

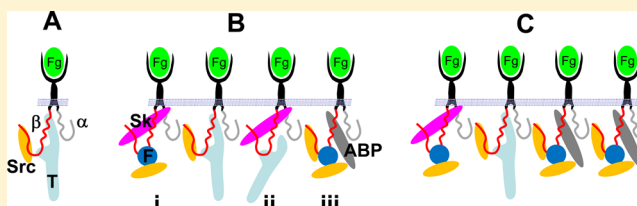
Skelemin in Integrin  $\alpha_{IIb}\beta_3$  Mediated Cell Spreading

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## Supporting Information

**ABSTRACT:** Skelemin, a myosin-associated protein in skeletal muscle, has been demonstrated to interact with integrin  $\alpha_{IIb}\beta_3$  in nonmuscle cells during initial stages of cell spreading. The significance of this interaction and the role of skelemin in integrin signaling and cytoskeletal reorganization were investigated in this study. We established a series of Chinese hamster ovary cell lines expressing wild-type or mutant  $\alpha_{IIb}\beta_3$  receptors in which skelemin binding residues at the membrane proximal region of integrin tails were mutated to alanine. Most cells displayed unimpaired adhesive capacity and spreading on immobilized fibrinogen at the early stages of cell spreading. In addition, they formed normal focal adhesions and stress fibers with no indication of impaired cell spreading. R995A/R997A/L1000A, H722A, and K716A exhibited the greatest cell spreading, which was associated with enhanced p-Src activation but was independent of FAK activation. Transfection of the cells with GFP-skelemin, containing only the C2 integrin binding domain, caused wild-type cells to round up, but had no effect on R995A/R997A/L1000A, H722A, and K716A cell spreading. Furthermore, the protrusions of the leading edge of K716A cells showed strong colocalization of talin with  $\alpha_{IIb}\beta_3$  which was associated with a loss in skelemin binding. Thus, we propose that during early stages of cell spreading, skelemin exerts contractile force on cell spreading and modulates the attachment of cytoskeletal proteins and Src to integrin clusters.



Integrins are noncovalently linked  $\alpha$ - $\beta$  heterodimeric transmembrane receptors that mediate cell–cell and cell–matrix interactions. They provide a mechanism of linking the extracellular matrix (ECM) to the cytoskeletal/contractile apparatus within a cell and also transmit signals that initiate cell cytoskeleton reorganization which enables the cell to adhere, spread, move, proliferate and differentiate.<sup>1</sup> Integrin  $\alpha_{IIb}\beta_3$  is a platelet-specific family member and plays a vital role in homeostasis and thrombosis. Its membrane-proximal domains of  $\alpha$ - and  $\beta$ -subunit interact in a default manner, constraining the integrin in a resting low affinity conformation to its ligands.<sup>2</sup> This association of integrin subunits can be interrupted by agonists, such as adenosine diphosphate (ADP), thrombin, or collagen, triggering conformational changes in integrin extracellular domain and driving integrin to a high affinity state for its ligands (a process termed integrin activation or inside-out signaling). Ligand binding to integrin, in turn, initiates a process termed outside-in signaling which alters the structure of the receptor triggering intracellular signals that control cell polarity, cytoskeletal reorganization, gene expression, and cell survival and proliferation.<sup>3</sup>

Skelemin is a cytoskeletal protein first identified in the periphery of the sarcomeric M-line of myosin thick filaments in striated muscles.<sup>4</sup> In muscle cells, skelemin cross-linked myosin filaments to maintain thick filament lattice<sup>5</sup> and to serve as a linker between M-band and intermediate filaments through a desmin binding domain.<sup>6</sup> Skelemin belongs to a member of a family of myosin associated proteins and is highly homologous to myomesin as they are encoded by the same gene, but alternative splicing gives rise to the insertion of serine/proline-

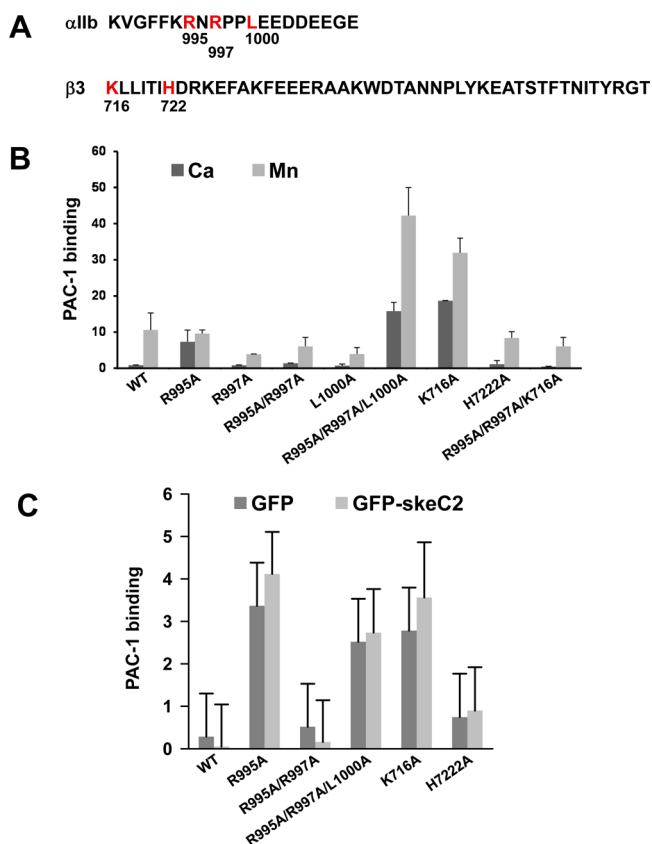
rich domain in the center of skelemin.<sup>7</sup> Recent studies have confirmed the presence of a skelemin in nonmuscle cells, such as platelets and Chinese hamster ovary (CHO) cells.<sup>8–10</sup> In addition, after adhering to immobilized ligand fibrinogen, skelemin can interact and colocalize with integrin  $\alpha_{IIb}\beta_3$  at the initial stage of cell spreading, suggesting that skelemin serves as a cross-linker between integrin and the myosin cytoskeleton in nonmuscle cells.<sup>8–10</sup>

Skelemin is one of very few proteins reported to bind to both the  $\alpha$  and  $\beta$  cytoplasmic tails of an integrin.<sup>8,11</sup> It contains five repeats of fibronectin type III motifs and seven repeats of immunoglobulin superfamily C2-like motifs.<sup>6</sup> The primary interaction of skelemin with  $\alpha_{IIb}\beta_3$  involves the skelemin immunoglobulin C2 motifs 5 and the membrane proximal regions of cytoplasmic tails of  $\alpha_{IIb}\beta_3$ , while there is an additional low affinity contact between the skelemin immunoglobulin C2 motifs 4 and the C-terminus of  $\beta_3$  tails.<sup>10,11</sup> However, the function significance of skelemin–integrin interactions has not been fully explored. In this paper, integrin affinity state, outside-in signaling, and related functions in CHO cells overexpressing mutant integrins lacking the binding capacity to skelemin were investigated. Our collaborators and we previously identified the critical residues in the  $\alpha_{IIb}$  and  $\beta_3$  tails involved in skelemin binding.<sup>8</sup> Here, we introduced alanine substitutions at Arg995, Arg997, and Leu1000 in  $\alpha_{IIb}$  tail, and Lys716 and His722 in  $\beta_3$  tail (Figure 1). We then established stably expressed single,

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**Figure 1.**  $\alpha_{IIb}\beta_3$  mutant sequences and PAC-1 binding in the presence of metal ions or GFP-skeC2. (A) Amino acid sequences of  $\alpha_{IIb}$  and  $\beta_3$  cytoplasmic tails. Residues targeted for alanine substitutions are underlined and sequence numbers are displayed. (B) Binding of PAC-1 to mutant cell lines in the presence of  $Ca^{2+}$  or  $Mn^{2+}$ . Cell treated with 2 mM  $Ca^{2+}$ , 200  $\mu$ M  $Mn^{2+}$  or 2 mM EDTA were incubated with PAC-1 and washed. Then the cells were incubated with a PE-conjugated secondary antibody and analyzed by flow cytometry. PAC-1 binding was expressed as a percentage of positive cells treated with  $Ca^{2+}$  or  $Mn^{2+}$  from flow cytometric histogram minus that obtained in the presence of EDTA. The data represent the mean  $\pm$  s.d. of three separate experiments. (C) Binding of PAC-1 to mutant cell lines following transfection with GFP-skeC2. Cells were transiently transfected with GFP or GFP-skeC2 and tested PAC-1 binding in the presence of  $Ca^{2+}$  or EDTA following the above method. PAC-1 binding was expressed as a percentage of positive cells treated with  $Ca^{2+}$  from flow cytometric histogram minus that seen in the presence of EDTA. The data represent the mean  $\pm$  s.d. of three separate experiments. The difference of PAC-1 binding with GFP or GFP-skeC2 expression for each cell line was not statistically significant ( $P < 0.05$ ).

double, or triple mutations in CHO cells, namely, R995A, R997A, R995A/R997A, L1000A, R995A/R997A/L1000A, K716A, H722A, and R995A/R997A/K716A. Integrin-mediated cell adhesion, cell spreading, activation of focal adhesion kinase (FAK), and Src were investigated, and the distribution of  $\alpha_{IIb}\beta_3$ , skelemin, and talin was measured in the protrusions of the cell leading edge.

## EXPERIMENTAL PROCEDURES

**Generation of Mutant Cell Lines.** The cDNAs for  $\alpha_{IIb}$  and  $\beta_3$  were cloned into pcDNA3.1 vectors with neomycin and hygromycin resistance genes respectively. Mutations were carried out with the use of site-directed mutagenesis strategy.

All sequences were verified by DNA sequencing. CHO cells were transfected using Lipofectamine (Invitrogen Corp.) with the respective plasmids for both  $\alpha_{IIb}$  and  $\beta_3$  subunits, and then followed by selection in 600  $\mu$ g/mL G418 and 500  $\mu$ g/mL hygromycin B. Control CHO cells were transfected with empty vectors. Stable CHO cell lines were maintained in the presence of 400  $\mu$ g/mL G418 and 300  $\mu$ g/mL hygromycin B and cultured in DMEM containing 10% FBS and antibiotics (penicillin, streptomycin).

**Flow Cytometry.** CHO cells were harvested and suspended in Tyrode's buffer containing 1 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , 0.1% glucose, and 0.1% BSA. For PAC-1 binding,  $5 \times 10^5$  cells were pretreated with Tyrodes buffer containing 2 mM  $Ca^{2+}$ , 200  $\mu$ M  $Mn^{2+}$ , or 2 mM EDTA at 37  $^\circ$ C for 15 min. Cells were then incubated with PAC-1 (10  $\mu$ g/mL, Becton Dickinson) at room temperature for 1 h, washed, and then incubated with PE-labeled goat antimouse Ig (BD Biosciences) for 1 h on ice. Cells were analyzed on a flow cytometer. The mean fluorescence intensity provided a measure of ligand binding affinity of integrins. To assess the effect of skelemin fragment expression on integrin affinity state, cells were transiently transfected with green fluorescence protein (GFP) or GFP-skeC2 and tested PAC-1 binding in the presence of 2 mM  $Ca^{2+}$  following the above method.

**Cell Adhesion Assay.** Tissue culture plates (96-well) were coated overnight at 4  $^\circ$ C with 50  $\mu$ L of 20  $\mu$ g/mL fibrinogen or heat-denatured 1% BSA, and blocked with 1% BSA at room temperature for 2 h before use. Cells were harvested and labeled by incubation with Calcein AM (10  $\mu$ M, Invitrogen) at 37  $^\circ$ C for 30 min in the dark. Labeled cells were washed twice and resuspended in Tyrode's buffer containing 1 mM  $CaCl_2$ , 1 mM  $MgCl_2$ . The cells were then added to each well ( $4 \times 10^5$  cells in 100  $\mu$ L) and incubated at 37  $^\circ$ C for 30 min. Unbound cells were removed by washing two times. Bound cells were counted on a microplate reader (SpectraMax M2e) using an excitation wavelength of 494 nm and an emission wavelength of 517 nm. The specific percentage of cells bound to ligand in each well was calculated as (FU from ligand-coated wells) – (FU from BSA-coated wells)  $\times$  100/total FU added to each well.

**Immunohistochemistry.** Falcon 4-well Culture Slides were treated with 1% SDS, rinsed with PBS, and then precoated with 20  $\mu$ g/mL of fibrinogen overnight at 4  $^\circ$ C. Cells were seeded and adhered to culture slides for different required times. Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized by 0.2% (V/V) Triton X-100 for 10 min, washed three times and blocked with 1% BSA. Filamentous actin (F-actin) was stained using Alexa Fluor 594 phalloidin (Invitrogen) for 30 min in 1:40 dilution. For visualization of integrin distribution, cells were fixed in 4% paraformaldehyde, incubated with  $\beta_3$ -specific mAb (AP3, GTI Diagnostics) overnight at 4  $^\circ$ C and then a PE-conjugated secondary antibody for 2 h. For visualization of integrin and skelemin/talin colocalization,  $\beta_3$  integrins were first stained as described above, and then talin (goat IgG from Santa Cruz) or skelemin (rabbit IgG from Dr. T. Ugarova) were stained with BD Cytofix/Cytoperm solution (BD Biosciences). This sequential approach preserved the cell surface antigens during the intracellular staining.

**Immunoprecipitation and Western Blot.** Cells were trypsinized and plated on fibrinogen-coated culture dishes for 1 h at 37  $^\circ$ C. Nonadherent cells were washed away and adherent cells were solubilized with lysis buffer (20 mM Tris-HCl (pH

7.4), 150 mM NaCl, 1% Triton X-100, 1 mM CaCl<sub>2</sub>, 1 mM PMSF, 100 μg/mL leupeptin, and 10 mM benzamide) for 1 h at 4 °C. For immunoprecipitation, lysates (600–1000 μg total proteins) were incubated with 2 μg of AP3 or 2 μL anti-GFP antibody (Invitrogen) for 2 h at 4 °C. The immunocomplexes were captured by incubation with 40 μL of Protein G-agarose (Santa Cruz) overnight at 4 °C and washed three times. The immunocomplex samples or total cell lysates were boiled in loading buffer and loaded onto 7.5% SDS-PAGE gels. Proteins were then transferred to nitrocellulose membrane (Whatman). The membrane was blocked with 5% milk-TBST or 5% BSA-TBST for detecting phosphorylated proteins, and incubated with primary antibody overnight at 4 °C. Anti-pY416-Src was obtained from cell signaling, and anti-β<sub>3</sub> antibody (N20), anti-pY397 FAK, and β-actin were obtained from Santa Cruz. Anti-Src (mAb327) was obtained from Calbiochem.

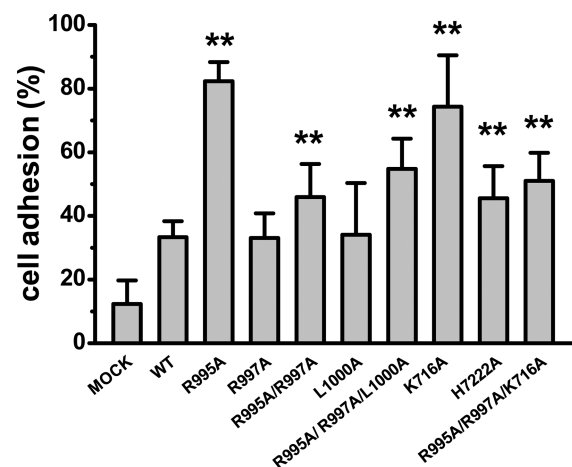
## RESULTS

**Integrin Affinity for Ligand.** The expression of wild-type and mutant integrin was first assessed by flow cytometry using a β<sub>3</sub>-specific antibody (Supporting Information). The levels of integrin expression and percentage of cells expressing receptors in the mutant cell lines were comparable to that of wild-type α<sub>IIb</sub>β<sub>3</sub> cells, except for K716A which had only 70% of cells expressing a comparable amount of integrins. The membrane proximal regions in the integrin cytoplasmic domains are important in integrin activation modulation, and point mutations within this region could enhance the affinity for integrin ligands and promote constitutive signaling.<sup>12,13</sup> To assess the activation state of α<sub>IIb</sub>β<sub>3</sub>, we tested these mutants for PAC-1 binding in the presence of 2 mM Ca<sup>2+</sup>, 200 μM Mn<sup>2+</sup>, or 2 mM EDTA by flow cytometry (Figure 1B). Nonspecific PAC-1 binding in the presence of EDTA was low and negligible (data not shown). In the presence of Ca<sup>2+</sup>, only R995A, K716A, and R995A/R997A/L1000A cells bound significant levels of PAC-1, showing that the three mutants are constitutively active. These data are consistent with previous mutational studies,<sup>12,14</sup> suggesting that the residues of K716 and R995 within β<sub>3</sub> tails are involved in the regulation of α<sub>IIb</sub>β<sub>3</sub> activation. PAC-1 binding under the condition of Mn<sup>2+</sup> treatment was also tested, which is assumed to be the maximal activation of integrin. Generally, Mn<sup>2+</sup> was able to activate wild-type and mutant α<sub>IIb</sub>β<sub>3</sub> leading to a much higher level of PAC-1 binding. However, Mn<sup>2+</sup> did not fully activate integrin as Mn<sup>2+</sup>-induced PAC-1 binding in wild-type α<sub>IIb</sub>β<sub>3</sub> cells is still significantly lower than that of K716A and R995A/R997A/L1000A cells treated with Ca<sup>2+</sup>. Even K716A and R995A/R997A/L1000A were in a constitutive activation state, Mn<sup>2+</sup> had an additional activating effect for these mutants and enhanced PAC-1 binding with 3–4 fold than wild-type α<sub>IIb</sub>β<sub>3</sub> in the presence of Mn<sup>2+</sup>. These results suggest that among all the mutants, R995A, K716A, and R995A/R997A/L1000A exhibited partial activation, and in the presence of Mn<sup>2+</sup> K716A and R995A/R997A/L1000A had higher maximal activation than that of wild-type α<sub>IIb</sub>β<sub>3</sub>.

To assess if knocking-down skelemin binding contributes to integrin activation, we investigated the effects of exogenous skelemin overexpression on integrin activation. We assume if lack of skelemin binding was responsible for constitutive activation of α<sub>IIb</sub>β<sub>3</sub>, introduction of exogenous skelemin would change the activation state of α<sub>IIb</sub>β<sub>3</sub>. In this assay, wild-type and mutant cells were transiently transfected with either GFP-tagged skelemin immunoglobulin C2 motifs 4–5 (SkeC2: the α<sub>IIb</sub>β<sub>3</sub> binding domain of skelemin) or GFP alone, and their

PAC-1 binding levels in the presence of Ca<sup>2+</sup> or EDTA were investigated (Figure 1C). Specific PAC-1 binding was measured by subtracting the nonspecific binding (in the presence of EDTA) from PAC-1 binding in the presence of Ca<sup>2+</sup>. For wild-type α<sub>IIb</sub>β<sub>3</sub> and inactive mutants, R995A/R997A and H722A, GFP-SkeC2 expression did not enhance PAC-1 binding. Similar results were also obtained in three active mutants, R995A, K716A, and R995A/R997A/L1000A. No statistically significant difference was found between cells expressing GFP-SkeC2 or GFP and their ability to bind PAC-1. Thus, skelemin expression did not appear to alter the affinity state of α<sub>IIb</sub>β<sub>3</sub>, suggesting that skelemin is not involved in α<sub>IIb</sub>β<sub>3</sub> activation.

**Adhesion to Immobilized Fibrinogen.** The strength of cell adhesion to ECM not only depends on integrin expression level and integrin affinity, but also relies on integrin-mediated cytoskeleton linkages. Previous studies demonstrated that skelemin and integrin association was an early response to integrin occupancy and clustering, being initiated between 30 min to 2 h after cell adherence. If their association is essential for linking ECM to the cell cytoskeleton, mutant cells may show decreased cell adhesion to ECM. Therefore we compared the capacity of the mutant and wild-type cells to adhere to fibrinogen over a 30 min time period (Figure 2). Fluorescently

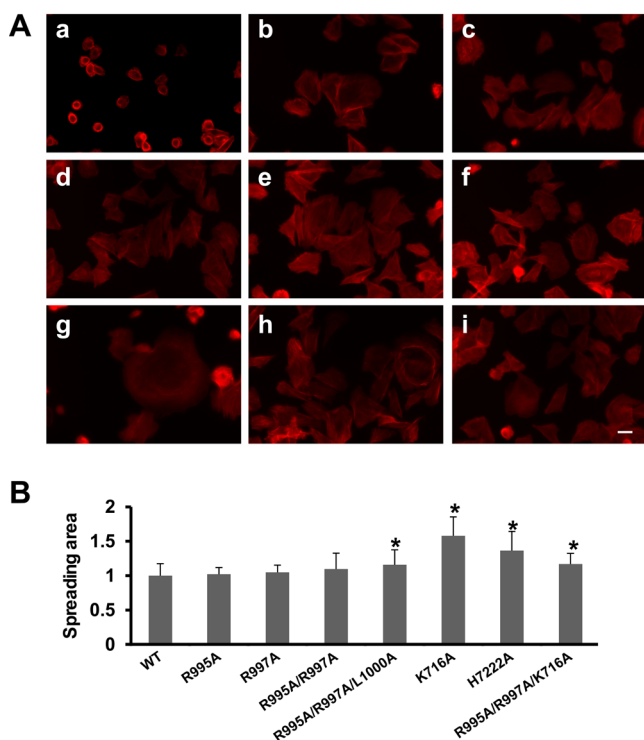


**Figure 2.** Adhesion of mutant cells to immobilized fibrinogen. Calcein-AM labeled cells were allowed to adhere to 20 μg/mL fibrinogen-coated wells for 30 min in Tyrode's buffer. After being washed, adherent cells were counted on a fluorescence microplate reader. Following subtraction of background adhesion to BSA-coated wells, cell adhesion was expressed as the percent of adherent cells to total cells added. The data represent the mean ± s.d. of three separate experiments. \*\**P* < 0.01 compared to wild-type cells.

labeled cells were allowed to adhere to fibrinogen-coated microplates. After washing, adherent cells were counted as a percent of the number of total cells added. Nonspecific cell adhesion was low as less than 5% cells adhered to BSA-coated wells for all cell lines tested. In addition, cell adhesion to fibrinogen was α<sub>IIb</sub>β<sub>3</sub>-mediated as adhesion of wild-type cells was 3-fold higher than that of mock-transfected cells. As shown in Figure 2, cell attachment of R995A, R995A/R997A, R995A/R997A/L1000A, K716A, H722A, and R995A/R997A/K716A to fibrinogen was higher than that of wild-type cells, while R997A and L1000A cell adhesion were comparable. The R995A, K716A, and R995A/R997A/L1000A mutations showed the strongest cell adhesion, in agreement with their constitutively active state as assessed by PAC-1 binding. In

general it appears that disruption of the skelemin binding sites in  $\alpha_{IIb}\beta_3$  did not impair stable cell adhesion, suggesting that skelemin- $\alpha_{IIb}\beta_3$  interaction may not be essential for the linkage of integrin-cell cytoskeleton.

**Cell Spreading and Membrane Protrusions.** Integrins and their associated proteins form focal adhesions in cultured cells, which link integrin clusters to the actin cytoskeleton and initiate actin assembly into stress fibers. Here, stress fibers were stained with fluorescence-labeled phalloidin after cell adhesion to fibrinogen-coated coverslips for 1 h (Figure 3A). Normal



**Figure 3.** Effect of  $\alpha_{IIb}\beta_3$  mutations on the actin cytoskeleton. (A) After being spread on fibrinogen-coated wells for 1 h, cells were fixed, permeabilized, and stained with rhodamine phalloidin to detect F-actin. a: CHO; b: wild-type; c: R995A; d: R997A; e: R995A/R997A; f: R995A/R997A/L1000A; g: K716A; h: H722A; and i: R995A/R997A/K716A. Scale bar: 100  $\mu$ m. (B) Quantitative analysis of cell spreading. The areas of cell spreading were measured using ImageJ analysis software. The area of wild-type cells was normalized to 1. Error bars are standard deviations. \* $P < 0.05$  compared to wild-type cells.

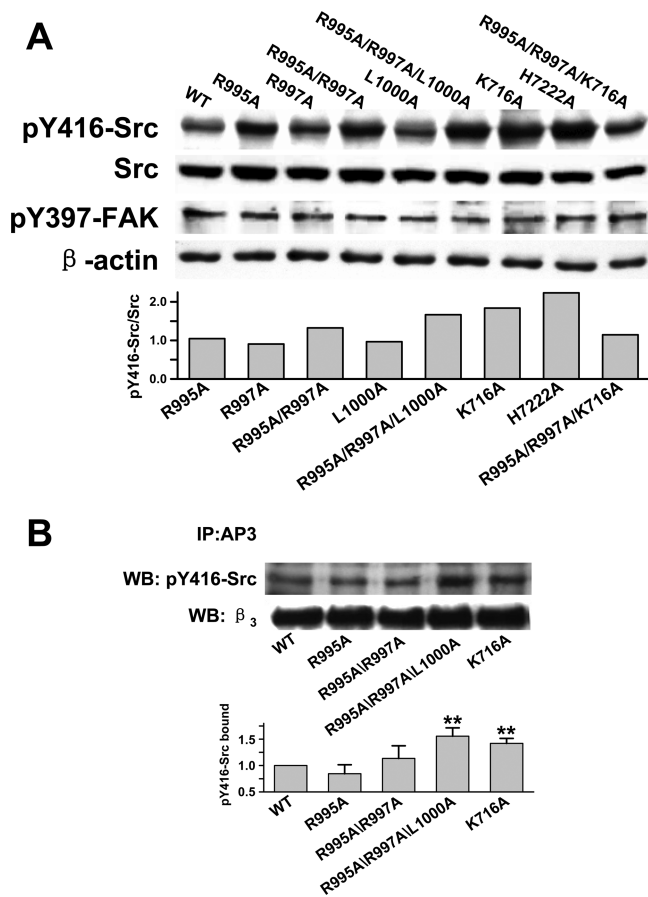
CHO cells lacking  $\alpha_{IIb}\beta_3$  expression were still round at this time point, whereas wild-type and mutant  $\alpha_{IIb}\beta_3$  transfected cells were spreading and already displaying strong formation of stress fibers. There was also obviously formation of lamellipodia and filopodia within the mutant cell lines. Quantitative measurements of cell areas clearly showed some mutant cells exhibited a greater extent of cell spreading, that is, R995A/R997A/L1000A, K716A, H722A and R995A/R997A/K716A (Figure 3B). We also stained cells with anti- $\beta_3$  antibody (AP3) and observed tremendous  $\alpha_{IIb}\beta_3$ -based focal adhesions, lamellipodia, and filopodia at the leading edges of the mutant cell lines (data not shown). Taken together, these results demonstrated that defective binding of skelemin to  $\alpha_{IIb}\beta_3$  does not disrupt actin cytoskeleton organization, membrane protrusion formation, and cell spreading, suggesting that other cytoskeletal proteins are capable of performing some of the functions performed by skelemin. Thus, different

cytoskeletal proteins may act in unison to prevent impaired integrin-cytoskeleton linkages.

**Src and FAK Activation Downstream of Integrin Signaling.** The increased cell adhesion ability and higher spreading level observed in some mutations suggested that there was an upregulation in integrin-downstream signaling. Therefore, we examined the activation of FAK and Src, two important tyrosine kinases in integrin signaling that are required for efficient adhesion and spreading of cells on integrin ligands.<sup>15</sup> Western blot analysis of total cell lysates showed that adhesion to fibrinogen did not affect the levels of total Src but increased the levels of activated, autophosphorylated Src (pY416-Src) in R995A/R997A/L1000A, K716A, and H722A mutant cells compared to wild-type cells (Figure 4A). Autophosphorylation and subsequent activation of Src could be a result of its direct binding to C-terminus of  $\beta_3$  tails via the Src SH3 domain upon cell adhesion,<sup>16</sup> or by interacting with  $\beta_3$  through its distal p-FAK binding site.<sup>17,18</sup> There were no differences in cellular levels of autophosphorylated FAK (pY397-FAK, active FAK form) among mutant and wild-type integrin-expressing cells (Figure 4A), in agreement with the previous study showing the membrane distal NPLY motif of  $\beta_3$  tails was required for FAK phosphorylation.<sup>19</sup> Thus, we speculate that the increase in p-Src levels in the mutant cell lines was a result of increased Src binding directly to the C-terminus of  $\beta_3$ , rather than interacting with  $\beta_3$  through p-FAK. With most mutant cell lines displaying an increase in total cellular pY416-Src, we determined if one could also detect an increase in pY416-Src associated with the  $\beta_3$  tail. Therefore, using the anti- $\beta_3$  antibody AP3, we immunoprecipitated  $\beta_3$  from wild-type and four mutant cell lines, and probed for pY416-Src (Figure 4B). In all cell lines tested, pY416-Src coimmunoprecipitated with  $\beta_3$  and in R995A/R997A/L1000A and K716A cell lines that contained high levels of total cellular pY416-Src, there was a significant increase in the levels of pY416-Src associated with the  $\beta_3$  tail compared to wild-type cells (Figure 4B,C). Here, the increased levels of pY416-Src in R995A/R997A/L1000A, K716A, and H722A cells after 1 h spreading on fibrinogen were in good agreement with their increased cell spreading.

#### Association of Skelemin with Mutant Integrins.

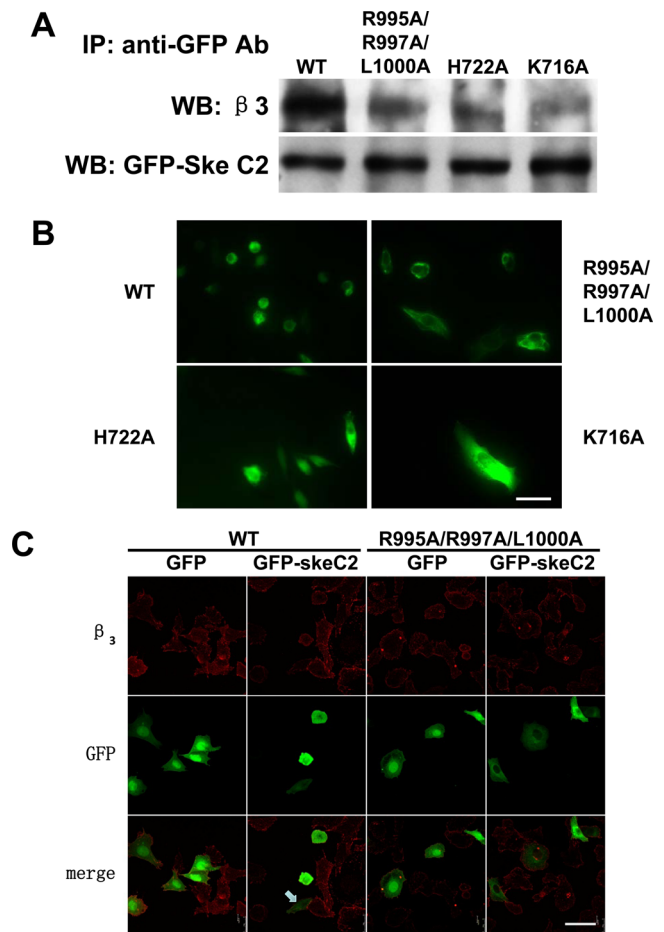
Experiments were then performed to confirm that our mutations resulted in a decrease association of skelemin with the expressed integrins. In cells, skelemin is present as either a soluble cytoplasmic protein or as an insoluble cytoskeletal-bound protein. The relative abundance of soluble endogenous skelemin in our cell lines was very low, and therefore the interaction between endogenous skelemin and  $\alpha_{IIb}\beta_3$  was difficult to elucidate and reproduce by coimmunoprecipitation and Western blot. Therefore, to overcome this problem, coimmunoprecipitation experiments were performed using recombinant GFP-skeC2 fusion protein. Wild-type, R995A/R997A/L1000A, H722A, and K716A cells transiently transfected with GFP-skeC2 plasmids or GFP as a control were subjected to immunoprecipitation with antibodies against GFP and Western blot analysis (Figure 5A). These three mutant cell lines were chosen as they all exhibited increased cell adhesion, spreading and active pSrc levels. Immunoblotting with an anti- $\beta_3$  antibody revealed that wild-type  $\alpha_{IIb}\beta_3$  coimmunoprecipitated with GFP-skeC2 (Figure 5A), but not with GFP (data not shown), confirming the specifics of interaction of  $\alpha_{IIb}\beta_3$  with skeC2. GFP-immunoreactive bands were consistent with the predicted size of the GFP-skeC2 fusion protein (~90 kDa) and



**Figure 4.** Effect of  $\alpha_{IIb}\beta_3$  mutations on Src signaling. (A) Increased levels of pY416-Src in mutant cell lines expressing similar levels of pY397-FAK. Cells were incubated with fibrinogen-coated plates for 1 h, lysed and total cell extracts subjected to SDS-Page. Blots were probed using antibodies against pY416-Src, Src, pY397-FAK, and  $\beta$ -actin.  $\beta$ -Actin staining demonstrated equal sample loading. The band ratio of pY416-Src/Src for each mutant cell line was shown in the lower panel. The band area was measured by ImageJ software and the band ratio of pY416-Src/Src for wild-type cells was normalized to 1. (B) Increased pY416-Src association with R995A/R997A/L1000A and K716A  $\alpha_{IIb}\beta_3$  mutants. Cells were plated on fibrinogen-coated culture dishes for 1 h and then lysed.  $\beta_3$  integrin was immunoprecipitated from the cell lysates using AP3 and Western blot analysis was performed using pY416-Src and  $\beta_3$  antibodies. Western blot analysis of total cell extracts using the  $\beta_3$  antibody demonstrated equal sample loading. Lower panel: Densitometric quantization of pY416-Src levels. Western blots of pY416-Src coimmunoprecipitated with  $\beta_3$  were quantitated by densitometric analysis. The data represent the mean  $\pm$  s.d. of three separate experiments. \*\* $P < 0.01$  compared to wild-type cells.

that equal amounts of fusion proteins were loaded in each lane. In comparison to wild-type cells, there was a marked decrease in the amount of  $\alpha_{IIb}\beta_3$  coimmunoprecipitated with R995A/R997A/L1000A, H722A, and K716A mutated integrins. Thus, we confirmed that the mutations did result in a decreased association of skelemin with  $\alpha_{IIb}\beta_3$ .

Previous studies have demonstrated that introduction of skeC2 into wild-type cells caused spread cells to round up.<sup>9,10</sup> We also investigated the spreading level of these cells transfected with GFP-skeC2 or GFP (Figure 5B). Wild-type, R995A/R997A/L1000A, H722A, and K716A cells transiently transfected with GFP-skeC2 or GFP were replated on fibrinogen, and cell areas were measured by GFP immuno-



**Figure 5.** Reduced skelemin binding to  $\alpha_{IIb}\beta_3$  mutants does not affect cell anchorage. (A) Association of GFP-skeC2 with  $\alpha_{IIb}\beta_3$ . Wild-type, R995A/R997A/L1000A, H722A, and K716A cells were transfected with GFP-skeC2 plasmids for 48 h, and then allowed to adhere to immobilized fibrinogen for 1 h. Cell lysates were immunoprecipitated with anti-GFP. Western blot analysis using anti- $\beta_3$  antibody revealed a decreased association between  $\beta_3$  mutants and GFP-skeC2 compared to wild-type  $\alpha_{IIb}\beta_3$ . (B) Immunofluorescence images reveal different effects of skelemin C2 transfection on wild-type (WT), R995A/R997A/L1000A, H722A, and K716A cells. Cells were transiently transfected with GFP-skeC2 and plated on fibrinogen for 2 h, fixed, and detected by GFP fluorescence. Scale bar: 100  $\mu$ m. (C)  $\beta_3$  integrin stained with AP3 (red) and GFP fluorescence were visualized by confocal microscopy. Wild-type and R995A/R997A/L1000A cells transfected with GFP or GFP-skeC2 were treated as described in (B). The cell that weakly expresses GFP-skeC2 is indicated by an arrowhead.

fluorescence on 2 h after spreading (Figure 5B). Compared to control transfections with GFP, GFP-skeC2 expression inhibited wild-type cells spreading, while it had little effect on the spreading of R995A/R997A/L1000A, H722A, and K716A cells. Figure 5C shows a representative confocal microscopy image of transfected wild-type and R995A/R997A/L1000A cells that were stained with AP3 (red) after 2 h spreading. Most wild-type cells expressing GFP-skeC2 were completely rounded up, whereas R995A/R997A/L1000A cells transfected GFP-skeC2 were fully spreading. It was noted that wild-type cells highly expressing GFP-skeC2 were rounded up but cells that were weakly expressing GFP-skeC2 had normal spreading morphology (indicated by arrowhead). A similar result was also observed in Hek293 cells. As shown in Supporting Information,

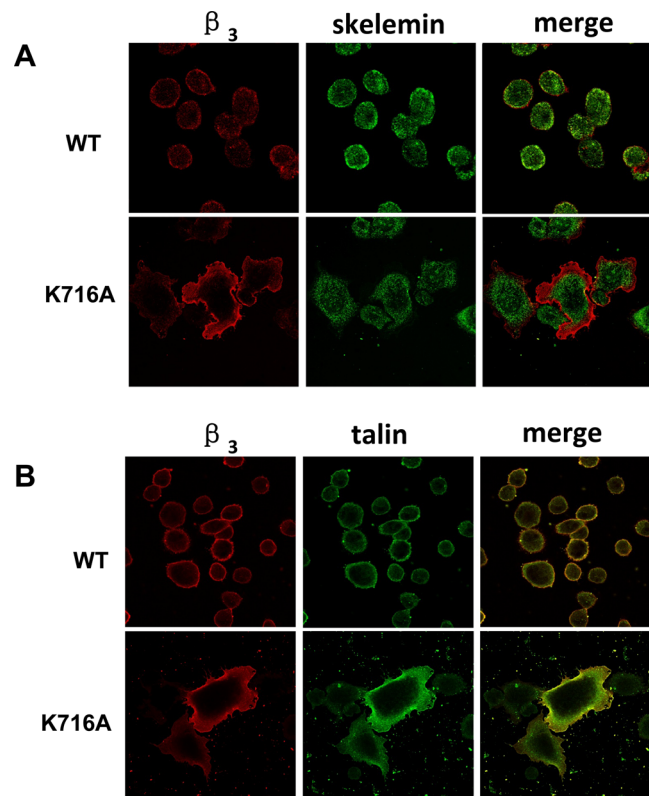
Figure 2, the transfection of Hek293 cells with GFP-skeC2 inhibited the  $\alpha_{\text{IIb}}\beta_3$ -mediated cell spreading only when the stoichiometry of GFP-skeC2 to  $\alpha_{\text{IIb}}\beta_3$  was high.

SkeC2 expression could abolish  $\alpha_{\text{IIb}}\beta_3$ -mediated cell spreading and had a more inhibitory effect for spreading in wild-type cells than mutant cells, at least in R995A/R997A/L1000A, H722A, and K716A cells. We conclude from these results that transfection of wild-type cells with GFP-skeC2 competes with endogenous skelemin for integrin binding, thus disrupting the binding of integrin tails to not only endogenous skelemin, but to other cytoskeletal proteins that physically link  $\alpha_{\text{IIb}}\beta_3$  to the cell cytoskeleton. However, the reduced affinity of GFP-skeC2 for mutant receptors allows for other cytoskeletal proteins to stabilize cell anchorage by providing the physical linkage of  $\alpha_{\text{IIb}}\beta_3$  to the cell cytoskeleton. Thus,  $\alpha_{\text{IIb}}\beta_3$  and skelemin, or other cytoskeletal proteins link the cell cytoskeleton to the ECM to stabilize cell anchorage and promote cell spreading.

**Co-Localization of Talin and Skelemin with Integrin  $\alpha_{\text{IIb}}\beta_3$  in Wild-Type and K716A Mutant Cells.** Among those integrin-cytoskeleton linkage candidates, talin is of interest as, similar to skelemin, it has a membrane-proximal binding region in the  $\beta_3$  tail, and it also has a distinct NPLY membrane-distal binding site.<sup>20</sup> In addition, talin plays critical roles in linking integrin to actin cytoskeleton and focal adhesion proteins during cell spreading, apart from being a direct activator of  $\alpha_{\text{IIb}}\beta_3$ .<sup>21</sup> Therefore, we used double labeling of integrin  $\alpha_{\text{IIb}}\beta_3$  (red) and talin (green) or skelemin (green) to compare the distribution of talin and skelemin in wild-type and mutant cells at the early time point of cell spreading (40 min). Among all the mutant cell lines, K716A was chosen to be shown here (Figure 6), as it developed enormous integrin-based membrane protrusions of filopodia and lamellipodia at the early cell spreading. Staining with AP3 (red) showed cell attachment to fibrinogen promoted translocation of integrin from the cytosol to the cell periphery in wild-type cells as well as K716A mutant cells. K716A mutant cells had a more profound spreading level, and filopodia and lamellipodia structures were strikingly obvious. Co-staining with skelemin showed that skelemin colocalized with  $\alpha_{\text{IIb}}\beta_3$  at the cell periphery in wild-type cells, while in K716A mutant cells it was not present in sheets of membrane protrusions at cell periphery but localized diffusely in the main cell body, thus losing the colocalization with  $\alpha_{\text{IIb}}\beta_3$  (Figure 6A). Compared with skelemin, both of the cell lines exhibited highly colocalization of  $\alpha_{\text{IIb}}\beta_3$  and talin at the cell periphery (Figure 6B). Wild-type cells displayed very strong ring-shaped staining for both  $\alpha_{\text{IIb}}\beta_3$  and talin at the cell periphery. This strong colocalization pattern is also clearly visualized in the sheets of lamellipodia in K716A mutant cells. It seems that colocalization of talin and integrin  $\alpha_{\text{IIb}}\beta_3$  supports active formation of cell membrane protrusions in K716A mutation, which could be a result of knocking down skelemin binding to  $\alpha_{\text{IIb}}\beta_3$  cytoplasmic tails.

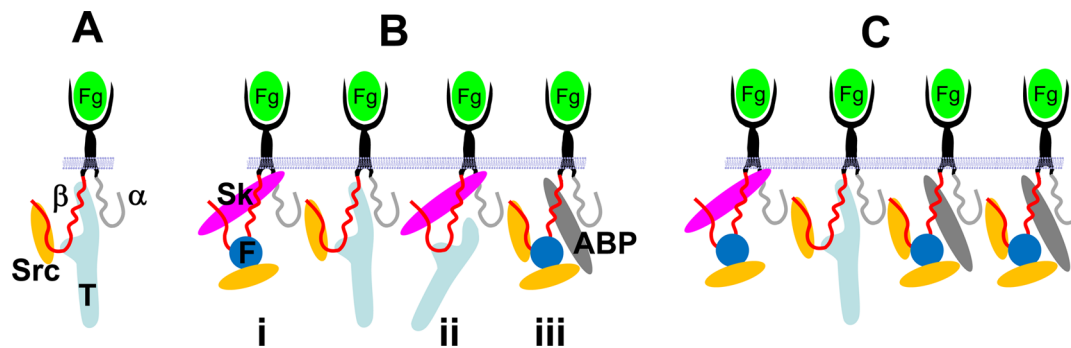
## DISCUSSION

The association of integrins with cytoskeletal proteins is crucial for the transmission of biochemical signals and mechanical force across these adhesion receptors and, thus, for integrin-mediated cell functions, such as spreading, migration, and gene expression.<sup>22</sup> The dynamic binding of skelemin to the cytoplasmic domains of integrin  $\alpha_{\text{IIb}}\beta_3$  during cell spreading process has been reported: skelemin did not bind to resting  $\alpha_{\text{IIb}}\beta_3$  in nonadhered platelets and CHO cells; cell adhesion and spreading to immobilized fibrinogen promoted skelemin



**Figure 6.** Distribution of skelemin and talin in wild-type and K716A cells. After spreading on fibrinogen-coated wells for 40 min, (A) wild-type (WT) or K716A cells were fixed and stained for  $\beta_3$  integrin and skelemin or (B)  $\beta_3$  integrin and talin. Images were collected on a confocal fluorescent microscope.

binding with  $\alpha_{\text{IIb}}\beta_3$ ; and, the two proteins dissociate in later stages of cell spreading.<sup>8–10</sup> Since skelemin is a family member of myosin-associated myomesin, it was previously speculated to exert a contractile force by linking integrin to myosin.<sup>10</sup> In the present study we tested this hypothesis and tried to elucidate the role of skelemin in integrin functions with the use of a series of stable CHO cell lines expressing mutant  $\alpha_{\text{IIb}}\beta_3$  integrins in which key residues involved in the binding of skelemin to  $\alpha_{\text{IIb}}\beta_3$  were mutated. We recognize that these mutations might also exhibit impaired interactions with other integrin binding proteins, and therefore we generated a number of mutants to reveal a general picture of the functional role of skelemin–integrin interactions. We found that most of the mutant cells defective in skelemin binding had unimpaired cell adhesion and spreading capacity at the early stages of cell spreading on immobilized fibrinogen. Some of the mutant cells also had increased membrane protrusion formation, a larger cell spreading area, and elevated levels of activated pY416-Src. These data lead us to conclude that engagement of skelemin to the cytoplasmic tail of  $\alpha_{\text{IIb}}\beta_3$  is not essential for the expansion of a cell protrusion during cell spreading. Instead, we propose that the binding of skelemin, talin, and other proteins to the tail of  $\alpha_{\text{IIb}}\beta_3$  is a mutually exclusive event and depending on what protein is bound, a cell will either spread or contract. As discussed below, when skelemin is bound to the cytoplasmic tail of  $\alpha_{\text{IIb}}\beta_3$ , a contractile force is generated which supports cell contraction. To initiate cell spreading, a cell must prevent or disrupt skelemin binding to allow for the recruitment of other proteins to integrin clusters that facilitate cell spreading, such as



**Figure 7.** Proposed model of skelemin interacting with  $\alpha_{IIb}\beta_3$  tails. (A) Activation. Integrin activation involves the binding of talin (T) to the membrane proximal and NPLY regions of  $\beta_3$  that results in unclasp of the integrin tails, leading to  $\alpha_{IIb}\beta_3$  binding to fibrinogen (Fg). Src is also directly bound to C-terminus of the  $\beta_3$  tail. (B) Early stages of cell anchorage and spreading. Following integrin activation, integrin clustering is initiated together with the activation of FAK (F). This results in the recruitment of additional integrins, skelemin and other cytoskeletal proteins to the integrin cluster and the formation of a focal adhesion. (i) Skelemin binds to the membrane-proximal regions of  $\alpha_{IIb}$  and  $\beta_3$  tails and to the C terminus of  $\beta_3$  tails, which displaces Src from the C-terminus of  $\beta_3$ , but still allows Src to bind to  $\beta_3$  via activated FAK bound to the NPLY region. (ii) In the presence of skelemin, talin can still bind to the  $\beta_3$  NPLY region but not to its  $\beta_3$  membrane-proximal binding domain. (iii) The binding of other actin-binding proteins (ABP) to integrin tails allows for maximal Src activation while providing a linkage to the actin cytoskeleton. Skelemin can also provide transient linkages to the actin cytoskeleton (i and ii). A combination of these binding scenarios would be present within a focal adhesion. (C) Late stages of cell anchorage and spreading. As cell adhesion and spreading progresses, the majority of the soluble skelemin is replaced by other actin-binding proteins, allowing for increased Src activation. The remaining skelemin makes firm contacts with the actin cytoskeleton bringing cell spreading and adhesion to completion.

talin and Src. Thus, modulating skelemin binding to integrin tails is one mechanism a cell can use to regulate the highly organized process of cell spreading.

We identified three constitutively active integrin mutants: R995A, K716A, and R995A/R997A/L1000A. To address whether the activation of the mutant is caused by knocking down skelemin binding, we characterized PAC-1 binding of cells overexpressing GFP-SkeC2. Overexpression of this fragment did not alter the integrin affinity state of either wild-type or active mutant cells (Figure 1C). Unlike talin which is able to unclasp the membrane-proximal interface of  $\alpha_{IIb}/\beta_3$  cytoplasmic tails and lead to integrin activation, skelemin cannot unclasp the interface even though skelemin also binds the membrane proximal regions of  $\alpha_{IIb}/\beta_3$  cytoplasmic tails as the head domain of talin. It has been demonstrated that skelemin and  $\alpha_{IIb}\beta_3$  association occurs after unclasp of the  $\alpha_{IIb}/\beta_3$  interface due to integrin–ligand ligation, which unmasked binding residues with skelemin.<sup>8</sup> Our data here support the view that the association of skelemin with  $\alpha_{IIb}\beta_3$  is a postligand event, but not involved in the process of integrin–ligand affinity regulation.

The binding of skelemin with integrin is dynamically regulated during cell spreading, suggesting its regulatory role for cell spreading. Our proposed model for skelemin in cell spreading and anchorage is displayed in Figure 7. Focusing on cytoskeletal proteins, activation of integrins involves talin binding (Figure 7A) that results in integrin clustering and activation of FAK, resulting in the recruitment of skelemin and other cytoskeletal proteins to integrin clusters (Figure 7B). During these early phases of cell adhesion and spreading, skelemin competes with other cytoskeletal proteins for binding to the  $\beta$  tail and prevents the activation of Src from occurring at the C-terminus of the  $\beta$  tail. In doing so, skelemin would counteract the activity of other proteins involved in cell adhesion, spreading and signaling, providing the cell with a mechanism to fine-tune its cell shape. During later stages of cell spreading, soluble skelemin either converts into insoluble skelemin, or dissociates from integrins,<sup>8</sup> being replaced by talin

or other actin-binding proteins and firm adhesion occurs (Figure 7C).

Previous studies reported that microinjection or overexpression of skeC2 fragments into cultured cells abolished cell spreading.<sup>9,10</sup> It was assumed that skelemin was essential for cell spreading based on the interpretation that skeC2 competes with endogenous skelemin binding to integrins but lack of a cytoskeletal binding site, and thus resulting in breakage of the integrin–cytoskeleton linkage. However, we found that mutant cells defective in skelemin binding had unimpaired adhesion and spreading capacity on integrin ligands, suggesting that skelemin binding to integrin was not essential for cell spreading. These apparent contradictory results can be resolved if one reinterprets the earlier study with the new data generated herein. We propose that the binding of skeC2 with integrin tails results in a loss of the control mechanisms governing the dynamics of cell adhesion and spreading by blocking the binding of endogenous skelemin and other cytoplasmic proteins to integrin tails that are important in regulating the formation of cellular protrusions and cell spreading. Whereas in our study, the use of cells expressing receptors containing site-specific point mutations impeded the binding of endogenous skelemin but not the interactions of other cellular proteins with the integrin tails. Furthermore, the stoichiometry of skelemin and integrin needs to be considered when using GFP-skeC2 since we found that GFP-skeC2 inhibited the  $\alpha_{IIb}\beta_3$ -mediated cell spreading only when the stoichiometry of GFP-skeC2 to  $\alpha_{IIb}\beta_3$  was high (Figure 5C and Supporting Information, Figure 2). The interaction between integrin and skelemin is robust. A previous NMR study showed that the association between skelemin C2 and integrin  $\beta_3$  tail is increased by increasing the ratio of skelemin C2 to  $\beta_3$ .<sup>11</sup> If the ratio of skelemin C2 to integrin is much higher in plasmid-transfected CHO cells than in wild type cells, such as platelets, one may overestimate or incorrectly define the role of skelemin in integrin biology. One problem with overexpressing skelemin C2 is that it would saturate the skelemin binding sites on integrins and not only block endogenous skelemin binding but also the association of other integrin binding partners that compete for the skelemin

binding site. This is likely why we were able to obtain some novel insights into skelemin function from experiments utilizing our wild-type and mutant integrin cell lines but not from skelemin C2 overexpression experiments.

The cytoplasmic tails of  $\alpha_{IIb}\beta_3$  are key structures for outside-in signaling in that they recruit a substantial number of cell signaling and cytoskeletal proteins.<sup>23</sup> Notable among these are Src and talin, which are obligatory for cell spreading.<sup>24,25</sup> The level of pY416-Src in total cell lysates and that coimmunoprecipitating with  $\beta_3$  increased in R995A/R997A/L1000A and K716A cells (Figure 5B), suggesting that a reduction in skelemin bound to integrins may allow more Src to be bound and priming it for activation. NMR spectroscopy study revealed that immunoglobulin C2-like repeats 4 (SkIgC4) interacts weakly with C terminus of  $\beta_3$  tails, which is also the binding site for Src involved in binding with integrin  $\alpha_{IIb}\beta_3$ .<sup>11,16</sup> Therefore, it is very likely that the binding of SkIgC4 with C terminus of  $\beta_3$  tails occupies the Src binding site and thereby reduces the capacity to maximally activate Src. However, it does not completely block Src activation as Src can still bind to and become activated at the membrane distal NPLY motif of  $\beta_3$  through activated FAK.<sup>19</sup>

Recent structural studies shed additional light on this topic in identifying the critical roles of K716 of  $\beta_3$  integrin in the  $\alpha_{IIb}\beta_3$  interface and its interactions with  $\alpha_{IIb}$  via hydrogen bonds and electrostatic interactions.<sup>14,26,27</sup> Our PAC-1 binding assay confirmed that K716A is an activated integrin mutant. Highly developed filopodia and lamellipodia were visualized at early cell spreading stage in K716A mutant cells (Figure 6). Furthermore, the residue of K716 appeared to be the most important for skelemin binding in *in vitro* studies.<sup>8</sup> Given the overlapping binding areas within the  $\beta_3$  membrane proximal region, we predict that binding of skelemin and talin to the  $\beta_3$  membrane proximal region are mutually exclusive events. This was supported in our study that talin is strongly colocalized with  $\alpha_{IIb}\beta_3$  in the ruffle structure of the K716A cell protrusions, but skelemin still remained in the main cell bodies (Figure 6). Disrupting skelemin binding in K716A cell may facilitate talin recruitment that promotes actin polymerization and membrane protrusion formation. Filopodia and lamellipodia are the two integrin and actin-based membrane protrusions formed at the leading edge of a moving cell or the periphery of a spreading cell that are prerequisite cell motility and spreading.<sup>28</sup> The elongation of these protrusions pushes the leading edge forward while the tail edge undergoes retraction enabling the cell to migrate.<sup>29</sup> Dynamic cell spreading requires that a cell balance its extending and contractile forces. In platelets, contractile forces also play an important role in blood clot retraction, where the fibrin meshwork is bound to  $\alpha_{IIb}\beta_3$  and pulled together by the platelet cytoskeleton. Contractile forces are provided by myosin II.<sup>30</sup> Similar to myomesin, skelemin was thought to regulate the organization of myosin filaments and mediate the interaction of myosin with integrins.<sup>10</sup> In our study, skelemin was not present in the sheets of lamellipodia, and reducing skelemin-integrin interactions promoted cell protrusion formation in K716A cell and cell spreading in other mutant cells. These results do not support a role for skelemin in generating an extending force but are consistent with skelemin exerting a contractile force.

In summary, our results extend the current understanding of skelemin function as an integrin–cytoskeleton linker. We propose a model that the two components of skelemin, soluble and insoluble, might differ in importance for particular integrin functions. During the initial stages of cell spreading, soluble

skelemin proteins bind to  $\alpha_{IIb}\beta_3$  integrin clusters at the leading edges of cells. These skelemin–integrin interactions function to coordinate the binding of different cytoskeletal proteins to the membrane proximal region of integrin tails, such as talin and Src (Figure 7B). However, the NPLY region remains exposed during skelemin binding and thus talin and Src can still bind to  $\beta_3$ . During this time period, talin can therefore function as a linker of integrin and actin filaments and maximal Src activation can be modulated by skelemin. This might afford a mechanism to dampen Src activation and consequently suppress integrin signaling. As cell adhesion and spreading progresses and large amounts of cell protrusion form, a majority of skelemin then dissociates from integrins to allow for other actin-binding proteins to bind to integrins, bringing cell spreading and adhesion to completion (Figure 7C). Concurrently, there is an increase in active Src levels and talin at the cell leading edges, due to skelemin dissociation. Some of soluble skelemin can remain bound to  $\alpha_{IIb}\beta_3$  and function as a linker between integrins and the myosin cytoskeleton, thereby transforming it into insoluble skelemin.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Figures showing the cell-surface expression levels of  $\alpha_{IIb}\beta_3$  and Hek293 cells cotransfected with  $\alpha_{IIb}\beta_3$  and GFP-Ske C2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS:

ADP, adenosine diphosphate; AP3, an activation-independent anti- $\beta_3$  antibody; CHO, Chinese hamster ovary; ECM, extracellular matrix; FAK, focal adhesion kinase; GFP, green fluorescence protein; PAC-1, an antibody specific for integrin  $\alpha_{IIb}\beta_3$  activation conformation; skeC2, immunoglobulin C2 motifs 4–5 of skelemin that contains the  $\alpha_{IIb}\beta_3$  binding domain

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