The glycosylphosphatidylinositol-linked urokinase-type plasminogen activator receptor (uPAR) interacts with the heterodimer cell adhesion molecules integrins to modulate cell adhesion and migration. Devoid of a cytoplasmic domain, uPAR triggers intracellular signaling via its associated molecules that contain cytoplasmic domains. Interestingly, uPAR changes the ectodomain conformation of one of its partner molecules, integrin \( \alpha_{\beta_1} \), and elicits cytoplasmic signaling. The separation or reorientation of integrin transmembrane domains and cytoplasmic tails are required for integrin outside-in signaling. However, there is a lack of direct evidence showing these conformational changes of an integrin that interacts with uPAR. In this investigation we used reporter monoclonal antibodies and fluorescence resonance energy transfer analyses to show conformational changes in the \( \alpha_M \beta_2 \) headpiece and reorientation of its transmembrane domains when \( \alpha_M \beta_2 \) interacts with uPAR.

The integrins are heterodimeric cell adhesion molecules, each formed by an \( \alpha \) and \( \beta \) subunit. Twenty-four specific pairs of integrins are expressed in humans. They serve as adhesion molecules and are bona fide signaling receptors. Integrin \( \alpha_M \beta_2 \) (Mac-1, CR3, CD11bCD18) is expressed primarily on cells of the myeloid lineage and natural killer cells (1, 2). Structural data of \( \alpha_M \beta_2 \) are lacking. A model of \( \alpha_M \beta_2 \) in a bent conformation was generated based on the crystal structure of \( \alpha_5 \beta_3 \) as template is shown to illustrate its domain organization in general (see Fig. 1). \( \alpha_M \beta_2 \) binds a multiplicity of ligands that include intercellular adhesion molecule 1, complement protein iC3b, blood coagulation protein fibrinogen, saccharides on microbes, and denatured proteins (4–7). It mediates adhesion, migration, phagocytosis, and degranulation of monocytes and neutrophils and has a role in immune tolerance (2, 8–12). In addition, the functional couplings of \( \alpha_M \beta_2 \) and partner molecules Fc\( \gamma \)RIIIB, Fc\( \gamma \)RIIA, low density lipoprotein receptor, and urokinase-type plasminogen activator receptor (uPAR) \( ^2 \) have been reported (13–16).

The glycosylphosphatidylinositol-anchored uPAR (CD87), its ligand urokinase plasminogen activator (uPA), and \( \alpha_M \beta_2 \) form a trimolecular complex that promotes fibrinolysis (17, 18). uPAR also interacts with the \( \beta_1 \) and \( \beta_3 \) integrins (19, 20). The uPAR-integrin complex is important in tumor biology and metastasis because of the proteolytic activity of uPA-uPAR and signaling capacity of the uPAR-integrin complex (21). Although uPAR does not contain a cytoplasmic domain, it can trigger cytosolic signaling by changing the shape of its integrin partner. uPAR changes the conformation of integrin \( \alpha_5 \beta_1 \), which promotes RGD-independent adhesion to fibronectin (22). Furthermore, the uPAR-\( \alpha_5 \beta_1 \) complex induces extracellular signal-regulated kinase signaling in tumor cells (23).

Integrins undergo marked conformational changes under specific cellular or extracellular conditions. One of these changes involves a switch-blade-like conversion from a bent to an extended conformation (24, 25). The integrin uPAR binding sites are located in the \( \beta_3 \)-propeller of the integrin \( \alpha_5 \) subunit, and the inserted (I)-like domain of the \( \beta_2 \) subunit (22, 26). The marked difference in the heights of uPAR and an extended integrin on a cell membrane does not favor such interaction as compared with an integrin that is bent (Fig. 1) (27, 28). uPAR changes the conformation of the \( \beta_1 \) ectodomain when it interacts with a bent \( \alpha_5 \beta_3 \) (22), but the molecular details of signal transmission at the C-terminal half of the integrin remain unclear. It was reported that the separation of the integrin \( \alpha_5 \beta_3 \) TMs is an important event for outside-in signaling (29). Here, we showed that the association of uPAR with a bent \( \alpha_M \beta_2 \) induces movement of the hybrid domain in the \( \beta_2 \) subunit and the reorientation of the \( \alpha_M \beta_2 \) TMs.

**EXPERIMENTAL PROCEDURES**

*Reagents and Antibodies*—The hybridoma of the mAb LPM19c (\( \alpha_M \)-specific, function blocking) (30, 31) was a generous gift from Dr. K. Pulford, LRF Diagnostic Unit, Oxford. The hybridomas of the mAbs KIM185 (\( \beta_2 \)-specific, activating) (32),

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\( ^2 \) The abbreviations used are: uPAR, urokinase-type plasminogen activator receptor; CFP, cyan fluorescent protein; mCFP, monomeric CFP; YFP, yellow fluorescent protein; mYFP, monomeric YFP; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; HA, hemagglutinin; PBS, phosphate-buffered saline; TMs, transmembrane domains; mAb, monoclonal antibody; DTSSP, 3,3’-dithiobis(sulfosuccinimidylpropionate); HRP, horseradish peroxidase; DMEM, Dulbecco’s modified Eagle’s medium.

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KIM127 (β2-specific, reporter mAB of extended β2 integrin) (33, 34), and IB4 (β2 integrins heterodimeric-specific) (35) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These mAbs were purified from culture supernatant using HiTrap™ protein A/G columns according to the manufacturer’s instructions (GE Healthcare). The mAb MEM148 (β2-specific, reporter mAB of β2 hybrid domain movement) (36) was purchased from AbD Serotec. The mAb VIM5 that recognizes domain 1 (D1) of uPAR was from BD Biosciences. The uPAR D2- and D3-specific mAbs and uPA (HMW-tc) were obtained from American Diagnostica Inc., Stamford, CT. The mouse anti-actin antibody was purchased from Sigma-Aldrich, CA. Human anti-vimentin antibody was purchased from Sigma-Aldrich, CA. Rabbit anti-HA antibody was purchased from Santa Cruz Biotechnology, CA. HRP-conjugated donkey anti-rabbit IgG, HRP-conjugated sheep anti-mouse IgG, and streptavidin-HRP were purchased from GE Healthcare. All general chemicals and reagents were purchased from Sigma-Aldrich unless stated otherwise.

**Expression Constructs**—The numbering of the integrin and uPAR amino acids is based on the mature protein as described in Barclay et al. (37). The amino acid numbering with the initiation Met considered as the first amino acid is also included and indicated in parentheses in Figs. 3, 4, and 6. The integrin αM and β2 pcDNA3 expression plasmids were described previously (35). uPAR cDNA that encodes the amino acid sequence Leu1–Thr313 having a stop codon introduced after Thr313 was amplified using the forward primer 5’-GAAGATCTCTCTCCGGGT-GCATGCACTGTG-3’ and reverse primer 5’-TCCCCGGCGTTCA-TTGGTTCCAGAAGGAG-3’ that contained the BglII and SacII restriction enzyme sites, respectively. The digested PCR product was ligated into the BglII and SacII sites of the pDisplay vector (Invitrogen) to allow the uPAR cDNA to be in-frame with the N terminus HA tag (in the pDisplay vector) to generate the expression plasmid HA-uPAR. The D1-deleted uPAR mutant referred to as D2D3 (Leu93–Thr313) was reported previously (35). It was cloned into the pDisplay vector to generate HA-D2D3 using the same procedure as for the full-length uPAR. The forward and reverse primers used were 5’-GAAGATCTCTCGAATGCATTTCCTGTGGC-3’ and 5’-TCCCCGGCTACTAGTGTCAGGAGAAGGAG-3’, respectively.

For fluorescence resonance energy transfer (FRET) experiments, the FRET pair fluorophores monomeric cyan fluorescent protein (mCFlp) and the monomeric yellow fluorescent protein (mYFP) were fused to the C termini of the integrin cytoplasmic tails. The αM mCFlp and β2 mYFP expression constructs were reported previously (38, 39). The αM mCFlp, αM mCFlp, αM mCFlp, αM mCFlp, αM mCFlp, and αM mCFlp constructs were generated using standard molecular biology procedures. All point mutations that introduce Cys into αM and β2 TMs were made using the QuikChange™ site directed mutagenesis kit (Stratagene, La Jolla, CA) with relevant primer pairs. All expression constructs were verified by sequencing (Research Biolabs sequencing service, Singapore).

**Cell Transfection—**293T and K562 cells (ATCC) were maintained in either DMEM medium or RPMI1640 medium, respectively, each containing 10% (v/v) heat-inactivated fetal bovine serum, and 100 IU/ml penicillin and 100 μg/ml streptomycin (Hyclone, Logan, UT) at 37 °C in a 5% CO2 atmosphere. Transient transfection of 293T was performed by the calcium phosphate method (35). 5 μg of plasmid was used for each indicated constructs unless otherwise stated. Transient transfection of K562 was performed by electroporation using the Amaza Nucleofector device and reagents as recommended by the manufacturer (Amaza Gmbh). αMmCFlp and variants (3.5 μg each), αM mCFlp (3.5 μg), β2 YFP and variants (3.5 μg each), HA-uPAR, and HA-D2D3 (2.7 μg each) were used unless otherwise indicated.

**Flow Cytometry and FRET Analyses**—Flow cytometry analyses of transfectants were performed essentially as described (35). Briefly, cells were stained with 20 μg/ml primary mAB followed by fluorescein isothiocyanate-conjugated sheep anti-mouse IgG (1:400 dilution) (Sigma). Stained cells were analyzed on a FACScalibur using the CellQuest software (BD Biosciences).

Photobleach FRET was performed essentially as reported (39). K562 transfectants expressing the integrin α and β sub-units with mCFlp or mYFP fused to their cytoplasmic tails were spun onto poly-l-lysine-coated glass slides. Photobleach FRET was performed on a Zeiss LSM510 confocal microscope with an oil immersion 63× objective (Carl Zeiss Inc., Thornwood, NY). mCFlp was excited with the 488-nm argon laser line, and the emission signal was detected with a BP 500–550-nm emission filter. mYFP was excited using the 514-nm argon laser line, and the emission signal was detected with a LP 530-nm emission filter. mCFlp and mYFP were excited, and emission signals were detected for 10 time points. The time interval between each time point was 3 s, except that between the fifth and sixth time points, when photobleaching of mYFP was performed. For mYFP photobleach, the entire cell was scanned 20 times with the 514 argon laser line set at the maximum intensity. The duration of the photobleach was ~60 s depending on the size of the cell that was scanned. For analysis, only the cell membrane was selected as the region of interest. Fluorescent signals that were detected in the perinuclear region of the cell (integrins in the Golgi) were not considered. The mCFlp signals in the region of interest at the fifth time point (pre-mYFP photobleach) and sixth time point (post-mYFP photobleach) were acquired. FRET efficiency (Ef) was calculated as a percentage based on the equation $E_f = \left( \frac{I_n - I_0}{I_0} \right) \times 100/100$, where $I_n$ is the mCFlp intensity at the nth time point. Similar analyses of cells that
were not subjected to mYFP photobleach (referred to as unbleached) were included and plotted against samples that were subjected to photobleach. The mean noise computed as \( N_p = (I_o - I_p) \times 100/I_p \) in which the mCFP signals at the fourth and fifth time points before the bleaching process was close to zero in all cases. For experiments involving uPA, transfectants were incubated in RPMI medium containing 0.1% (w/v) bovine serum albumin and 100 nM uPA for 45 min at 37 °C (35). Thereafter, unbound uPA was removed by centrifuging cells through a heat-inactivated fetal bovine serum cushion. Cells were later spun onto poly-l-lysine slides and subjected to FRET analyses.

**Cell Adhesion Assays**—Adhesion of K562 transfectants to human fibrinogen (Sigma) was performed as reported (35). Briefly, Polysorb microtiter well (Nunc, Roskilde, Denmark) was coated with fibrinogen (250 μg/ml) in 50 mM bicarbonate buffer, pH 9.2, overnight at 4 °C. Nonspecific binding sites were blocked with 0.2% (w/v) polyvinylpyrrolidone (PVP) buffer, pH 9.2, overnight at 4 °C. The labeling reaction was terminated by washing the cells in PBS containing 10 mM Tris-HCl, pH 8.0, and 0.1% (w/v) bovine serum albumin. For reporter mAb immunoprecipitation analysis, the labeled cells were incubated in DMEM medium containing 5% (v/v) heat-inactivated fetal bovine serum albumin and 100 nM uPA for 45 min at 37 °C (35). Thereafter, unbound uPA was removed by washing the cells twice in the medium containing 5% (v/v) heat-inactivated fetal bovine serum albumin. For reporter mAb immunoprecipitation analysis, the labeled cells were incubated in DMEM medium containing 5% (v/v) heat-inactivated fetal bovine serum albumin and 100 nM uPA for 45 min at 37 °C (35). Thereafter, unbound uPA was removed by washing the cells twice in the medium containing 5% (v/v) heat-inactivated fetal bovine serum albumin.

**Cell Surface Protein Biotinylation, Immunoprecipitation, and Detection**—Labeling of cell surface proteins with biotin was performed as described previously (39). 293T transfectants were washed twice in PBS and incubated in PBS containing 0.5 mg/ml sulfo-NHS-biotin (Pierce) for 15 min at room temperature. The labeling reaction was terminated by washing the cells in PBS containing 10 mM Tris-HCl, pH 8.0, and 0.1% (w/v) bovine serum albumin. For reporter mAb immunoprecipitation analysis, the labeled cells were incubated in DMEM medium containing 5% (v/v) heat-inactivated fetal bovine serum and 10 mM HEPES (DMEM-FH) with the relevant mAb (3 μg each) in the presence or absence of 2 mM MnCl₂ (referred to as Mn²⁺ in main text) for 30 min at 37 °C. The unbound mAbs were removed by washing the cells twice in the DMEM-FH and lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet-P 40) with protease inhibitors (Roche Applied Science) for 30 min at 4 °C. Integrins bound to the mAbs were precipitated with protein A-Sepharose beads (GE Healthcare). Precipitated proteins were resolved on 7.5% SDS-PAGE under reducing conditions, electroblotted onto polyvinylidene difluoride membrane (Millipore, Billerica, MA), and probed with streptavidin-conjugated HRP (GE Healthcare). Protein bands were visualized by enhanced chemiluminescence (ECL) using the ECL kit (GE Healthcare) according to the manufacturer’s instructions.

For the cross-linking study, transfectants were incubated in PBS containing 0.5 mg/ml cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce) for 30 min at room temperature. The reaction was quenched by washing cells in PBS containing 1 mM Tris, pH 7.5. Cells were incubated in DMEM-FH with 3 μg each of control mouse IgG or KIM185 for 30 min at 37 °C. Unbound antibodies were removed by washing cells twice in DMEM-FH. Cells were lysed, and bound mAbs were precipitated as described above. Precipitated proteins were resolved on 6% SDS-PAGE under nonreducing (−dithiothreitol (−DTT)) conditions or on 10% SDS-PAGE under reducing (+DTT) conditions. The HA-uPAR cross-linked to α₅β₃ was detected by immunoblotting with anti-HA antibody. Protein bands were detected using HRP-conjugated donkey anti-rabbit IgG followed by ECL.

For examining integrins with cysteine-lock TMs, transfectants were labeled with sulfo-NHS-biotin as described above. Instead of using reporter mAbs, 3 μg of mAb IB4 was added to cell lysate and incubated for 1 h at 4 °C to immunoprecipitate the integrins. Thereafter, proteins were precipitated with protein A-Sepharose and resolved on 6% SDS-PAGE under either nonreducing conditions or on 7.5% SDS-PAGE under reducing conditions followed by ECL detection aforementioned. For HA-uPAR communoprecipitation analyses, transfectants were incubated in DMEM-FH with the relevant mAb (3 μg each) for 30 min at 37 °C. After washing in DMEM-FH, cells were lysed in lysis buffer. The HA-uPAR-integrin complex was precipitated as described above and resolved on 10% SDS-PAGE under reducing conditions. To detect HA-uPAR that co-precipitated with the integrins, blots were probed with anti-HA antibody followed by HRP-conjugated donkey anti-rabbit IgG. Protein bands were visualized by ECL.

For the detection of actin in cell lysates of transfectants, lysates were immunoblotted with mouse anti-actin antibody followed by HRP-conjugated sheep anti-mouse IgG. Protein bands were visualized by ECL.

**RESULTS**

**Interaction with uPAR Induces the Movement of the α₅β₃ Hybrid Domain**—The association of uPAR with α₅β₃ has been reported (14, 17, 26, 35, 40). Here, we further verify the association of uPAR with α₅β₃ in 293T transfectants. 293T cells were transiently transfected with α₅β₃ alone, α₅β₃ with HA-uPAR, or α₅β₃ with HA-D2D3 (uPAR without D1). HA-D2D3 was included as a control because we reported previously that D1 of uPAR is critical for its association with α₅β₃ (35). Flow cytometry was performed, and the expression of α₅β₃ was detected by mAb KIM185 that is β₃ subunit-specific and an activating mAb (32), and the expressions of HA-uPAR and HA-D2D3 were detected by anti-uPAR D2-specific mAb (Fig. 2A). Comparable levels of α₅β₃ were detected in all transfectants, and a high level of HA-uPAR and HA-D2D3 was detected in respective transfectants.

Next we performed chemical cross-linking experiment using the water-soluble and membrane-impermeable compound DTSSP containing two NHS-ester functional groups that react with primary amines and which has a thiol-cleavable spacer (37 Å). The close proximity of uPAR with the integrins would allow cross-linking of both molecules by DTSSP. 293T transfectants were incubated in PBS containing DTSSP followed by immunoprecipitation as described under “Experimental Procedures” (Fig. 2B). Immunoprecipitated proteins were resolved on denaturing SDS-PAGE under nonreducing (−DTT) or reducing (+DTT) conditions. Under nonreducing conditions an apparent high molecular weight HA-uPAR was detected only in the KIM185 precipitate of α₅β₃/HA-uPAR transfectants. We conjectured
FIGURE 2. Conformation of αMβ2 when it interacts with uPAR. A, flow cytometry analyses of 293T cells transiently transfected with αMβ2, αMβ2, with HA-uPAR, or αMβ2 with HA-D2D3. To detect αMβ2 expression, cells were stained with mAb KIM185 (β2-specific). To detect HA-uPAR and HA-D2D3 expressions, cells were stained with anti-uPAR mAb (D2-specific). Cells were subsequently stained with fluorescein isothiocyanate-conjugated secondary antibody followed by flow cytometry analyses. Shaded histograms represent staining with KIM185 or anti-D2 (uPAR). Open histograms, irrelevant mAb. B, cross-linking of αMβ2 and HA-uPAR on 293T transfectants. Cells expressing αMβ2, αMβ2 with HA-uPAR, or αMβ2 with HA-D2D3 were incubated in PBS containing the chemical DTSSP for 30 min at room temperature. Thereafter, cells were subjected to immunoprecipitation with KIM185 as described under “Experimental Procedures.” An irrelevant mAb was included as control. Precipitated proteins were resolved on a 6% SDS-PAGE under nonreducing conditions (–DTT) or on a 10% SDS-PAGE under reducing conditions (DTT). Detection of HA-uPAR was performed by anti-HA immunoblotting followed by ECL. C, flow cytometry analyses of 293T transfectants expressing αMβ2, with or without HA-uPAR. mAb KIM185 was employed to detect αMβ2 expression, and mAb VIM5 (D1-specific) was used for HA-uPAR detection. Shaded histograms represent staining with KIM185 or VIM5. Open histograms, irrelevant mAb. D, the conformation of αMβ2, interacting with uPAR was assessed by immunoprecipitation analyses. 293T transfectants expressing αMβ2, or αMβ2 and HA-uPAR were surface-biotinylated, lysed, and immunoprecipitated with mAbs KIM127, MEM148, or KIM185. Immunoprecipitated proteins were resolved on a 7.5% SDS-PAGE gel under reducing conditions. Biotin-labeled αMβ2 subunits were probed with streptavidin-IRP and detected by ECL. E, conformational change in αMβ2 when it interacts with uPAR was assessed by a different approach. 293T transfectants bearing αMβ2 and HA-uPAR were immunoprecipitated with the indicated mAbs, and HA-uPAR that co-precipitated with αMβ2 was detected by immunoblotting with anti-HA antibody. Proteins were resolved on a 10% SDS-PAGE gel under reducing conditions. IP, immunoprecipitation.

that the high molecular weight signal detected for HA-uPAR was because of its association with αMβ2 covalently cross-linked by DTSSP (uPAR ~ 50 kDa, αMβ2 ~ 170 kDa, β2 ~ 95 kDa; uPAR-αMβ2 ~ 315 kDa). When DTT was included, HA-uPAR was dissociated from the KIM185-immunoprecipitated αMβ2, and it migrated as an ~50-kDa protein band. By contrast, HA-D2D3 was not detected in the KIM185 precipitate of αMβ2/HA-D2D3 transfectants, which was consistent with the requirement of D1 for the interaction of uPAR with αMβ2 (35). Thus, the physical association of uPAR with αMβ2 can be detected in the 293T transfection system.

The integrin headpiece comprises from the α subunit the inserted (I) domain (for I domain-containing integrins that include αMβ2; this domain is also referred to as the A domain), β-propeller, and thigh domain and from the β subunit the I-like domain, hybrid domain, plexin-semaphorin-integrin, and integrin epidermal growth factor 1 (Fig. 1) (24). Three distinct integrin conformations have been reported based on electron microscopy analyses of αMβ2 and αMβ2. These are the bent conformer, the extended conformer with a closed headpiece, and an extended conformer with an open headpiece (41).

One of the key features distinguishing a closed headpiece from an open headpiece lies in the orientation of the hybrid domain. In the closed headpiece the hybrid domain and plexin-semaphorin-integrin are in juxtaposition to the α subunit, and in the open headpiece they “swing-out” (42) by 62° in the crystal structure of ligand-mimetic bound platelet integrin αMβ2 (43).

We examined whether αMβ2 interaction with uPAR induces conformational change to the integrin headpiece. 293T cells were transfected with αMβ2 or αMβ2/HA-uPAR. Cell surface expressions of αMβ2 and HA-uPAR were determined by flow cytometry analyses using the mAb KIM185 and mAb VIM5, another uPAR-specific mAb, but it recognizes D1 of uPAR (Fig. 2C). Comparable expressions of αMβ2 were detected in both transfectants, and high levels of HA-uPAR were detected in αMβ2/HA-uPAR transfectants.

We then performed immunoprecipitation analyses with β2 integrin activation reporter mAbs. 293T cells expressing αMβ2 or αMβ2/HA-uPAR were surface-labeled with biotin, and αMβ2 was immunoprecipitated with mAbs MEM148, KIM127, and
uPAR Induces αMβ2 Conformational Changes

KIM185. The mAb MEM148 was shown to recognize a neoepitope in the hybrid domain of the β2 subunit that is masked in the absence of hybrid domain movement (36). Hybrid domain movement is a key feature of an integrin with an open headpiece (24). The mAb KIM127 recognizes a neo-epitope in the I-EGF2 of the β2 subunit and is masked in the bent conformation but expressed in the extended conformation (33, 34). KIM185 was also included in the analysis as a control, and its epitope lies in the β2 I-EGF4/β-tail domain region (44). Transfectants were incubated in media containing these mAbs with or without Mn2+, which activates αmβ2 (45), for 30 min at 37 °C before immunoprecipitation was performed. KIM127 and MEM148 did not immunoprecipitate a significant level of αmβ2 heterodimer from lysate of cells transfected with αmβ2 (Fig. 2D). The β2 signal detected in the MEM148 sample was attributed to unassociated β2 as described previously (46). When supplemented with Mn2+, a high level of αmβ2 was precipitated by KIM127 and MEM148. The control mAb KIM185 precipitated αmβ2 without requirement of Mn2+ treatment. These data showed that under resting conditions, αmβ2 is in a bent conformation with a closed headpiece because of the lack of reactivity of αmβ2 with KIM127 and MEM148. When cells co-expressing αmβ2 and HA-uPAR were analyzed, the profiles of KIM127 and KIM185 samples were similar to that of cells expressing αmβ2 only, but significant αmβ2 signal was detected by MEM148 even without Mn2+ treatment. The addition of Mn2+ further increased the amount of αmβ2 precipitated by MEM148. These data suggest that when uPAR interacts with αmβ2, the hybrid domain of β2 was displaced as reported by MEM148, although it was also apparent that the population of αmβ2 with hybrid domain displacement was relatively small in the presence of uPAR interaction.

To further verify that the interaction with uPAR induces hybrid domain movement in αmβ2, co-immunoprecipitation analysis was performed (Fig. 2E). The rationale of this experiment was that if the interaction with uPAR induces shape changes in αmβ2, uPAR would be co-precipitated with the αmβ2 that has undergone conformational change using the relevant reporter mAb. 293T cells transfected with αmβ2 and HA-uPAR were lysed and subjected to immunoprecipitation with the indicated mAbs. HA-uPAR that co-precipitated was probed and detected with anti-HA immunoblotting. The mAb LPM19c that recognizes an epitope in the αm I domain was also included as a control (47). Expression of HA-uPAR was detected in total cell lysate of transfected but not untransfected cells. HA-uPAR was not detected in the control Ig lane and was poorly detected in lanes of LPM19c and KIM127. The lack of interacting HA-uPAR with αmβ2 in samples containing LPM19c was consistent with a previous report and possibly due to the masking of αm I domain by the partner uPAR (35). The lack of HA-uPAR co-precipitating with αmβ2 in the sample of KIM127 and the presence of significant HA-uPAR in samples of KIM185 and MEM148 were consistent with that aforementioned. Thus, these data lend support to the concept that uPAR interaction with αmβ2 induces an open headpiece with hybrid domain displacement, but it retains an overall bent conformation.

Interaction with uPAR Induces the Separation of the αmβ2 TMs—We next examined whether interaction of uPAR with αmβ2 could induce the separation or reorientation of the αmβ2 TMs, which is an important event in integrins αmβ2 and αmβ3 outside-in signaling (29, 38), and this is relevant to the possible mechanism by which uPAR signals through its partner integrins. To test this hypothesis we made use of photobleach FRET detection method by fusing mCFP and mYFP to the C termini of integrin cytoplasmic tails (Fig. 3A) (38). mCFP and mYFP will be referred to as CFP and YFP henceforth. In the integrin α subunit expression construct, a five-amino acid linker was engineered between the mCFP and the last amino acid of the cytoplasmic tail. In the integrin β subunit, a six-amino acid linker was introduced. It was conceived that the close proximity of the cytoplasmic tails, which suggest proximity of the TMs, would allow effective FRET. A decrease in FRET would ensue if the TMs are separated, which leads to the separation of cytoplasmic tails.

When first tested, we observed that the integrin FRET pair αmCFP and β2YFP in a K562 transfectant system described previously (38, 39) failed to show a significant increase in the fluorescence intensity of CFP after YFP photobleaching when compared with cells that were not subjected to photobleach (unbleach) (Fig. 3B). This could be attributed to the marked length difference of the αm and β2 cytoplasmic tails. We noted that FRET was used to demonstrate constitutive heterodimerization of αmβ2 in adherent CHO and 293T cells and was proposed to be cell-type-independent (48). However, in our hands, we were unable to obtain interpretable plasma membrane FRET data because of the uneven morphology of 293T cells. Furthermore, the authors also reported possible conformational differences between αmβ2 expressed in Chinese hamster ovary and 293T cells (48). Thus, we retained the use of non-adherent K562 cells but with re-engineered integrin cytoplasmic tails. Previously, we analyzed FRET of αmβ2 cytoplasmic tails in transfected K562 (39). Hence, we re-engineered αm with its cytoplasmic tail substituted with that of αL having a C terminus CFP, referred to as αmL (Fig. 3A). Cells transfected with αmLCFPβ2YFP showed an increase in CFP signal when YFP was photobleached (Fig. 3B), suggesting proximity of the cytoplasmic tails and, reasonably, the association of the αmβ2 TMs. To provide ease in comparison and with more cells analyzed, we plotted % FRET efficiency as described under “Experimental Procedures,” and these plots were used in all subsequent figures. When compared with cells expressing αmLCFPβ2YFP, cells expressing αmLCFPβ2YFP showed higher FRETs (Fig. 3C). Unbleached sample was also plotted for comparison. To exclude the possibility that FRET was detected due to micro-clustering of the integrins in K562 or due to sample preparation, we transfected K562 cells with αmLCFP, αmLYFP, and β2. If micro-clustering occurs, it is likely that the close proximity of αmLCFPβ2 and αmLYFPβ2 will lead to detectable FRET. Included for comparison were cells transfected with αmCFP, αmYFP, and β2. Whereas significant FRET was detected for cells expressing αmLCFPβ2YFP, markedly less FRET was detected in cells expressing αmLCFP, αmLYFP, and β2 and expressing αLCFP, αLYFP, and β2. These data suggest that the FRET detected in αmLCFPβ2YFP was due primarily to intramolecular
cytoplasmic tails interaction rather than microclustering. In addition, we could infer that the TMs of the integrin are in close juxtaposition.

We then analyzed the effect of uPAR on \( H_2M_2 \) TMs. uPAR contains three domains, D1, D2, and D3 (Fig. 1). We showed previously that D1 is required for uPAR interaction with \( \alpha_\text{M} \beta_2 \) because the deletion of D1 abrogated association between uPAR and \( \alpha_\text{M} \beta_2 \). The expression and identity of full-length HA-uPAR and HA-D2D3 were re-assessed in K562 transfectants with a panel of uPAR domain-specific mAbs followed by flow cytometry analyses (Fig. 4A). Although cells expressing full-length HA-uPAR stained positive with all three mAbs, cells bearing HA-D2D3 only showed positive staining with anti-D2 and anti-D3 mAbs. Next, FRET analyses were performed on K562 expressing full-length HA-uPAR or HA-D2D3 with \( \alpha_\text{M}_{\text{L}} \text{CFP}\beta_2\text{YFP} \) (Fig. 4B). Cells expressing only the integrins and cells expressing integrins with HA-D2D3 showed comparable levels of FRET. By contrast, a significant diminution of FRET was observed for cells expressing integrins with full-length HA-uPAR. These data suggest the separation of the integrin cytoplasmic tails in uPAR co-expressing cells, and they suggest reorientation or separation of the \( \alpha_\text{M} \beta_2 \) TMs in these cells.

The reduction in FRET signal of \( \alpha_\text{M}_{\text{L}} \text{CFP}\beta_2\text{YFP} \) in the presence of HA-uPAR was not an aberrant effect of HA-uPAR on the \( \alpha_\text{M} \) cytoplasmic tail that was engineered into \( \alpha_\text{M} \) because HA-uPAR had minimal effect on the FRET signal of \( \alpha_1 \) CFP\( \beta_2 \) YFP on K562 transfectants (Fig. 4C). It was also noted that in \( \alpha_\text{M}_{\text{L}} \text{CFP}\beta_2\text{YFP} \), the cytoplasmic tail substitution was made from \( \alpha_\text{M} \) Lys1114 onward (Figs. 3A and 4D). Recently, NMR studies reveal that the first charged residue Lys on the intracellular side of \( \alpha_\text{IIb} \) and \( \alpha_\text{III} \) does not demarcate the C-terminal end of the transmembrane domains (49, 50). In \( \alpha_\text{M} \), two residues that are located after Lys989, namely Phe992 and Phe993, loop back into the intracellular side of the plasma membrane (49). Comparison of \( \alpha_\text{M} \) and \( \alpha_1 \) membrane proximal sequences reveals an amino acid difference (shaded in black) between \( \alpha_\text{M} \) Lys1114–Arg1120 and \( \alpha_1 \) Lys1088–Arg1094 (Fig. 4D). The \( \alpha_\text{M}_{\text{L}} \text{CFP} \) may have a disrupted membrane insertion due to the substitution of \( \alpha_\text{M} \) Leu1115 with Val. This may complicate the analyses of \( \alpha_\text{M}_{\text{L}} \text{CFP}\beta_2\text{YFP} \) inter-

**FIGURE 3. FRET analyses to detect \( \alpha_\text{M}/\beta_2 \) TM movement.** A, schematic illustrating integrins with different mCFP and mYFP fusions. The linker residues between the cytoplasmic tails and the FRET pair fluorophores are shown. For ease of reference, the amino acids flanking each cytoplasmic tail are numbered according to the mature protein (top row) and numbered by considering the initiation Met as the first amino acid (bottom row and in parentheses). B, representative fluorescence intensity plots of K562 transfectants expressing integrins with FRET pair fusions pre- and post-YFP photobleach. The time interval between each point is 3 s, except that between the 5th and 6th time points, when the photobleaching was conducted the duration was 60 s as it varies with cell size. C and D, % FRET efficiency plots of integrin cytoplasmic tail mutants. Data are representative of two independent experiments. Data show the mean ± S.E. for 15 cells analyzed. Student’s t test, assuming unequal variance, was used for statistical analyses. *, p < 0.001.
**FIGURE 4.** uPAR induces reorientation or separation of the αMβ2 TMs. A, expressions of HA-uPAR and HA-D2D3 on 293T transfectants were determined by immunostaining with domain-specific mAbs (shaded histograms) followed by flow cytometry analyses. Open histogram (irrelevant mAb). B, FRET analyses of αMβ2 TM reorientation or separation in K562 transfectants. Cells were transiently transfected with αM CFPβ2 YFP followed by FRET detection. Included in the analyses was co-expression of HA-uPAR or HA-D2D3 with αM CFPβ2 YFP. Data show the mean ± S.E. for 20 cells analyzed. C, FRET analyses of K562 cells transfected with αM CFPβ2 YFP in the presence or absence of HA-uPAR co-expression. Data show the mean ± S.E. for 14 cells analyzed. D, FRET analyses of K562 cells expressing αM CFPβ2 YFP with the co-expression of HA-uPAR or HA-D2D3. In αM CFPβ2, the cytoplasmic tail substitution of αM with that of αL was made after Arg1120. Data show the mean ± S.E. for 15 cells analyzed. In the schematic illustrating the membrane proximal sequences of αM and αL cytoplasmic tail, αM Leu1115 and αL Val1089 are in black boxes. Amino acid numbers according to the mature protein (top row) and with initiation Met as the first amino acid (bottom row, parentheses) are shown. E, FRET analyses of K562 cells expressing αM CFPβ2 YFP and HA-uPAR in the presence of uPA (100 nM). Data show the mean ± S.E. for 20 cells analyzed. Data are representative of two independent experiments. Student’s t test, assuming unequal variance, was used for statistical analyses. F, 293T transfectants expressing αMβ2 and HA-uPAR were incubated in medium containing uPA (100 nM) for 45 min at 37°C. Immunoprecipitation analyses were performed with the indicated mAbs, and HA-uPAR that co-precipitated with αMβ2 was detected by immunoblotting with anti-HA antibody. Proteins were resolved on 10% SDS-PAGE under reducing conditions. IP, immunoprecipitation. *, p < 0.001.
uPAR Induces $\alpha_M^\beta_2$ Conformational Changes

acting with uPAR. Thus, we have generated an additional chimera $\alpha_{ML}^*$CFP in which the cytoplasmic tail exchange between $\alpha_M$ and $\alpha_l$ was made after the GFFKR sequence. FRET analyses of $\alpha_{ML}^*$CFP$\beta_2$YFP in the presence of HA-uPAR or HA-D2D3 were performed. The FRET profile of $\alpha_{ML}^*$CFP$\beta_2$YFP (Fig. 4D) was similar to that of $\alpha_{ML}^*$CFP$\beta_2$YFP (Fig. 4B). Thus, we reasoned that $\alpha_M$, Leu$^{115}$ replaced by Val in $\alpha_{ML}$CFP had minimal precocous effect at least on the $\alpha_{ML}^*$CFP$\beta_2$YFP FRET studies performed herein.

uPA forms a trimolecular complex with uPAR and $\alpha_M^\beta_2$, and it initiates $\text{Ca}^{2+}$ signaling in neutrophils (51). The addition of uPA to cells expressing $\alpha_{ML}^*$CFP$\beta_2$YFP and HA-uPAR did not show any significant FRET difference from cells without uPA, albeit avid uPA binding on these cells (Fig. 4E and data not shown). We went further to examine the effect of uPA on $\alpha_M^\beta_2$ that is associated with HA-uPAR by the method of immunoprecipitation with the reporter mAbs MEM148 and KIM127 (Fig. 4F). The profile of the immunoprecipitation data was similar to that of $\alpha_{ML}^\beta_2$/HA-uPAR transfectants in the absence of uPA (Fig. 2E). However, we do not preclude uPA inducing further structural changes in the $\alpha_M^\beta_2$ TMs and ectodomain that may not be detected using our present method of FRET analyses and immunoprecipitation assays with the reporter mAbs available to us.

Next, the expression of uPAR is low on $\alpha_M^\beta_2$-expressing monocytes, but its expression can be up-regulated markedly when these cells are treated with *Escherichia coli* lipopolysaccharide or *Mycobacterium tuberculosis* lipoarabinomannan (52). In this study the expression of uPAR is comparatively high. We asked whether reduction of uPAR expression would also exert a similar effect on the conformation of $\alpha_M^\beta_2$, as shown in previous sections but at a reduced level. To this end we performed FRET analyses of K562 expressing $\alpha_{ML}^*$CFP$\beta_2$YFP and different levels of HA-uPAR (by transfecting the same amount of $\alpha_{ML}^*$CFP$\beta_2$YFP but different amounts of HA-uPAR). A reduction of HA-uPAR expressed was detected in cell lysates of transfectants with decreasing amounts of plasmid HA-uPAR transfected (Fig. 5A). An anti-actin blot was included as a control. The expressions of $\alpha_{ML}^*$CFP$\beta_2$YFP in these transfectants were comparable as determined by confocal microscopy analyses (data not shown). When these cells were subjected to FRET analyses, a decrease in HA-uPAR expression had a lesser effect on $\alpha_{ML}^*$CFP$\beta_2$YFP FRET signal (Fig. 5B). We extended the analyses by performing reporter mAb immunoprecipitation of $\alpha_M^\beta_2$ when co-expressed with HA-uPAR using 293T transfectants. A reduction in HA-uPAR plasmid transfected led to a decrease in HA-uPAR expression in 293T transfectants as determined by flow cytometry analyses using mAb VIM5 and immunoblotting with anti-HA antibody (Fig. 5C). The anti-actin control blot was included. The expressions of $\alpha_M^\beta_2$ in these transfectants were comparable as determined by flow cytometry analyses (data not shown).
**uPAR Induces α_Mβ_2 Conformational Changes**

Disulfide Clasp in the α_Mβ_2 TMs Prevent Their Separation Induced by uPAR—To further validate the separation of the α_Mβ_2 TMs induced by uPAR, we sought to generate disulfide clasp α_Mβ_2 mutants. In these mutants, the extracellular membrane proximal residues of α_Mβ_2 TMs were mutated singly to Cys to allow disulfide bond formation between permissible pairs of introduced Cys as indicated (Fig. 6A). Clasping of TMs by engineered disulfide bonds has been shown to attenuate α_iβ_j outside-in signaling (29, 53). Transfectants bearing the α_MιCFβ_2YFP Cys mutants were surface-labeled with biotin, lysed, and immunoprecipitated with the mAb IB4 that is specific to heterodimeric β integrins (35). Integrins precipitated were resolved on SDS-PAGE under nonreducing (−DTT) or reducing (+DTT) conditions (Fig. 6B). Under nonreducing conditions, high molecular weight protein bands were detected that corresponded to integran heterodimers that were covalently linked. Except for the cysteine mutant pair that contained α_MιL1091C, the α_MιP1092C and α_MιL1093C showed a propensity to form disulfide-bonded heterodimer with β_1679C, β_2A680C, β_2A681C, and β_2J682C. Under reducing conditions, only three of these heterodimers were separated into their individual α_MιCF and β_2YFP subunits. It was not clear why the others showed a much lower level of individual subunits. However, one of these pairs, α_MιL1093C β_2A680C, was selected for subsequent analyses. This cysteine clasp will be referred to as c-c in subsequent discussion.

Before proceeding to test the effect of c-c clasp on the α_Mβ_2 TMs induced by uPAR, we verified that the clasp did not alter the ligand binding function and conformation of the α_Mβ_2. K562 cells transfected with wild-type α_Mβ_2 or α_Mβ_2c-c (Fig. 6C) were allowed to adhere to the α_Mβ_2 ligand fibrinogen (Fig. 6D). Cells expressing α_Mβ_2c-c showed a similar adhesion profile to cells expressing wild-type α_Mβ_2 with minimal adhesion to fibrinogen in the absence of activation, and a high level of adhesion was detected when the β_2 activating mAb KIM185 was included. Adhesion specificity was demonstrated with the heterodimeric-specific and function-blocking mAb IB4. Thus, the c-c clasp did not affect the cell surface expression and the ligand binding function of the α_Mβ_2 ectodomain.

Next, we analyzed the ectodomain conformation of the α_Mβ_2c-c co-expressed with HA-uPAR (Fig. 7A). The profile of HA-uPAR co-precipitating with α_Mβ_2c-c was similar to that with wild-type α_Mβ_2 (Fig. 2E). α_Mβ_2c-c interacting with HA-uPAR most possibly retained a bent conformation because

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**FIGURE 6. Generation of α_Mβ_2 with a disulfide clasp in its TM.** A, illustration of possible disulfide bonds that can be formed between the 4 β_2 and 3 α_M membrane proximal TM residues when mutated to Cys. For ease of

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**reference two sets of amino acid numbering are shown based on the mature protein (top row) or the initiation Met being considered as the first amino acid (bottom row and in parentheses).** B, assessing the formation of disulfide bond in integrin TM mutants. Cell surface-biotinylated α_MιCF β_2YFP TM mutants were immunoprecipitated with the β_2 integrins heterodimer-specific mAb IB4. Proteins were resolved on a 6% SDS-PAGE gel under nonreducing conditions (−DTT) and on a 7.5% SDS-PAGE gel under reducing conditions (+DTT). Proteins were probed with streptavidin-HRP and detected by ECL. The asterisk denotes the pair of integrin TM mutants that were used in subsequent analyses. This disulfide clasp is referred to as c-c henceforth. C, flow cytometry analyses of K562 transfectants expressing α_Mιβ_2 or α_Mιβ_2 with the disulfide clasp c-c. The mAb used was IB4 (shaded histogram). Open histogram, irrelevant mAb. D, K562 transfectants were examined for their adhesive properties to fibrinogen. For activation of α_Mιβ_2, the activating mAb KIM185 was used. Cell adhesion specificity was demonstrated using mAb IB4, which is also a function-blocking mAb.
KIM127 failed to co-precipitate HA-uPAR, and it adopted an open headpiece conformation because it showed reactivity with MEM148. Thus, the introduction of the c-c clasp had also no apparent effect on the ectodomain conformation of α₅β₂ interacting with HA-uPAR.

We then examined whether c-c clasp prevents reorientation of the α₅β₂ TMs induced by HA-uPAR (Fig. 7B). K562 transfectants expressing the indicated constructs were subjected to FRET analyses. Cells expressing α₅L.CFPβ₂YFP showed a significant reduction in FRET efficiency in the presence of HA-uPAR co-expression. Cells expressing α₅L.CFPβ₂YFP with c-c clasp showed comparable FRETs to those without the clasp. However, co-expressing HA-uPAR with α₅L.CFPβ₂YFP c-c clasp did not show significant reduction in FRET signal. This was not attributed to poor interaction of HA-uPAR with the c-c clasp integrin because HA-uPAR was co-precipitated effectively with the integrins by KIM185 and MEM148 aforementioned (Fig. 7A). Taken together, these data suggest that the TM c-c clasp prevents the separation or reorientation of the α₅β₂ TMs induced by HA-uPAR, lending further support that uPAR interacting with α₅β₂ not only induces the opening of the α₅β₂ headpiece but the “activation” signal is propagated to the C-terminal tail of the α₅β₂ that leads to the reorientation of the TMs.

**DISCUSSION**

The integrin α₅β₂ is a primary receptor that promotes the adhesion and migration of polymorphonuclear leukocytes and mononuclear phagocytes (2). The activity of α₅β₂ that is conformation-dependent is regulated at several levels. Extracellular divalent cation Mn²⁺ stimulates adhesion of monocytes to endothelial cells mediated by α₅β₂ and enhances α₅β₂ ligand binding (45). From the intracellular compartment, the large cytoskeletal network protein talin interacts with the β₂ cytoplasmic tail to regulate α₅β₂-mediated phagocytosis in the mouse macrophage line (54). On the plasma membrane α₅β₂ interacts with FcγRIIB, FcγRIIA, low density lipoprotein receptor, and uPAR (13–16). uPAR is well reported to form a functional complex with α₅β₂ in fibrinolysis, and it modulates the activity of α₅β₂ (17, 18, 26, 35, 55, 56). uPAR-deficient mice also showed impaired α₅β₂-mediated recruitment of neutrophils in response to infections (57, 58). However, it is not well characterized how interaction with uPAR affects the conformation of the α₅β₂.

Our data suggest that interaction of uPAR with α₅β₂ changes the conformation of the integrin headpiece from a closed to an open conformation. A displaced β₂ hybrid domain was detected using the reporter mAb MEM148. Indeed, our observation is in line with a study made on uPAR and integrin α₅β₂ that showed conformational changes in the integrin using two reporter mAbs HUTS-21 and 9EG7 (22). It was observed that suppressing expression of uPAR by siRNA in human fibrosarcoma and breast cancer cell lines enhanced the epitope expressions of HUTS-21 and 9EG7, which recognize ligand-induced epitopes in β₁ subunit (22). Thus, it was inferred that uPAR directly affects the conformation of its partner α₅β₂.

Interestingly, the epitope of HUTS-21 lies in the region spanning residues 355–425 of the β₁ subunit that is in the hybrid domain (59). The epitope of 9EG7 lies in region 495–602 of the β₁ integrin epidermal growth factor 2, -3, and -4 (60). Together, these data suggest that conformational changes are transmitted from the headpiece of an integrin involving hybrid domain movement to the C-terminal tail of the integrin ectodomain when it interacts with uPAR.

A key feature of uPAR interaction with an integrin is the overall bent conformation of the integrin because both molecules are juxtaposed on the same membrane, and the interaction sites on both molecules disfavor association of uPAR with an extended integrin as discussed in the Introduction. Indeed, we were unable to detect extended α₅β₂ interacting with uPAR using the reporter mAb KIM127, although association of uPAR with α₅β₂ was verified using KIM185. However, uPAR induces displacement of the α₅β₂ hybrid domain, which may be remi-

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**FIGURE 7.** The disulfide clasp prevented reorientation or separation of the α₅β₂ but did not attenuate the conformational changes in the α₅β₂ ectodomain induced by uPAR. A, the ectodomain conformation of the α₅β₂ c-c interacting with HA-uPAR was analyzed by co-immunoprecipitation analyses. Cell lysate of transfectants were immunoprecipitated with the mAbs indicated, and HA-uPAR was detected by immunoblotting with anti-HA antibody. Proteins were resolved on a 10% SDS-PAGE gel under reducing conditions. IP, immunoprecipitation. B, FRET analyses of transfectants co-expressing the indicated integrins with or without the disulfide clasp (c-c) and with or without HA-uPAR. Data are representative of three independent experiments. Data show the mean ± S.E. for 20 cells. Student's t test, assuming unequal variance, was used for statistical analyses. *, p < 0.001.
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FIGURE 8. Hypothetical model of $\alpha_M\beta_2$ conformational changes induced by uPAR. In cis interaction of $\alpha_M\beta_2$ with uPAR induces hybrid domain movement in an overall bent conformation, subsequently triggering the reorientation or separation of the TM’s.

niscent to that of a ligand-bound $\alpha_\gamma\beta_1$ (61) and $\alpha_\delta\beta_3$ (43). Thus, uPAR may be considered as an in cis ligand of $\alpha_M\beta_2$.

An interesting observation made in this study is the movement of the $\alpha_M\beta_2$ TM’s when it is associated with uPAR. This is inferred from the FRET analyses of $\alpha_M\beta_2$ with FRET pair fluorophore fused to the cytoplasmic tails, which report movement of the TM’s. We were unable to detect significant FRET in $\alpha_M\beta_2$ with its native cytoplasmic tails; hence, we have made substitution of $\alpha_M$ cytoplasmic tail with that of $\alpha_L$, which was successful in the FRET system we employed previously (39). We acknowledge that there can be a limitation in the use of $\alpha_L$ cytoplasmic tail in an $\alpha_M$ construct to report $\alpha_M\beta_2$ conformational changes; however, we reasoned that this approach is still relevant and useful in demonstrating TM separation of $\alpha_M\beta_2$.

The reorientation of TM’s in $\alpha_M\beta_2$ interacting with uPAR is further verified by clamping the TM’s with a disulfide bond. This also eliminates the possibility of uPAR directly inducing spatial changes in the integrin cytoplasmic tails via other factors, which would complicate the readout and the interpretation of the data with regards to TM movement. The separation of the integrin TM’s is important for outside-in signaling as shown directly in $\alpha_M\beta_2$ (29). Thus, our data suggest that uPAR changes the ectodomain conformation of its partner integrin, which is sufficient to trigger movement of the integrin TM’s as illustrated in the model (Fig. 8). However, we should be cautious with the interpretation because the reorientation of the TM’s or their complete separation when an integrin is activated remains to be fully characterized by structural studies. Nonetheless, the movement of the integrin TM’s induced by uPAR can be one of the mechanisms by which uPAR trigger integrin-mediated cytosolic signaling. Other mechanisms may involve dimerization of uPAR and its potential to partition into membrane rafts (62).

Our data and that of others (22) suggest that uPAR induces shape changes in its integrin partner that retain an overall bent conformation. Although integrin extension is one of the hallmarks of integrin activation, there are also reports of activated bent integrins (24, 25, 41, 63–65). A model was proposed for non-L domain-containing integrins in which engagement of a bent integrin with its ligand induces a certain degree of unbending, and in I domain containing integrins the swing out of the hybrid domain is an event of outside-in signaling in the dead-bolt model of activation (25). Thus, it will be interesting to obtain structural data of an uPAR-integrin complex in future work to obtain direct measurements of these conformational changes. These will add to the ensemble of conformations adopted by the integrins under different physiological conditions that are required for fine-regulating integrin-mediated biological processes.

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REFERENCES

1. Plow, E. F., and Zhang, L. (1997) J. Clin. Investig. 99, 1145–1146
2. Ehlers, M. R. (2000) Microbes Infect. 2, 289–294
3. Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) Science 296, 151–155
4. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J., and Smith, J. W. (2000) J. Biol. Chem. 275, 21785–21788
5. Forsyth, C. B., Plow, E. F., and Zhang, L. (1998) J. Immunol. 161, 6198–6205
6. Cywes, C., Godenir, N. L., Hoppe, H. C., Scholle, R. R., Steyn, L. M., Kirsch, R. E., and Ehlers, M. R. (1996) Infect. Immun. 64, 5373–5383
7. Davis, G. E. (1992) Exp. Cell Res. 200, 242–252
8. Rosenkranz, A. R., Coxon, A., Maurer, M., Gurish, M. F., Austen, K. F., Friend, D. S., Galli, S. J., and Mayadas, T. N. (1998) J. Immunol. 161, 6463–6467
9. Andrew, D. P., Spellberg, J. P., Takimoto, H., Schmits, R., Mak, T. W., and Zukowski, M. M. (1998) Eur. J. Immunol. 28, 1959–1969
10. Lu, H., Smith, C. W., Perrard, J., Bullard, D., Tang, L., Shappell, S. B., Entman, M. L., Beaudet, A. L., and Ballantyne, C. M. (1997) J. Clin. Investig. 99, 1340–1350
11. Behrens, E. M., Sriram, U., Shivers, D. K., Gallucci, M., Ma, Z., Finkel, T. H., and Gallucci, S. (2007) J. Immunol. 178, 6268–6279
12. Skoberne, M., Somersans, S., Almodovar, W., Truong, T., Petrova, K., Hensohn, P. M., and Bhardwaj, N. (2006) Blood 108, 947–955
13. Annenkov, A., Ortlepp, S., and Hogg, N. (1996) Eur. J. Immunol. 26, 207–212
14. Bohuslav, J., Horejsi, V., Hansmann, C., Stockl, J., Weidle, U. H., Majdic, O., Bartke, I., Knapp, W., and Stockinger, H. (1995) J. Exp. Med. 181, 1381–1390
15. Spijkers, P. P., da Costa Martins, P., Westein, E., Gahmberg, C. G., Zwagila, G., and Bentin, B. (2005) Blood 105, 170–177
16. Zhou, M., Todd, R. F., III, van de Winkel, J. G., and Petty, H. R. (1993) J. Immunol. 150, 3030–3041
17. Simon, D. I., Rao, N. K., Xu, H., Wei, Y., Majdic, O., Rorne, E., Kobzik, L., and Chapman, H. A. (1996) Blood 88, 3185–3194
18. Puskota, E., Soloviev, D. A., and Plow, E. F. (2003) Blood 101, 1582–1590
19. Xue, W., Mizukami, I., Todd, R. F., III, and Petty, H. R. (1997) Cancer Res. 57, 1682–1689
20. Tarui, T., Mazar, A. P., Cines, D. B., and Takada, Y. (2001) J. Biol. Chem. 276, 3983–3990
21. Blasi, F., and Carmeliet, P. (2002) Nat. Rev. Mol. Cell Biol. 3, 932–943
22. Wei, Y., Czekay, R. P., Robillard, L., Kugler, M. C., Zhang, F., Kim, K. K., Xiong, J. P., Humphries, M. J., and Chapman, H. A. (2005) J. Cell Biol. 168, 501–511
23. Wei, Y., Tang, C. H., Kim, Y., Robillard, L., Zhang, F., Kugler, M. C., and Chapman, H. A. (2007) J. Biol. Chem. 282, 3929–3939
24. Luo, B. H., Carman, C. V., and Springer, T. A. (2007) Annu. Rev. Immunol. 25, 619–647
25. Arnaout, M. A., Goodman, S. L., and Xiong, J. P. (2007) Curr. Opin. Cell Biol. 19, 495–507
26. Simon, D. I., Wei, Y., Zhang, L., Rao, N. K., Xu, H., Chen, Z., Liu, Q., Rosenberg, S., and Chapman, H. A. (2000) J. Biol. Chem. 275, 10228–10234
27. Llinas, P., Le Du, M. H., Gardsvoll, H., Dano, K., Ploug, M., Gilequin, B., Stura, E. A., and Menez, A. (2005) *EMBO J.* 24, 1655–1663
28. Arnason, M. A., Mahalingam, B., and Xiong, J. P. (2005) *Annu. Rev. Cell Dev. Biol.* 21, 381–410
29. Zhu, J., Carman, C. V., Kim, M., Shimaoka, M., Springer, T. A., and Luo, B. H. (2007) *Blood* 110, 2475–2483
30. Uciechowski, P., and von Schimidt, R. E. (1989)
31. Shaw, J. M., Al-Shamkhani, A., Boxer, L. A., Buckley, C. D., Dodds, A. W., Klein, N., Nolan, S. M., Roberts, I., Roos, D., Scard, S. L., Simmonns, D. L., Tan, S. M., and Law, S. K. (2001) *Clin. Exp. Immunol.* 126, 311–318
32. Andrew, D., Shock, A., Bell, E., Ortlepp, S., Bell, J., and Robinson, M. (1993)
33. Stephens, P., Romer, J. T., Spitali, M., Shock, A., Ortlepp, S., Figdor, C. G., and Robinson, M. K. (1995) *Cell Adhes. Commun.* 3, 375–384
34. Beglova, N., Blacklow, S. C., Takagi, J., and Springer, T. A. (2002) *Nat. Struct. Biol.* 9, 282–287
35. Tang, M. L., Kong, L. S., Law, S. K., and Tan, S. M. (2005) *J. Biol. Chem.* 280, 29208–29216
36. Barclay, A. N., Brown, M. H., Law, S. K., McKnight, A. J., Tomlinson, M. G., and van der Merwe, P. A. (1997) *The Leukocyte Antigen Facts Book*, 2nd Ed., pp. 158–160, 177–178, and 347–348, Academic Press, Inc., London, UK
37. Kim, M., Carman, C. V., and Springer, T. A. (2003) *Science* 301, 1720–1725
38. Vararattanavech, A., Tang, M. L., Li, H. Y., Wong, C. H., Law, S. K., Torres, J., and Tan, S. M. (2007) *Biochem. J.* 410, 495–502
39. Xue, W., Kindzelskii, A. L., Todd, R. F., III, and Petty, H. R. (1994) *J. Immunol.* 152, 4630–4640
40. Nishida, N., Xie, C., Shimaoka, M., Cheng, Y., Walz, T., and Springer, T. A. (2006) *Immunity* 25, 583–594
41. Mould, A. P., Barton, S. J., Askari, J. A., McEwan, P. A., Buckley, P. A., Craig, S. E., and Humphries, M. J. (2003) *J. Biol. Chem.* 278, 17028–17035
42. Xiao, T., Takagi, J., Coller, B. S., Wang, J. H., and Springer, T. A. (2004) *Nature* 432, 59–67
43. Lu, C., Ferkly, M., Takagi, J., and Springer, T. A. (2001) *J. Immunol.* 166, 5629–5637
44. Altieri, D. C. (1991) *J. Immunol.* 147, 1891–1898
45. Cheng, M., Foo, S. Y., Shi, M. L., Tang, R. H., Kong, L. S., Law, S. K., and Tan, S. M. (2007) *J. Biol. Chem.* 282, 18225–18232
46. Violette, S. M., Rusche, J. R., Purdy, S. R., Boyd, J. G., Cos, J., and Silver, S. (1995) *J. Immunol.* 155, 3092–3101
47. Fu, G., Yang, H. Y., Wang, C., Zhang, F., You, Z. D., Wang, G. Y., He, C., Chen, Y. Z., and Xu, Z. Z. (2006) *Biochim. Biophys. Res. Commun.* 346, 986–991
48. Lau, T. L., Dua, V., and Ulmer, T. S. (2008) *J. Biol. Chem.* 283, 16162–16168
49. Lau, T. L., Partridge, A. W., Ginsberg, M. H., and Ulmer, T. S. (2008) *Biochemistry* 47, 4008–4016
50. Cao, D., Mizukami, I. F., Garni-Wagner, B. A., Kindzlzskii, A. L., Todd, R. F., III, Boxer, L. A., and Petty, H. R. (1995) *J. Immunol.* 154, 1817–1829
51. Juffermans, N. P., Dekkers, P. E., Verbon, A., Speelman, P., van Deventer, S. J., and van der Poll, T. (2001) *Infect. Immun.* 69, 5182–5185
52. Luo, B. H., Springer, T. A., and Takagi, J. (2004) *PLoS Biol.* 2, e153
53. Lim, J., Wiedemann, A., Tzicritocs, G., Monrkey, S. J., Critchley, D. R., and Caron, E. (2007) *Mol. Biol. Cell* 18, 976–985
54. Pluskota, E., Soloviev, D. A., Bdeir, K., Cines, D. B., and Plow, E. F. (2004) *J. Biol. Chem.* 279, 18063–18072
55. Zhang, H., Colman, R. W., and Sheng, N. (2003) *Inflammation* 36, 86–93
56. Gyetko, M. R., Aizenberg, D., and Mayo-Bond, L. (2004) *J. Leukocyte Biol.* 76, 648–656
57. Gyetko, M. R., Sud, S., Kendall, T., Fuller, J. A., Newstead, M. W., and Standiford, T. J. (2000) *J. Immunol.* 165, 1513–1519
58. Luque, A., Gomez, M., Puizon, W., Takada, Y., Sanchez-Madrid, F., and Cabanas, C. (1996) *J. Biol. Chem.* 271, 11067–11075
59. Bazzoni, G., Shih, D. T., Buck, C. A., and Hemler, M. E. (1995) *J. Biol. Chem.* 270, 25570–25577
60. Takagi, J., Strokovich, K., Springer, T. A., and Walz, T. (2003) *EMBO J.* 22, 4607–4615
61. Cunningham, O., Andolfo, A., Santovito, M. L., Iuzzolino, L., Blasi, F., and Sidenius, N. (2003) *EMBO J.* 22, 5994–6003
62. Adair, B. D., Xiong, J. P., Maddock, C., Goodman, S. L., Arnason, M. A., and Yeager, M. (2005) *J. Cell Biol.* 168, 1109–1118
63. Chigaev, A., Blenc, A. M., Braaten, J. V., Kumaraswamy, N., Kepley, C. L., Andrews, R. P., Oliver, J. M., Edwards, B. S., Prossnitz, E. R., Larson, R. S., and Sklar, L. A. (2001) *J. Biol. Chem.* 276, 48670–48678
64. Larson, R. S., Davis, T., Bologa, C., Semenuk, G., Vijayan, S., Li, Y., Oprea, T., Chigaev, A., Buranda, T., Wagner, C. R., and Sklar, L. A. (2005) *Biochemistry* 44, 4322–4331
65. Huai, Q., Mazar, A. P., Kuo, A., Parry, G. C., Shaw, D. E., Callahan, J., Li, Y., Yuan, C., Bian, C., Chen, L., Furie, B., Furie, B. C., Cines, D. B., and Huang, M. (2006) *Science* 311, 656–659
66. Baldwin, E. T., Sarver, R. W., Bryant, G. L., Jr., Curry, K. A., Fairbanks, M. B., Finzel, B. C., Garlick, R. L., Heinrikson, R. L., Horton, N. C., Kelley, L. L., Mllerner, A. M., Moon, J. B., Mott, J. E., Mutchler, V. T., Tomich, C. S., Watenpaugh, K. D., and Wiley, V. H. (1998) *Structure* 6, 923–933
67. Shi, M., Foo, S. Y., Tan, S. M., Mitchell, E. P., Law, S. K., and Lescar, J. (2007) *J. Biol. Chem.* 282, 30198–30206