Effect of Cytoplasmic Domain Mutations on the Agonist-stimulated Ligand Binding Activity of the Platelet Integrin αIIbβ3*

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The platelet integrin αIIbβ3 is regulated by agonist-generated signals interacting with its cytoplasmic tails. When αIIbβ3 is expressed in Epstein-Barr virus-transformed B lymphocytes, stimulation of the cells with phorbol 12-myristate 13-acetate results in αIIbβ3-mediated lymphocyte adherence to immobilized fibrinogen, as well as soluble fibrinogen binding to αIIbβ3, indicating that agonists increase the affinity of αIIbβ3 for fibrinogen in these cells. To address the contribution of the αIIb and β3 cytoplasmic tails to this process, we mutated each tail and expressed the mutants in B lymphocytes. Truncation of the αIIb tail did not impair unstimulated or stimulated lymphocyte adherence to fibrinogen, regardless whether the truncation was proximal or distal to the conserved GFFKR sequence. However, deleting GFFKR or replacing it with alanines markedly reduced αIIbβ3 expression due to impaired intracellular assembly of αIIbβ3 heterodimers, probably due to a mutation-induced change in the conformation of αIIb. Introducing β3 mutations known to impair αIIbβ3 function in platelets into the cytoplasmic tail of β3 in lymphocytes also impaired αIIbβ3 function in these cells. These studies demonstrate that the cytoplasmic tail of αIIb is not required for αIIbβ3 function in lymphocytes, although the presence of GFFKR in the αIIb tail is required for αIIb to interact with β3. Additionally, they indicate that signals interacting with the β3 cytoplasmic tail are responsible for the ability of agonists to stimulate αIIbβ3 function.

The ability of naturally occurring mutations involving the cytoplasmic tail of β3 to abrogate αIIbβ3 function in platelets (3, 4) indicates that this portion of β3 is essential in the process of αIIbβ3 activation. The role of the αIIb cytoplasmic tail is less certain. Although no naturally occurring αIIb mutations involving the αIIb tail have been reported, studies of recombinant αIIbβ3 expressed in Chinese hamster ovary (CHO) cells suggest that the conserved membrane-proximal sequence GFFKR in the αIIb cytoplasmic tail regulates the affinity of αIIbβ3 for ligands by maintaining unstimulated αIIbβ3 in an inactive state (5, 6).

To develop an in vitro model to study the agonist-induced function of wild-type and mutant αIIbβ3, we have expressed αIIbβ3 in human Epstein-Barr virus (EBV)-transformed B lymphocytes and found that stimulation of these cells by phorbol 12-myristate 13-acetate (PMA) resulted in their adherence to immobilized fibrinogen, suggesting that PMA stimulation had increased either the avidity or affinity of αIIbβ3 for fibrinogen (7). In this paper, we report that PMA stimulation resulted in the binding of soluble fibrinogen to lymphocytes expressing αIIbβ3, indicating that agonist stimulation increased the affinity of the expressed αIIbβ3 for fibrinogen. We then used the lymphocyte system to address the roles of the αIIb and β3 cytoplasmic tails in αIIbβ3 activation. We found that although deletion of the αIIb tail did not affect the ability of αIIbβ3 to interact with fibrinogen or to respond to PMA stimulation, deletion or replacement of GFFKR markedly reduced the expression of αIIbβ3 on the lymphocyte surface by impairing the intracellular association of αIIb with β3. In contrast, introduction of the naturally occurring β3 mutations that impair αIIbβ3 function in platelets into the cytoplasmic tail of β3 expressed in lymphocytes also impaired αIIbβ3 function in these cells. These results suggest that intracellular signals interacting with the cytoplasmic tail of β3, but not the cytoplasmic tail of αIIb, are responsible for the ability of agonists to stimulate αIIbβ3 function.

EXPERIMENTAL PROCEDURES

Construction of αIIb and β3 Mutants—Mutations were introduced into the cytoplasmic tail of αIIb and into residue 752 of β3 (Fig. 1) using a modified overlap polymerase chain reaction procedure as described previously (7) with oligonucleotides encoding the desired mutations plus additional conservative mutations that generated diagnostic restriction sites (7). Wild-type αIIb and β3 in the EBV-based episomal plasmids pREP9 and pREP4 (8), respectively, were used as templates for the polymerase chain reaction. The amplified DNA fragments were then subcloned into pBS, sequenced, and assembled into the expression plasmids. The β3 mutation Arg-724 → stop was produced using the QuickChange site-directed mutagenesis method (Stratagene) and using β3 inserted into the Bluescript vector as a template. The identity of

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† The abbreviations used are: CHO, Chinese hamster ovary; EBV, Epstein-Barr virus; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; ER, endoplasmic reticulum.
Expression of Wild-type αIIbβ3 and αIIbβ3 Mutants in Human B Lymphocytes—pREP vectors containing cDNAs for wild-type and mutant αIIb and β3 were introduced into 7.5 × 10⁶ GM1000 B lymphocytes by electroporation (250 V and 960 millifarads). Stable cotransfectants were selected by growth in RPMI medium containing 20% fetal calf serum and both G418 (750 μg/ml) and hygromycin (250 μg/ml). The presence of αIIbβ3 on the lymphocyte surface was detected by flow cytometry. Cells were stained with the αIIbβ3-specific murine monoclonal antibody (mAb) A2A9 (9), the β3-specific mAb SSA6 (10), and the class-specific control antibody OKT3 (ATCC), followed by fluorescein-conjugated anti-murine IgG as described previously (11). Flow cytometry was performed using a FACSscan flow cytometer (Becton-Dickinson). The assembly of αIIbβ3 heterodimers in lymphocytes was studied by pulse-chase analysis. Briefly, transfected lymphocytes were pulse-labeled with [35S]methionine (500 μCi/ml) for 60 min in methionine-free medium. The medium was then replaced with complete medium containing 1 mM unlabeled methionine, and the incubation was continued for various periods of time after which the cells were extracted with 2.5 mM Tris buffer, pH 7.4, containing 1% Triton X-100. Pro-αIIb and αIIbβ3 were then immunoprecipitated from the extracts using polyclonal rabbit anti-αIIb antisera and the αIIbβ3-specific mAb A2A9, followed by SDS-polyacrylamide gel electrophoresis and autoradiography as described previously (12). The polyclonal anti-αIIb antisera used in these experiments only immunoprecipitates αIIb and pro-αIIb and not αIIbβ3 heterodimers (data not shown). Integration of wild-type αIIb and the various αIIb mutants into the membranes of the lymphocyte endoplasmic reticulum (ER) was studied by treating isolated lymphocyte membranes with sodium carbonate, as described previously (13). Briefly, transfected lymphocytes were pulse-labeled with [35S]methionine and chased with methionine-free medium for 4 h as described above. The labeled cells were then disrupted by 30 strokes in a Dounce homogenizer fitted with a tight pestle and the homogenate centrifuged for 10 min at 2000 rpm to sediment remaining intact cells and nuclei (14). Membranes were sedimented from the postnuclear supernatant by centrifugation at 230,000 × g for 15 min and resuspended in 0.5-ml 100 mM sodium carbonate, pH 11.5. Following a 30-min incubation on ice, extracted proteins were separated from membranes by an additional centrifugation at 230,000 × g for 2 h. After neutralization, 0.5% Triton X-100 was added to the samples and αIIb was immunoprecipitated using rabbit polyclonal anti-αIIb antisera. As a positive control for the sodium carbonate extraction, αIIbα₃, a soluble αIIb mutant produced by introducing a stop codon into an αIIbα₃ transmembrane domain, was expressed in COS-1 cells (15). Forty-eight hours after transfection, the COS-1 cells were carried through the sodium carbonate treatment protocol concurrently with the lymphocytes.

Interaction of Lymphocytes Expressing αIIbβ3 with Soluble Fibrinogen—Previously, we demonstrated that stimulation of EBV-transformed B lymphocytes expressing αIIbβ3 with the phorbol ester PMA results in the adherence of these cells to immobilized fibrinogen (7). However, it was uncertain whether this response to PMA results simply from more avid lymphocyte adherence to fibrinogen or from an actual increase in the affinity of αIIbβ3 for fibrinogen. To address this question, we tested the ability of soluble fibrinogen to inhibit lymphocyte adherence, based on the premise that soluble fibrinogen must bind to αIIbβ3 to inhibit adherence. Accordingly, we coated the wells of microtiter plates with four different concentrations of fibrinogen (1, 2.5, and 10 μg/ml) and measured PMA-stimulated lymphocyte adherence in the presence of increasing concentrations of soluble fibrinogen. As shown in Fig. 2A, the adherence of PMA-stimulated lymphocytes expressing αIIbβ3 decreased as the concentration of soluble fibrinogen increased for each concentration of immobilized fibrinogen, suggesting that there was competition between the soluble and immobilized fibrinogen for binding to αIIbβ3. At the highest concentration of soluble fibrinogen tested (14 μg/ml or 44 μM), lymphocyte adherence was inhibited by ~90% to plates that had been coated with fibrinogen at 5 and 10 μg/ml and by ~95% to plates that had been coated at 1 and 2 μg/ml. To verify that the inhibitory effect of soluble fibrinogen was specific, we tested the ability of equimolar concentrations of a number of other soluble proteins to inhibit lymphocyte adherence. As shown in Fig. 2B, other proteins including the β3-specific mAb SSA6, the αIIb-specific mAb B1B5, albumin, and transferrin failed to inhibit PMA-stimulated lymphocyte adherence at the maximum concentration of soluble fibrinogen examined, 44 μM.

The ability of soluble fibrinogen to inhibit PMA-stimulated lymphocyte adherence to immobilized fibrinogen is indirect evidence that soluble fibrinogen can bind to αIIbβ3 on these cells. Therefore, to directly demonstrate soluble fibrinogen binding to lymphocyte αIIbβ3, we labeled fibrinogen with FITC and measured FITC-labeled fibrinogen binding to untransfected lymphocytes and lymphocytes expressing αIIbβ3 by fluorescence-activated flow cytometry (16). As shown in Fig. 3, there was no change in the position of the fluorescence histogram when untransfected lymphocytes incubated with FITC-
labeled fibrinogen were stimulated with PMA, demonstrating that there were no detectable binding sites for FITC-labeled fibrinogen on the surface of these cells. Additionally, the positions of these histograms coincided with the position of the fluorescence histogram of lymphocytes expressing αIbβ3 that had been incubated with FITC-labeled fibrinogen in the absence of PMA stimulation. However, following PMA stimulation, the position of the latter histogram was shifted to the right, indicating that PMA had exposed fibrinogen binding sites on the lymphocyte surface. To verify that these binding sites corresponded to αIbβ3, the fibrinogen binding measurements were repeated in the presence of A2A9, a mAb that specifically inhibits fibrinogen binding to αIbβ3 on stimulated platelets (9). The presence of A2A9 shifted the position of the histogram back to that of unstimulated cells, confirming that the FITC-labeled fibrinogen was bound to αIbβ3. Chelation of divalent cations using EDTA gave an identical result, verifying that the measured fibrinogen binding was cation-dependent (data not shown). Thus, these experiments indicate that PMA stimulation results in an increase in the affinity of lymphocyte αIbβ3 for soluble, as well as immobilized, fibrinogen. Moreover, they argue that the adherence of lymphocytes expressing αIbβ3 to immobilized fibrinogen is a reflection of this increased affinity rather than a post-receptor event.

**Effect of Mutations Involving the αIb Cytoplasmic Tail on the Expression of αIbβ3 by Lymphocytes**—To examine the role of the αIb cytoplasmic tail in agonist-stimulated αIbβ3 function, we truncated αIb proximal and distal to its GFFKR motif by replacing the codons for Gly-991 and Asn-996 in an αIb cDNA with stop codons. The resulting αIb mutants were designated αIbβ3G991P and αIbβ3G995P, after their carboxyl-terminal residue (Fig. 1). In addition, we replaced GFFKR with AAAAA and Gly-991 with the helix-breaking amino acid Pro (αIbβ3A5) based on predictions derived from homology modeling that the membrane-proximal region of the αIb tail including GFFKR assumes an α helical configuration (6).2 The resulting αIb mutants and wild-type αIb were then coexpressed with β3 in EBV-transformed B lymphocytes. To assess the level of expression of each αIbβ3 mutant on the lymphocyte surface, the cell lines were stained with the αIbβ3-specific mAb A2A9 and examined by flow cytometry. As shown in Fig. 4, there was similar staining of wild-type αIbβ3, αIbβ3G991P, and αIbβ3G995P, whereas the staining of lymphocytes expressing αIbβ3A5 and αIbβ3G995P was markedly decreased. In six separate transfections of αIbβ3G995P and three separate transfections of αIbβ3A5, there was only a 2.6 ± 0.5-fold and a 1.9 ± 0.3-fold increase in MFI, respectively, when the cells were

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1 T. Keiber-Emmons and J. S. Bennett, unpublished data.
stained with A2A9 compared with staining with the class-
matched control antibody OKT3. In contrast, the increases in
MFI after staining cells expressing wild-type αIIbβ3, αIIb995β3, and αIIb991Pβ3 were 15.3 ± 2-fold, 5.9 ± 0.7-fold, and 9.7 ± 2.8-fold, respectively. Staining the cells with the β3-specific mAb SSA6 instead of A2A9 gave similar results, indicating that the reduction in A2A9 staining was not due to absence of the epitope for A2A9 on cells expressing αIIb995β3 and αIIb99Pβ3 (data not shown). Thus, these data indicate that the GFFKR motif plays an important role in αIIbβ3 expression, a conclusion that is consistent with previous observations of the role played by GFFKR in the expression of β1 and β2 integrins (20–22).

FIG. 4. Histograms from the flow cytometric analysis of lymphocytes expressing wild-type αIIbβ3 and the indicated αIIbβ3 mutants. Transfected cells were incubated sequentially with either the αIIbβ3-specific mAb A2A9 or the class-matched control mAb OKT3 and then with fluorescein-conjugated anti-murine IgG. Labeled cells were analyzed by flow cytometry as described under “Experimental Procedures.”

To examine the basis for the reduced expression of αIIb995β3 and αIIb99Pβ3 on the lymphocyte surface, we performed pulse-chase studies, comparing the biosynthesis of these heterodimers with that of wild-type αIIbβ3 and αIIb99Pβ3. Lymphocytes were pulsed with [35S]methionine for 1 h and at intervals throughout the subsequent 8-h chase, aliquots of lymphocytes were extracted with Triton X-100 and pro-αIIb and αIIbβ3 heterodimers were immunoprecipitated. As seen in Fig. 5, there was no discernible difference in either the synthesis or stability of pro-αIIb, pro-αIIb995, pro-αIIb99P, or pro-αIIbA5 throughout the 8-h period of chase. However, whereas heterodimers were clearly present by 2 h in detergent extracts of the αIIbβ3 and αIIb99Pβ3 transfectants, only traces of heterodimer were detectable in extracts from the αIIb995β3 and αIIb99Pβ3 transfectants at any time point up to 8 h. Thus, these studies indicate that the reduced expression of αIIb995β3 and αIIb99Pβ3 resulted from decreased assembly of αIIb99Pβ3 heterodimers and suggest that deletion or replacement of GFFKR impairs the ability of αIIb to interact with β3.

Assembly of αIIb and β3 into heterodimers involves sequences located in the extracellular domain of each subunit (15, 23), whereas GFFKR is located in the αIIb cytoplasmic tail adjacent to its transmembrane sequence. Thus, it is unlikely that absence of GFFKR directly impairs the ability of αIIb to associate with β3. On the other hand, it is conceivable that the absence of GFFKR impairs the stop-transfer mechanism that anchors αIIb in membranes, thereby shifting a fraction of nascent αIIb from the membrane to the lumen of the ER, where it would be unavailable to associate with nascent β3. Alternatively, absence of GFFKR could affect the folding of nascent αIIb such that it was no longer able to be recognized by β3. To address the first possibility, we studied the association of αIIb, αIIb95, αIIb990, and αIIbA5 with lymphocyte membranes by isolating membranes from [35S]methionine-labeled transfected cells and treating the membranes with 0.1 M sodium carbonate, pH 11.3 (13). This treatment converts closed membrane vesicles into open sheets, thereby releasing their luminal contents and peripheral membrane proteins in soluble form. As concurrent positive and negative controls, membranes were also prepared from [35S]methionine-labeled COS-1 cells transiently expressing αIIbX, an αIIb mutant truncated proximal to the αIIb transmembrane domain (15), and from [35S]methionine-labeled untransfected lymphocytes. As shown in Fig. 6, soluble αIIbX was present in the sodium carbonate extract of COS-1 cell membranes, but neither αIIb, αIIb995, αIIb990, nor αIIbA5 were detected in the sodium carbonate extracts of lymphocyte membranes. Thus, these experiments indicate that absence of GFFKR does not prevent αIIb anchoring in lymphocyte membranes, implying that each form of nascent αIIb is available to associate with nascent β3 in the lymphocyte ER. Consequently, the failure to detect αIIbβ3 in lymphocytes expressing αIIb990 and αIIbA5 suggests that absence of GFFKR results in a conformational change in αIIb that impairs its ability to be recognized by β3.

Effect of Mutations Involving the αIIb Cytoplasmic Tail on the Adherence of Lymphocytes to Immobilized Fibrinogen—Next, we compared the ability of mock-transfected lymphocytes and lymphocytes expressing αIIbβ3 heterodimers containing wild-type αIIb, αIIb990, or αIIb995 to adhere to immobilized fibrinogen. As shown in Fig. 7, there was no adherence of mock-transfected lymphocytes to fibrinogen, either before or after PMA stimulation. By contrast, we found that 4–8% of lymphocytes expressing wild-type αIIbβ3 were adherent to fibrinogen in the absence of PMA stimulation and adherence increased to 35–40% following stimulation by 200 ng/ml PMA. Moreover, deleting the 13 amino acids in the αIIb tail distal to
GFFKR had no effect on lymphocyte adherence because 12–20% of lymphocytes expressing αIIb995β3 were adherent in the absence of PMA stimulation and stimulation increased adherence to 35–50%. In addition, although only 1–2% and 8–9% of lymphocytes expressing αIIb990β3 were adherent in the absence and presence of PMA stimulation, respectively, when lymphocyte adherence was normalized for the level of heterodimer expression by calculating a ratio of adherence (Fig. 7) to MFI (Fig. 4), the ratios for cells expressing αIIb990β3 (0.25) and αIIbβ3 (0.17) were similar. The adherence of lymphocytes expressing wild-type αIIbβ3 and each of the αIIb mutants was inhibited completely by the mAb A2A9, confirming that it was mediated by the extracellular domain of αIIbβ3 (data not shown). Thus, these data indicate that lymphocytes expressing αIIb990β3 adhere to fibrinogen at least as avidly as cells expressing wild-type αIIbβ3. In addition, they demonstrate that truncation of the αIIb cytoplasmic tail, whether proximal or distal to GFFKR, neither impairs αIIbβ3 function in lymphocytes nor precludes the ability of αIIbβ3 to respond to PMA stimulation in these cells.

The results described above suggest that the presence or absence of GFFKR in the cytoplasmic tail of αIIb may affect the conformation of αIIb and its ability to interact with β3. To pursue this observation, we measured the unstimulated and PMA-stimulated adherence of lymphocytes expressing αIIbA5β3 and αIIbG991Pβ3 to fibrinogen. Like αIIb990β3, we found the ability of αIIbA5β3 to mediate lymphocyte adherence was commensurate with its level of expression on the lymphocyte surface, since 4–5% of the cells were adherent in the absence of PMA stimulation. In addition, they demonstrate that
absence of PMA stimulation and adherence increased to 13–14% following exposure to PMA (Fig. 7). It is noteworthy, however, that while the adherence of PMA-stimulated lymphocytes expressing αIbβ3(3991-Pro) (38–41%) was identical to that of cells expressing wild-type αIbβ3, the adherence of unstimulated cells (17–21%) was significantly increased (p < 10^-7). This suggests that replacing Gly-991 with proline resulted in an increase in the fraction of αIbβ3 heterodimers that reside on the lymphocyte surface in a constitutively active state.

**Effect of Mutations Involving the β3 Cytoplasmic Tail on αIbβ3 Function**—Our previous studies of the function of αIbβ3-αβ2 chimeras suggested that the β3 cytoplasmic tail is specifically involved in αIbβ3 activation in lymphocytes (7). To test this suggestion, we introduced two naturally occurring mutations known to impair αIbβ3 function, Ser-752 → Pro (4) and Arg-724 → stop (3), into the cytoplasmic tail of β3 by in vitro mutagenesis (Fig. 1) and measured the adherence of lymphocytes expressing αIbβ3 heterodimers containing these mutations to immobilized fibrinogen. As shown by the flow cytometry histograms of cells stained with the mAb A2A9 in Fig. 8, comparable levels of wild-type αIbβ3, αIbβ3 containing the Ser-752 → Pro mutation (αIbβ3ser752pro), and αIbβ3 containing β3 truncated at amino acid 724 (αIbβ3724) were expressed on the surface of transfected lymphocytes. Nevertheless, whereas 4.8% of lymphocytes expressing wild-type αIbβ3 were adherent to fibrinogen in the absence of PMA stimulation and adherence increased to 37.8% in the presence of PMA, the corresponding values for lymphocytes expressing αIbβ3ser752pro and αIbβ3724 were 0.7% and 11.7% and 0.7% and 15.2%, respectively (Fig. 9). Thus, these experiments confirm the importance of the β3 cytoplasmic tail in agonist-stimulated αIbβ3 function.

Despite levels of expression comparable with wild-type αIbβ3, αIbβ3ser752pro and αIbβ3724 were only 25–30% as effective in mediating lymphocyte adherence to fibrinogen, indicating that their function was impaired. On the other hand, each mutant is unable to support platelet aggregation and fibrinogen binding (4) and when expressed in CHO cells, they greatly reduce ligand binding by constitutively active αIbβ3 (6). Accordingly, we had anticipated that the adherences of lymphocytes expressing αIbβ3ser752pro and αIbβ3724 would be essentially absent. Previously, we noted that B lymphocytes express a small amount of endogenous β3 as the β subunit of αβ3 (7). Thus, it is possible that when we transfected lymphocytes with αIb and β3 or with a cDNA for αIb alone. As seen in Fig. 10A, transfection with αIb alone resulted in the substantial accumulation of αIbβ3 on the lymphocyte surface (MFI of 98 versus 167 for the αIbβ3 transfectant). Moreover, as seen in Fig. 10B, the αIbβ3 expressed by these cells mediated adherence to fibrinogen at a level that was commensurate with the amount of αIbβ3 expressed on their surface. Thus, it is plausible that the reduced adherence of cells expressing αIbβ3ser752pro and αIbβ3724 was actually mediated by coincidentally expressed wild-type αIbβ3.
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determined and PMA-stimulated adherence of the transfected lymphocytes to

FIG. 10. αIβb3 expression on the surface of lymphocytes transfected both αIb and β3 or with αIb alone. A, histograms from the flow cytometry of mock-transfected cells, cells transfected with αIb and β3, and cells transfected with αIb alone. Cells were stained with the αIb3-specific mAb A2A9 and analyzed as described in Fig. 2 and under “Experimental Procedures.” B, comparison of the unstimulated and PMA-stimulated adherence of the transfected lymphocytes to fibrinogen. Lymphocyte adherence was measured as described in Fig. 7 and under “Experimental Procedures.”

DISCUSSION

In platelets, agonist-initiated signals convert αIbβ3 from an inactive to an active state by increasing its affinity for ligands such as fibrinogen and von Willebrand factor (1). We studied this process in vitro using a B lymphocyte expression system based on the premise that agonist-initiated inside-out signaling in lymphocytes can up-regulate the function of the hetero-
determined and PMA-stimulated adherence of the transfected lymphocytes to fibrinogen. Lymphocyte adherence was measured as described in Fig. 7 and under “Experimental Procedures.”

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3 Additionally, studies of PMA-stimulated soluble ICAM-1 binding to αLβ2 on T lymphocytes suggest that PMA stimulation converts an average of only 16% of the αLβ2 to a high affinity state (29). Similarly, it has been observed that a mAb that binds exclusively to the activated conformation of αMβ2 binds only to 10–20% of the αMβ2 molecules on activated neutrophils (30). Thus, it is possible that, unlike platelets, where there is a 1:1 correlation between fibrinogen binding and the number of αIbβ3 molecules (9), only a fraction of the αIbβ3 molecules on transfected B lymphocytes bind fibrinogen following PMA stimulation. Nevertheless, our data clearly demonstrate that, like platelets, agonist stimulation of lymphocytes increases the affinity of αIbβ3 for fibrinogen and provide evidence that this increase in affinity is responsible for the adherence of the cells to immobilized fibrinogen.

The mechanism by which inside-out signaling increases the affinity of αIbβ3 and other integrins for ligands is not known. Although the responsible intracellular signals interact with the cytoplasmic portions of integrins, the relative contribution of each integrin subunit to the signaling process is uncertain. Thus, while there is convincing evidence for the participation of the β subunit tail in the response of many integrins to agonists (29), the contribution of the α subunit tail appears to depend on the integrin studied and the cell in which the integrin is expressed. For example, truncation of the cytoplasmic tails of α2, α4, and α6A immediately distal to their GFFKR motifs abolished the constitutive and phorbol ester-stimulated ligand binding activity of β1 integrins in RD (20), K562 (21), and P388D1 (30) cells, whereas replacement of the α2 tail with tails of α4 or α5 (20) and the α4 tail with those of α2 or α5 had no effect (21). Thus, these data indicate that an α subunit tail can be required for integrin function, but this requirement may be fulfilled by the tails of a number of subunits. Further, the addition of only 3–4 amino acids on the carboxyl side of GFFKR may be sufficient to restore the ligand binding activity of integrins containing truncated α subunits (31). In contrast to these observations, deletion of the αL tail distal to GFFKR had no effect on the ability of αLβ2 expressed in COS cells to interact with ICAM-1 (22), nor did the deletion of the αM tail impair the interaction of αMβ2 with iC3b-coated erythrocytes (32).

αIbβ3 function has been studied extensively in vitro using adherent CHO fibroblasts (5, 6). Although CHO cells expressing αIbβ3 spontaneously adhere to and spread on immobilized fibrinogen (33), they are unable to bind the activation-dependent, αIbβ3-specific mAb PAC1 (34), suggesting that their αIbβ3 is in a low affinity state. However, truncation of either the αL tail before GFFKR or the β tail before the membrane proximal sequence LLITIHD results in PAC1 binding, implying that each truncation shifts αIbβ3 from a low affinity to a high affinity state (5, 6). These experiments suggest a model of αIbβ3 activation in which GFFKR and LLITIHD act as a “hinge,” interacting in unstimulated cells to maintain αIbβ3 in a low affinity state and separating following agonist stimulation to propagate signals from the cytoplasmic to the extracel-

ular portions of the molecule (6). In support of the model, disruption of either the GFFKR or LLITIHD sequence by alanine substitutions resulted in PAC1 binding, as did αIbβ3D295G and β3D723R mutations (35). Moreover, the “reversal” mutant αIbβ3D295Gβ3D723R was inactive, arguing that a salt bridge between Arg-995 in αIb and Asp-723 in β3 in unstimulated cells constrains αIbβ3 function. Nevertheless, replacing the αIb cytoplasmic tail with the tails of α2, α5, α6A, or α6B also induces PAC1 binding, despite preservation of the GFFKR

3 E. Loh, W. Qi, G. Vilaire, and J. S. Bennett, unpublished observations.
motif (6). Thus, other features of the αIIb tail in CHO cells, besides the presence of GFFKR, may regulate αIIbβ3 function.

We have examined the contribution of the αIIb and β3 cytoplasmic tails to αIIbβ3 function by expressing αIIbβ3 in the B lymphocytes (7). We found that despite deletion of the αIIb cytoplasmic tail, αIIbβ3 retained the ability to mediate lymphocyte adherence to fibrinogen, regardless of whether the tail was deleted before or after the GFFKR motif. Thus, like αβ2 (22) and αMβ2 (32), the presence of an αIIb tail is not required for αIIbβ3 function in these cells. Moreover, we found that αIIbβ3 retained the ability to be up-regulated by PMA despite the absence of a GFFKR motif. Thus, in contrast to αIIbβ3 in CHO cells (5), our data indicate that the bulk of the αIIbβ3 in unstimulated lymphocytes remains in an inactive state despite the absence of GFFKR and suggest it unlikely that GFFKR functions as part of a hinge in lymphocytes, although it possibly could do so in other cells. Nevertheless, the proportion of lymphocytes expressing αIIb(b990)β3 that were adherent to fibrinogen in the absence of PMA stimulation was significantly greater than the proportion of unstimulated lymphocytes expressing wild-type αIIbβ3 (p < 10−6). Thus, mutations involving GFFKR in lymphocytes, as in CHO cells, appear to increase the fraction of lymphocytes expressing αIIbβ3 in an activated form.

We found that the major consequence of removing the GFFKR motif from αIIb was a marked reduction in αIIbβ3 expression on the lymphocyte surface. A similar effect of GFFKR deletion on integrin expression has been observed when α2, α4, α5, and αL were truncated proximal to GFFKR (20, 21, 30, 36) and when αIIb, truncated proximal to GFFKR, was expressed in transient expression systems (6). Although a number of explanations for the impaired αIIbβ3 expression are possible, including a truncation-related decrease in αIIb synthesis and/or stability or the intracellular retention of misfolded or activated αIIbβ3 heterodimers, we found that removing GFFKR impaired the ability of αIIb to associate with β3. This was unexpected because αIIbβ3 assembly involves sequences located in the αIIb and β3 extracellular domains at considerable distances from their cytoplasmic tails (15, 23). However, GFFKR is located adjacent to the αIIb transmembrane domain. Thus, it is conceivable that its absence impairs the stop-transfer mechanism that anchors αIIb in membranes (37), thereby shifting a fraction of nascent αIIb from the membrane to the lumen of the ER, where it might be unavailable to associate with nascent β3. We addressed this possibility by treating isolated lymphocyte membranes with 0.1 M sodium carbonate, pH 11.5, to extract proteins confined to the ER lumen. In control experiments, we found that αIIb truncated proximal to its transmembrane domain was extracted from isolated COS cell membranes by this treatment, and in agreement with previous studies using isolated dog pancreas micromeres (38), we found that intact αIIb was not extractable from isolated lymphocyte membranes. We were never able to detect even traces of αIIb(b990)β3 in αIIb(b990) or αIIbαL in sodium carbonate extracts. Thus, these results indicate that both αIIb(b990) and αIIbαL were integrated into lymphocyte membranes, eliminating the possibility that removal of GFFKR shifts αIIb from the ER membrane to the ER lumen.

A third possible explanation for our results is that the absence of GFFKR induces a change in the conformation of αIIb that is propagated to its extracellular domain, thereby impairing its recognition by β3. The inability of membrane-associated αIIb lacking GFFKR to recognize β3 provides prima facie evidence that such an event occurs when GFFKR is either deleted or mutated. Moreover, propagation of conformational change across the length of the molecule is a property of integrins that accounts for both inside-out and outside-in signaling (2, 39, 40). In addition, we observed that when αIIb(b990) lacking its 18 carboxyl-terminal amino acids, was extracted from lymphocyte membranes using sodium carbonate, it migrated as doublet on SDS gels whose mobility was retarded compared with the mobility of wild-type αIIb (Fig. 6). The migration of the related α subunit, αv, truncated distal to GFFKR at residue 995, was also found to be retarded on SDS gels (41). This was thought to be due to altered αv folding because of an accompanying change in the pattern of αv cleavage by chymotrypsin. Thus, it is possible that the retarded migration of αIIb(b990) also resulted from a truncation-induced alteration in αIIb folding. Loss of GFFKR, however, cannot be the entire explanation because the electrophoretic mobility of wild-type αIIb and αIIbαL were identical. Nevertheless, the absolute conservation of GFFKR, and the related GFFRR sequence (42), among integrin α subunits implies that this motif supplies an indispensable function. Our data suggest that GFFKR is required for proper α subunit folding. Perhaps the shift toward increased αIIbβ3 activity observed when GFFKR is replaced or mutated reflects an impairment of this requirement.

Our finding that αIIbβ3 can be activated and adhere to fibrinogen, despite the absence of an αIIb tail, suggests that the β3 tail is primarily involved in agonist-mediated regulation of αIIbβ3 function, at least in lymphocytes. The diminished adherence of lymphocytes expressing αIIbβ3(b224), and αIIbβ3(b24), despite the presence of intact αIIb, is consistent with this conclusion, as are previous studies of αIIbβ2 function (22, 43). Accordingly, our data are consistent with a model of αIIbβ3 activation in which signals interacting with the cytoplasmic tail of β3 are propagated to the extracellular domain of the heterodimer to expose its ligand binding site. However, differences in αIIbβ3 function in various cells, for example lymphocytes and CHO cells, also suggest that host cell factors interacting with αIIbβ3 may differ. Because lymphocytes are of hematopoietic origin and normally express β3, the factors in lymphocytes may be the same or related to those present in platelets. The recent identification of a protein from T cells, cytohesin-1, related to the yeast SEC7 gene product, that specifically interacts with the β2 cytoplasmic tail to induce β2 integrin-dependent adherence of Jurkat cells to ICAM-1 (44), provides support for our model and suggests that the same or a similar protein could interact with the β3 cytoplasmic tail to activate αIIbβ3. A polypeptide called β3-endonexin that interacts specifically with the β3 cytoplasmic tail has been isolated from a B cell cDNA library (45). Whether this polypeptide functions in manner analogous to cytohesin-1 is not yet known.

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