significantly more on coated collagen than nonactivated CHO cells. This is consistent with a study demonstrating that DTT stimulation (outside) is required to induce cell spreading of allb3-expressing CHO cells on coated von Willebrand factor (33). Second, and rather unexpected, we observed that $\alpha_2\beta_1$-expressing CHO cells manipulated with Mn$^{2+}$ spread significantly more than $\alpha_2\beta_1$-expressing CHO cells activated via inside-out signaling. There is some evidence for other integrins that certain conformational states are likely to be more suited for a transient adhesion like cell spreading, rolling, and migration, whereas other conformations favor a more stable adhesion. For example, it has been demonstrated that the integrin $\alpha_1\beta_2$ can support such a transient adhesion of leukocytes (namely rolling), together with selectins and $\alpha_4$ integrins, during inflammation in vivo (52). More recently, this observation could be linked to a certain conformational state of $\alpha_1\beta_2$. It was shown that an intermediate conformation of $\alpha_1\beta_2$, consisting of the extended state with closed headpiece, was responsible for a transient adhesion of leu-

FIGURE 8. Outside activation of $\alpha_2\beta_1$ on PBMC and Jurkat cells. Human PBMC or Jurkat cells were incubated without an agonist (gray shaded) or with Mn$^{2+}$ (1 mM, black line) or Co$^{2+}$ (1 mM, dotted gray line) for 30 min at room temperature. Next, cells were incubated with either collagen-FITC (40 $\mu$g/ml, gray bars) or IAC-1-FITC (10 $\mu$g/ml, white bars) for 30 min at room temperature. A representative histogram is shown for each cell type (A). Data are presented as the mean ± S.E. of the MFI of triplicate assays (B).
kocytes, whereas the fully open conformation with open headpiece appeared to be required for firm adhesion (53). Along the lines of these studies on αβ2, we now assume that the intermediate conformational state of αβ1 induced after outside manipulation is in a more suitable orientation to induce spreading on collagen than the fully activated state induced after inside-out stimulation. However, we still need a better insight in the physiological role of these conformational states of αβ1, especially in regard to the involvement of the different conformations of αβ1 during platelet thrombus formation in vivo.

Ultimately, we could link the flow cytometric observations on IAC-1 binding after the different stimulations to the observed effects on cell adhesion, because we could observe IAC-1 binding on attached cells after inside-out stimulation but no IAC-1 binding to spread cells after outside manipulation. The fact that cell adhesion was observed without IAC-1 binding has already been described previously for platelet adhesion to collagen under flow conditions (54).

Apart from studying the effect of outside manipulation on platelets and αβ1-expressing CHO cells, we also performed experiments with PBMC and Jurkat cells. Also, inside-out signaling of αβ2 always resulted in both collagen and IAC-1 binding. Most unexpectedly, outside manipulation with divalent cations not only resulted in collagen binding but was accompanied by an increase in IAC-1 binding, which is in contrast to what was seen upon outside manipulation of platelets or αβ1-expressing CHO cells. Although this discrepancy in IAC-1 binding after outside manipulation between platelets and αβ1-expressing CHO cells on the one hand and PBMC and Jurkat cells on the other hand remains to be clarified, it is known that integrins can display different ligand binding properties and functions, depending on the cell type on which the integrin is expressed (55). Similar to our findings with IAC-1, a very recent study reported that the exposure of the neo-epitope of MEM148 (already mentioned above) could vary after stimulation, depending on the cell type where the integrin is expressed (50). This study demonstrated that MEM148 could bind to αβ2-expressing MOLT-4 cells after Mg²⁺/EGTA stimulation but not after PMA treatment (50), which is in contrast to experiments performed previously, where αβ2-expressing myeloid cells did bind MEM148 after PMA stimulation (56).

In conclusion, the data presented here show that at least two different functionally active conformational states of αβ1 exist depending on the way the integrin is stimulated and on the cell type where αβ1 is expressed, and furthermore that these different conformations correlate with the different activities of the integrin.

Acknowledgments—We are grateful to Dr. N. Kieffer (Laboratoire de Biologie et Physiologie Intégrée, Université du Luxembourg, Luxembourg) for the αβ2-expressing CHO cells, Dr. T. Edgington (Scripps Institute, La Jolla, CA) for the antibody 7E3, and Dr. M. Kahn (University of Pennsylvania, PA) for the antibody TS2/16. We also acknowledge C. Boukaert and B. Strynck for histological assistance and P. Vandenberge for help with the cell cultures.

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Two Functional Conformations of the Integrin αβ1

In conclusion, the data presented here show that at least two different functionally active conformational states of αβ1 exist depending on the way the integrin is stimulated and on the cell type where αβ1 is expressed, and furthermore that these different conformations correlate with the different activities of the integrin.
Two Functional Conformations of the Integrin $\alpha_2\beta_1$

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