Analysis of the tetraspanin CD9–integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) complex in platelet membranes and transfected cells

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The platelet integrin, $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), and the tetraspanin, CD9, are integral membrane proteins that are abundant in platelet membranes. We have identified several proteins, including CD9, which were co-precipitated by anti- $\alpha_{IIb}\beta_3$ antibody from untreated, resting platelets that were solubilized with the poly(oxyethylene) non-ionic detergent, Brij-35. Immunoblot and quantitative immunoprecipitation showed that the association of $\alpha_{IIb}\beta_3$ with CD9 is specific and stoichiometric. The interaction between CD9 and $\alpha_{IIb}\beta_3$ is probably hydrophobic, as Triton X-100 and hydrophobic detergents of the Brij series completely

dissociated the CD9– $\alpha_{IIb}\beta_3$ complex. Recombinant CD9 and $\alpha_{IIb}\beta_3$ can associate after transfection into Chinese hamster ovary cells, as seen by co-immunoprecipitation and co-localization in the periphery of spreading cells and in the lamellipodia of cells plated on fibrinogen. This co-localization is absent from focal adhesions. Furthermore, anti-CD9-coated latex beads co-clustered $\alpha_{IIb}\beta_3$ with CD9. This work indicates that the tetraspanin, CD9, is associated with β_3 integrins in resting platelets and transfected cells.

INTRODUCTION

The platelet-specific integrin, $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), is the receptor for fibrinogen, and mediates platelet aggregation and adhesion [1]. It is composed of two type-I transmembrane subunits. $\alpha_{IIb}\beta_3$ and other integrins can regulate their ligand binding affinity in a dynamic process termed ‘activation’ or ‘inside-out’ signalling [2,3]. As shown by mutagenesis studies, the cytoplasmic domains of integrin are involved in these signalling events [4,5]. In addition, protein factors have been implicated in integrin affinity modulation and propagation of integrin signalling [6–8]. However, the intracellular events involved in these processes are poorly understood.

CD9 is an integral membrane protein of the recently described tetraspanin, or transmembrane 4 family, that share a 25–30 % homology and similar structural features [9]. CD9 possesses four transmembrane domains, which makes up approx. half of the molecule, with most of the remainder in two extracellular domains. The three short cytoplasmic domains include the nine-amino-acid-residue N-terminal domain and the 12-amino-acid-residue C-terminal domains [10]. CD9 is an abundant platelet membrane protein, of the same order as $\alpha_{IIb}\beta_3$ (approx. 45000 molecules per cell) [11,12], but with an uncertain function. CD9 and $\alpha_{IIb}\beta_3$ also share a similar cellular distribution in the membrane of platelet α -granules containing fibrinogen and von Willebrand’s Factor [13].

An involvement of CD9 in integrin $\alpha_{IIb}\beta_3$ function has been suggested by the ability of anti-CD9 monoclonal antibodies (mAbs) to cause platelet aggregation and fibrinogen binding [12,14], both known properties of $\alpha_{IIb}\beta_3$. However, these antibody-induced phenomena were mediated by the Fc γ R_{II} via the Fc portion of the anti-CD9 mAbs [15]. An association of CD9 with $\alpha_{IIb}\beta_3$ has been preserved by chemically cross-linking

platelets after pretreatment with anti-CD9 mAbs and subsequent platelet activation [16]. The question of whether this interaction exists in non-treated, resting platelets has not been investigated, nor has there been any investigation of the biochemical basis of this interaction.

In order to determine if this association exists in non-treated, resting platelets, we examined platelets and nucleated cells by immunoprecipitation and immunocytochemistry. We found that CD9 is part of a protein complex that co-precipitates with $\alpha_{IIb}\beta_3$ under native conditions and provides evidence of CD9 and $\alpha_{IIb}\beta_3$ associating in resting, inactive platelets. We observed a stoichiometric association of CD9– $\alpha_{IIb}\beta_3$ when non-ionic detergents of a relatively hydrophilic nature were used to solubilize platelets, and our data suggest that these two platelet integral membrane proteins physically associate with each other via hydrophobic interactions. Furthermore, in nucleated cells expressing recombinant $\alpha_{IIb}\beta_3$ and CD9, the association persisted and the two proteins co-localize in lamellipodia during cell spreading.

EXPERIMENTAL

Materials

The anti-human $\alpha_{IIb}\beta_3$ mAbs D57 and 2G12 and the anti- α_{IIb} mAb PMI-1 have been described previously [4,17]. The rabbit polyclonals 2308 and 8053 are anti-human GPIIb and IIIa respectively (M. H. Ginsberg, unpublished work). Anti-human CD9 mAbs MM/2-57 and PL4 were purchased from Tago (Burlingame, CA, U.S.A.) and Pi/33/2 from Dako (Carpinteria, CA, U.S.A.). The anti-platelet CD9 antibody P10 and the anti-human GPIX mAb 8.1.1 were kindly given by Dr. Z. A. Ruggieri (The Scripps Research Institute). P10 was purified on a Protein A–Sepharose 4B CL column according to the manufacturer’s instructions (Pharmacia, Uppsala, Sweden). The anti-human GPIIb

Abbreviations used: CHO, Chinese hamster ovary cell line; ECL, enhanced chemiluminescence; HLB, hydrophile–lipophile balance; HRP, horseradish peroxidase; IP, immunoprecipitate; mAb, monoclonal antibody; NEM, *N*-ethylmaleimide; NHS, *N*-hydroxysuccinimide ester; SA, streptavidin; TBS, Tris-buffered saline; TTBS, TBS containing 0.05 % (v/v) Tween-20; TX-100, Triton X-100.

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rabbit polyclonal has been described elsewhere [18]. The anti-human HLA-I mAb W2/32 and anti-human interleukin-2 receptor mAb 7G7B6 were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). The control mAbs MOPC-21 (IgG_{1,κ}), UPC-10 (IgG_{2a,κ}) and MOPC-141 (IgG_{2b,κ}) were purchased from Sigma (St. Louis, MO, U.S.A.). Horseradish peroxidase (HRP)-, rhodamine- and FITC-conjugated secondary mAbs were from Tago. Sulpho-NHS-biotin (NHS = *N*-hydroxysuccinimide ester) and NHS-biotin were from Pierce (Rockford, IL, U.S.A.) and antibodies were biotinylated according to manufacturer's instructions. Cy3-conjugated streptavidin (SA) was purchased from Jackson ImmunoResearch (West Grove, PA, U.S.A.).

Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and the $\alpha_{\text{ITb}}/\beta_3$ -expressing CHO cell line, A5, was described previously [19,20].

Brij-35 [polyoxyethylene (23) lauryl ether, C₁₂E₂₃] was from US Biochemicals (Cleveland, OH, U.S.A.); polyoxyethylene (9) lauryl ether (C₁₂E₉) and polyoxyethylene (10) lauryl ether (C₁₂E₁₀) were from Aldrich (Milwaukee, WI, U.S.A.). Triton X-100 (TX-100; octylphenoxy polyethoxyethanol) was from Fisher Scientific (Fair Lawn, NJ, U.S.A.), CHAPS from Pierce (Rockford, IL, U.S.A.), Tween-20 (polyoxyethylene sorbitan monolaurate) from Mallinckrodt (Paris, KY, U.S.A.); and SDS from Bio-Rad (Hercules, CA, U.S.A.). Digitonin and the protease inhibitors leupeptin, PMSF, E64, chymostatin, pepstatin A, and calpain inhibitors I and II were purchased from Calbiochem (La Jolla, CA, U.S.A.). The thiol alkylating reagent, *N*-ethylmaleimide (NEM), was from Sigma. All other chemicals were of the highest analytical quality from Sigma or Fisher Scientific, except where noted.

Platelet preparation and solubilization

Washed platelets were obtained from freshly drawn blood as described previously [21]. Platelets were then resuspended in Hepes/saline [3.8 mM Hepes/137 mM NaCl/2.7 mM KCl/3.3 mM Na₂HPO₄/5.6 mM D-glucose (pH 7.4)]. Platelets (10⁹ per ml) were biotinylated with 1 mM NHS-biotin or sulpho-NHS-biotin for 30 min, then incubated for 5 min with 50 mM Tris/HCl, pH 8.0. Platelets were washed and resuspended in Tris-buffered saline [20 mM Tris/HCl (pH 7.4)/150 mM NaCl] (TBS), in the presence of (final concentrations) 0.25 mM leupeptin, 2 mM PMSF, 0.1 mM E64, 0.1 mM chymostatin, 0.01 mM pepstatin and 2 mM NEM. After 5 min, the indicated detergents were added to the desired concentration (v/v) and the tubes were transferred into ice for 1–2 h, mixing occasionally.

Immunoprecipitation

Detergent-insoluble material from the platelet lysates (approx. 1.0 mg/ml total protein) was pelleted at 10000 *g*, and supernatant (0.5 ml) was transferred to tubes containing 50 μ l of Pansorbin cells (Calbiochem) and 0.2 ml of Sepharose 4B CL (Sigma) for overnight incubation (4 °C). After pelleting the adsorbents, supernatants were transferred to fresh tubes, incubated at 4 °C with primary antibodies, 10–20 μ g/ml of mAbs or 10–20 μ l/ml of polyclonals for 2–4 h. Immune complexes were cleared by overnight incubation with 20 μ l of equilibrated Protein G-Sepharose 4B CL (Sigma). Immunoprecipitates (IPs) were washed three times with TBS containing the appropriate detergent. Proteins were recovered by heating IPs for 10 min, 90 °C in 0.1 ml of $\times 2$ SDS/PAGE sample buffer containing 2 mM NEM.

SDS/PAGE, immunoblotting and quantification

Proteins were loaded on to 4–20% polyacrylamide (Bio-Rad) gradient gels, electrophoresed and electroblotted on to PVDF (Millipore) overnight in Towbin's buffer [25 mM Tris/192 mM glycine (pH 8.6)/20% (v/v) methanol]. Blots were blocked in 5% (w/v) non-fat milk in TTBS [TBS containing 0.05% (v/v) Tween-20] for 1 h, then reacted with avidin–HRP (ABC Kit from Vector, Burlingame, CA, U.S.A.) for 30 min and washed six times in TTBS. The biotinylated protein pattern was visualized by enhanced chemiluminescence (ECL; Amersham) and luminograms were produced by exposing to film (Kodak, Rochester, NY, U.S.A.). Immunoblots were blocked as above, incubated for 1–2 h with primary antibody, washed three times in TTBS, incubated with appropriate HRP-conjugated secondary antibody (1:7500 for 30 min), washed extensively with TTBS and visualized by ECL. Some blots were stripped of antibodies according to instructions (Amersham), blocked and reprobed.

For quantification, the luminograms of the IPs were scanned and evaluated using the NIH Image program. The luminograms used for densitometry were those of short exposure (usually 10–30 s) in which the bands of interest were not saturated. Gels and the PVDF blots (after ECL) were stained in order to verify equal IgG loading and transfer. The intensity ratio between the proteins measured (i.e. A = α_{ITb} , B = CD9) was derived as follows:

$$\text{Intensity ratio } A/B = \frac{\text{area (A)} \times \text{mean intensity (A)}}{\text{area (B)} \times \text{mean intensity (B)}}$$

Assuming similar biotinylation and immunoprecipitation efficiencies, the intensity ratio is an indirect measure of the molar ratio. If the molar ratio A/B is 1.0, for A = α_{ITb} (molecular mass = 140 kDa) and B = CD9 (molecular mass = 24 kDa), the intensity ratio is expected to be: molecular mass_(A)/molecular mass_(B) = 140/24 = 5.8.

Cell culture, transfection and immunoprecipitation

Cells were cultured and transfected as described previously [22]. The α_{ITb} , β_3 and Tac α_3 cDNAs were in CDM8 expression vectors constructed as described previously [4,23,24]. The full-length CD9 cDNA [10] was isolated from a human B-cell library and subcloned into a CDM8 expression vector [25] by reverse-transcriptase PCR. Cells were transfected with either 3 μ g of CD9 cDNA or 5 μ g of Tac α_3 cDNA, as described for α_{ITb} and β_3 cDNAs [22]. Transfection efficiency was monitored by flow cytometry [26,27] and was between 5 and 20%.

For the immunoprecipitation experiments, cells were detached with trypsin (0.5 g/l)/0.5 mM EDTA (Irvine Scientific, Santa Ana, CA, U.S.A.), washed and resuspended in PBS, pH 7.3, containing 1.0% (w/v) dextrose and then biotinylated with sulpho-NHS-biotin as for platelets. Cells were replated on tissue culture plates coated with 20 μ g/ml fibrinogen [(2–3) $\times 10^6$ cells per 100 mm plate]. After 2 h at 37 °C cells were washed in PBS, pH 7.3, inhibitors added as above and cells were lysed *in situ* with 1% (v/v) Brij-35 at 4 °C for 2 h. Lysates were immunoprecipitated as above.

Immunocytochemistry

Immunofluorescence staining was performed on 18 mm circular, glass coverslips. Clean, sterile coverslips were coated overnight at 4 °C with 20 μ g/ml purified fibronectin or fibrinogen in PBS, followed by blocking with 1% (w/v) BSA for 60 min at 37 °C.

Cells were detached as above, washed twice with serum-free medium and seeded on to coverslips in serum-free medium at 37 °C for 30–120 min, as indicated in Figures 5 and 6. The cells attached to the coverslips were fixed for 10 min with 3.7 % (v/v) formaldehyde (methanol free) (Polysciences, Warrington, PA, U.S.A.) in PBS and permeabilized for 5 min with 0.2 % (v/v) TX-100 in PBS at room temperature. To block non-specific binding, coverslips were incubated with 10 % (v/v) normal goat serum (Gibco–BRL, Bethesda, MD, U.S.A.) in PBS for 1 h at room temperature. Coverslips were incubated with the appropriate mixture of antibodies (mAbs at 10 μ g/ml, 1:200 for polyclonals) in 10 % normal goat serum for 60 min at 37 °C. After washing five times with PBS, pH 7.3, coverslips were incubated for another 30 min at 37 °C with a species-appropriate mix of FITC- or rhodamine-conjugated IgG F(ab')₂ (Tago) in 10 % normal goat serum. The coverslips were then washed five times with PBS, pH 7.3 and mounted in 25 % (w/v) glycerol in 0.1 M Tris/HCl, pH 8.5, containing 10 % (w/v) Mowiol 4-88 (Calbiochem) and 2.5 % (w/v) 1,4-diazabicyclo[2.2.2]octane (DABCO) (Aldrich). Slides were photographed with a Leica microscope on Kodak Ektachrome 1600 or Kodak TMAX Professional 3200.

Immunobead clustering

Tosyl-activated 4.5 μ m polystyrene beads (too large to be internalized by cells) were coated with mAbs exactly as recommended by the manufacturer (Dynal, Late Success, NY, U.S.A.). Cells were prepared and reacted with the beads as described previously [28], except that 2×10^5 beads were mixed with 1×10^5 cells and incubated for 1–2 h at 37 °C. Cells were then plated on coverslips coated with 20 μ g/ml fibrinogen for 30 min at 37 °C and processed for immunofluorescence as above.

RESULTS

$\alpha_{IIb}\beta_3$ co-immunoprecipitates with CD9

To identify integrin-associated proteins, we analysed immunoprecipitates of integrin $\alpha_{IIb}\beta_3$ by SDS/PAGE. In platelets solubilized with 1 % Brij-35, a prominent 24 kDa band was detected in precipitates formed with two different anti- $\alpha_{IIb}\beta_3$ mAbs (D57, Figure 1; 2G12, results not shown) and one rabbit polyclonal anti- $\alpha_{IIb}\beta_3$ (Figure 2A). The precipitated p24 protein was identified as CD9 by immunoblotting with anti-CD9 mAbs (Pi/33/2, PL4 and MM/2-57, Figure 2; P10, Figure 4). The integrin–CD9 association was verified by the reciprocal co-precipitation of $\alpha_{IIb}\beta_3$ in CD9 precipitates (Figure 1). The identity of the 140 kDa and 91 kDa bands (Table 1) as α_{IIb} and β_3 was established by immunoblotting with specific antibodies and by observing the electrophoretic mobility shift in apparent molecular mass occurring under reducing conditions [29].

Several other proteins co-precipitated with $\alpha_{IIb}\beta_3$ in Brij-35 lysates (Table 1; Figures 1 and 3). Some corresponded to surface proteins (132 kDa, 64 kDa), because they were labelled with the cell-impermeable sulpho-NHS–biotin. Three other proteins (73 kDa, 35 kDa and 29 kDa) were likely to be intracellular proteins because they failed to label with sulpho-NHS–biotin (Table 1).

Specificity and stoichiometry of the $\alpha_{IIb}\beta_3$ –CD9 association

The specificity of the CD9– $\alpha_{IIb}\beta_3$ association was further tested by probing CD9 and $\alpha_{IIb}\beta_3$ precipitates with antibodies against other integral platelet membrane proteins. Whereas CD9 and

$\alpha_{IIb}\beta_3$ were enriched severalfold in anti-CD9 or anti- $\alpha_{IIb}\beta_3$ IPs, other membrane proteins (GPIb, GPIX, HLA-I) were not, although these proteins were present in the lysates (Figures 1 and 2). Similarly, we failed to detect CD9 in anti-HLA and anti-GPIX IPs (Figure 2B). IPs performed with control antibodies, such as mouse IgGs and pre-immune rabbit serum, show that only a low level of non-specific interactions took place in the Brij and TX-100 lysates (Figures 2–4). These experiments confirmed that the CD9– $\alpha_{IIb}\beta_3$ complex in Brij-solubilized platelet membranes was not part of a large aggregate of membrane proteins that may form when inadequate solubilization takes place. In addition, we affirmed that no association of CD9 with $\alpha_{IIb}\beta_3$ took place post-lysis (results not shown) and that the CD9– $\alpha_{IIb}\beta_3$ complex existed before solubilization by Brij-35 (Figures 4 and 5).

The association of CD9 with $\alpha_{IIb}\beta_3$ in transiently transfected CHO cells was confirmed by immunoprecipitation. Transfected cells were plated on fibrinogen and, after spreading, were lysed *in situ* with Brij-35, and immunoprecipitated. CD9 could only be detected in immunoblots of anti- $\alpha_{IIb}\beta_3$ precipitates from transfected cells (Figure 4, lanes 3 and 9). Similarly, anti-CD9 precipitated $\alpha_{IIb}\beta_3$ only from transfected cells (Figure 4, lane 7). The identity of $\alpha_{IIb}\beta_3$ was verified by immunoblotting (results not shown). Therefore, the association of CD9 with $\alpha_{IIb}\beta_3$ did not require the presence of additional platelet-specific factors.

The stoichiometry of the CD9– $\alpha_{IIb}\beta_3$ relationship was assessed by sequential immunoprecipitation experiments. Brij-solubilized platelet lysate was subjected to three rounds of immunoprecipitation with anti- $\alpha_{IIb}\beta_3$ antibodies. CD9 was depleted quantitatively by anti- $\alpha_{IIb}\beta_3$ (Figure 2C, IP1–IP3 and S3). The ratio of CD9 detected in these precipitates versus CD9 in the starting cell lysate was found to decrease from 6.0 to 2.5, and finally 0.3. No such depletion was observed for the platelet-membrane protein GPIb, or when TX-100 was the solubilizing agent (Figure 2C). When the same blots were reprobed with anti- $\alpha_{IIb}\beta_3$, quantitative depletion of integrin was observed in both detergents (results not shown). The depletion experiments indicate that all of the CD9 is associated with $\alpha_{IIb}\beta_3$ in platelet membranes.

We quantitatively evaluated the ratio of GPIIb to CD9 in co-precipitates (Figure 1), as explained in the Experimental section. The intensity ratio of GPIIb to CD9 found (average $\pm$ S.E.M., $n = 7$) was 5.03 ± 0.85 when an anti- $\alpha_{IIb}\beta_3$ mAb (D57) was used and 4.57 ± 1.71 when an anti-CD9 mAb (MM/2) was used as immunoprecipitating antibody. As these measurements are close to the equimolar ratio, they suggest that most of the GPIIb was associated with co-precipitating CD9.

Biochemical nature of the $\alpha_{IIb}\beta_3$ –CD9 association

The possible nature of the CD9– $\alpha_{IIb}\beta_3$ interaction was indicated by experiments using poly(oxyethylene ether) detergents of different relative hydrophobicity (hydrophile–lipophile balance, HLB [30]). When platelets were solubilized with hydrophobic detergents of a similar HLB, such as TX-100, C₁₂E₉ or C₁₂E₁₀ (see the Experimental section), no CD9 was visible in anti- $\alpha_{IIb}\beta_3$ precipitates (Figures 1 and 3), nor detected by immunoblot (results not shown). In contrast, when the relatively hydrophilic (higher HLB) Brij-35 (C₁₂E₂₃) was used, the CD9– $\alpha_{IIb}\beta_3$ complex was precipitated (Figures 1–4; Table 1). The zwitterionic detergent CHAPS, which possesses both hydrophobic and hydrophilic groups, dissociated most, but not all, of the CD9– $\alpha_{IIb}\beta_3$ complex (Figure 1). As CHAPS is known to interact with hydrophobic domains and is used as an eluant in hydrophobic interaction chromatography [31,32], this provides further evidence for the hydrophobicity of the CD9– $\alpha_{IIb}\beta_3$ binding.

Figure 1 CD9 co-immunoprecipitates with $\alpha_{IIb}\beta_3$ in Brij-35- (BRIJ), but not TX-100-solubilized platelets

Resting, washed platelets were biotinylated with NHS-biotin and then solubilized with a 1% (v/v) concn. of the indicated detergent. Equal volumes of total cell lysates (1 mg/ml), L, were immunoprecipitated with anti-CD9 mAb MM/2 (CD9) or anti- $\alpha_{IIb}\beta_3$ (D57) mAb. Immunoprecipitated proteins (15 μ l/lane) were electrophoresed on a 5–20% polyacrylamide gradient gel, then electroblotted to PVDF membranes. After incubation with HRP-SA, proteins were visualized by ECL. Proteins identified by immunoblot are indicated on the right and molecular masses in kDa are on the left; L', shorter film exposure of L.

Recombinant CD9 co-localizes and clusters with $\alpha_{IIb}\beta_3$ in nucleated cells

We examined potential physical associations of CD9 with $\alpha_{IIb}\beta_3$ in the intact cell membrane. Due to the small size of platelets, we used CHO cells transfected with recombinant CD9 and $\alpha_{IIb}\beta_3$ to address this question by immunofluorescence. CD9 was expressed throughout the cell membrane and showed a pattern of intense localization in lamellipodia (Figure 5). When the transfectants were plated on fibrinogen-coated coverslips, both CD9 and $\alpha_{IIb}\beta_3$ heavily stained the cell periphery during the early stages of cell adhesion and spreading (results not shown). At later stages of cell spreading, $\alpha_{IIb}\beta_3$ was localized primarily to focal adhesions (Figure 5B) and was heavily co-localized with CD9 on the leading edge of lamellipodia (Figure 5C). At the anchored part of the cell, CD9 and $\alpha_{IIb}\beta_3$ were separated. CD9 was not present in focal adhesions and appeared to localize in the distal part of filopodia (Figure 5A), whereas $\alpha_{IIb}\beta_3$ stained in the filopodia closer to the cell body (Figure 5B). CD9 expression in transfected cells did not significantly affect the formation of focal adhesion complexes in these cells. A chimera of the interleukin-2-receptor extracellular domain and the integrin α_5 transmembrane and cytoplasmic domains, Tac α_5 , known not to affect $\alpha_{IIb}\beta_3$ function [9,29], was used as the control for a transfected protein that does not associate with $\alpha_{IIb}\beta_3$ (Figures 5E and 5F).

A5 cells are CHO cells stably expressing $\alpha_{IIb}\beta_3$ [19,20]. A5 cells transiently transfected with CD9 or Tac α_5 were incubated with microbeads previously coated with either anti-CD9 (MM/2), anti- $\alpha_{IIb}\beta_3$ (D57) or anti-Tac mAbs. Beads that have clustered cell-surface antigen will show a bright ring or pattern of intensely fluorescing antigen around it. The specificity of these interactions was verified with beads coated with BSA or mouse IgG, which did not bind to the transfected cells (cells with beads < 5%), and with anti-CD9, anti-Tac and anti- $\alpha_{IIb}\beta_3$ coated beads, which did not bind to untransfected CHO cells (cells with beads < 10%). Anti- $\alpha_{IIb}\beta_3$ staining showed the antigen clustering around the anti-CD9-coated beads (Figure 6A). Similarly, anti-CD9 staining showed that CD9 was clustered by the anti-CD9-coated beads

Figure 2 Specificity of the CD9- $\alpha_{IIb}\beta_3$ interaction

(A) Rabbit anti- $\alpha_{IIb}\beta_3$ IPs of total platelet lysates (L) were treated as described in Figure 1 and probed for Western blot (WB) with either anti-CD9 (0.1 μ g/ml), anti-GPIX (2 μ g/ml) or anti-HLA (1.5 μ g/ml) mAbs, followed by visualization by using ECL. Molecular masses in kDa are shown on the left for CD9 and GPIX and on the right for HLA. Overexposure (5 min) resulted in the visualization of several non-specific bands that occasionally appear when secondary mAbs interact with rabbit polyclonal antibodies used for IP. (B) Platelets were treated as described in Figure 1, precipitated with the indicated mAbs and then immunoblotted (except lane 1) with 1 μ g/ml anti-CD9 mAb PL4 (lane 2) and 1 μ g/ml mAb Pi/33/2 (lanes 3–7). All samples were Brij-35-solubilized, except one (lane 4) in which TX-100 was used. Lane 1, anti-mouse IgG (mlgG) precipitate biotinylated protein pattern. All immunoblotted lanes were overexposed (lane 2, 5 min; lanes 3–7, 10 min); therefore several faint, non-specific bands are present. Molecular masses in kDa are shown on the left. (C) Resting, washed platelets were lysed in 1% (v/v) Brij-35 and then subjected to three rounds of immunoprecipitation (IP1, IP2, IP3) with rabbit anti- $\alpha_{IIb}\beta_3$. Proteins were separated by SDS/PAGE (15 μ l/lane), then electroblotted on to PVDF membranes, followed by immunoblot with anti-CD9 mAb MM/2

Table 1 CD9– $\alpha_{IIb}\beta_3$ associated protein complex

Resting platelets were surface labelled with sulpho-NHS–biotin or the permeable analogue, NHS–biotin. Anti- $\alpha_{IIb}\beta_3$ (D57) precipitates from Brij-lysed platelets were run on non-reducing 4–15% and 4–20% gradient gels, as described in the Experimental section. The apparent molecular mass (Mol. mass) of proteins was estimated by linear regression analysis of molecular mass standards from Bio-Rad ($n \geq 10$ gels, $r^2 = 0.99$). ID, identification of major bands by immunoblot.

Mol. mass (kDa)	ID	Sulpho-NHS–biotin	NHS–biotin
140.5 ± 3.0	α_{IIb}	+	+
131.8 ± 4.3	–	+	+
91.3 ± 4.1	β_3	+	+
73.1 ± 2.6	–	–	+
64.1 ± 1.7	–	+	+
46.8 ± 1.8	–	+	+
35.3 ± 1.3	–	–	+
29.4 ± 1.6	–	–	+
24.4 ± 1.2	CD9	+	+

Figure 3 CD9 co-immunoprecipitates with $\alpha_{IIb}\beta_3$ in relatively hydrophilic, but not hydrophobic, non-ionic poly(oxyethylene ether) detergents

Biotin-labelled platelet lysates were immunoprecipitated and proteins were fractionated as described in Figure 1. Sample volume loaded was equalized according to lysate protein content. The solubilizing detergent (1%, v/v) is shown at the top of the lanes, while their HLB number is given at the bottom. The positions of CD9 (verified by immunoblot) and molecular masses in kDa are shown on the left. Figure is a composite of two gels: IP (rabbit anti- $\alpha_{IIb}\beta_3$ precipitates) and C (anti-mouse IgG precipitates), in which C lanes were exposed 10-fold longer than IP lanes.

(Figure 6B), and by anti- $\alpha_{IIb}\beta_3$ -coated beads as well (results not shown). A5 cells transfected with Tac α_5 showed neither clustering of $\alpha_{IIb}\beta_3$ with anti-Tac α_5 -coated beads (Figure 6C), nor was Tac α_5 clustered by anti- $\alpha_{IIb}\beta_3$ beads in those cells (results not shown), although anti-Tac beads did cluster the Tac α_5 protein (Figure 6D).

(20 ng/ml) or anti-GPIIb mAb (1 μ g/ml) and visualization by using ECL. S3, the soluble fraction remaining after the third IP; L, total platelet lysate. Lanes were overexposed (6 min). Molecular masses in kDa are shown on the left.

Figure 4 Co-immunoprecipitation of CD9 and $\alpha_{IIb}\beta_3$ from transfected cells

A5 and CHO cells were transfected and treated as described in the Experimental section. Cells were lysed *in situ* with Brij-35, immunoprecipitated and proteins visualized as described. Cell concentration in lysates was 1×10^7 /ml for CHO, 6×10^6 /ml for A5 and 3×10^6 /ml for CD9-transfected A5. Luminograms of the biotinylated proteins pattern were reproduced in lanes 1, 2 and 6–8 (SA), whereas lanes 3–5 and 9–11 were immunoblotted with anti-CD9 (CD9). Both blots had long exposures. Lanes 1 and 3, CD9-transfected A5 lysate; lanes 2 and 4, untransfected A5 lysate; lane 5, platelet lysate; lanes 6, 7, 9 and 10, lysate of CHO cells transfected with both $\alpha_{IIb}\beta_3$ and CD9; lanes 8 and 11, untransfected CHO lysate. Lysates (Lys; except for lanes 1 and 2) were precipitated with the indicated antibodies: rabbit anti- $\alpha_{IIb}\beta_3$ ($\alpha_{IIb}\beta_3$), anti-CD9 mAb (CD9) or mouse IgG (Ig). Positions of α_{IIb} , β_3 and CD9 are shown on the left and molecular masses in kDa are shown on the right.

DISCUSSION

The major finding of this work is that the integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) and the tetraspanin CD9 can physically associate in a protein complex that is present in resting platelet membranes. This conclusion was based on the specific (Figures 1–3) and quantitative (Figure 2C) co-immunoprecipitation of these membrane proteins. Furthermore, it was supported by the ability of recombinant CD9 and $\alpha_{IIb}\beta_3$ to associate in transfected CHO cells, as detected by immunoprecipitation, co-localization and co-clustering experiments (Figures 4–6). This indicates that no additional platelet-specific factors are required for CD9 and $\alpha_{IIb}\beta_3$ association.

Our work established a constitutive and direct association of $\alpha_{IIb}\beta_3$ with CD9 in Brij-35 solubilized, resting platelets without requiring pre-treatment with antibodies (Figure 1). Both anti- $\alpha_{IIb}\beta_3$ and anti-CD9 antibodies immunoprecipitated a similar pattern of protein bands (Table 1), including the co-precipitation of $\alpha_{IIb}\beta_3$ and CD9. Slupsky and et al. reported the association of CD9 with $\alpha_{IIb}\beta_3$ in activated platelets pre-treated with anti-CD9 mAb [16]. The activation of $\alpha_{IIb}\beta_3$ by anti-CD9 mAbs was presumed to be mediated by the Fc receptor [15]. In contrast, our use of the more hydrophilic Brij-35 enabled the preservation of the CD9– $\alpha_{IIb}\beta_3$ interaction in non-activated, resting platelets. Thus there is no need to activate the association of CD9 with $\alpha_{IIb}\beta_3$; the complex may exist as a normal component of the platelet membrane (Figure 1). Recently, CD9 was reported to associate with β_1 integrins in certain cell lines [33–35]. Thus CD9 can associate with integrins of multiple families.

Figure 5 Co-localization of CD9 and $\alpha_{IIb}\beta_3$ in transfected cells

A5 cells (CHO cells stably expressing $\alpha_{IIb}\beta_3$) were transfected with CD9 (3 μ g) or Tac α_5 (5 μ g) cDNAs, as described in the Experimental section, and plated on coverslips coated with 20 μ g/ml fibrinogen for 120 min. Fixed and permeabilized cells were double-stained with rabbit anti- α_{IIb} and either anti-CD9 or anti-Tac mAb and visualized with rhodamine- or FITC-conjugated secondary mAbs. Transparencies were scanned with a Nikon LS-1000 scanner and images merged electronically to assess co-localization (yellow). (A)–(C) Cells transfected with CD9 and plated for 120 min, stained for CD9 (A) or α_{IIb} (B). (D)–(F) A5 cells transfected with Tac α_5 and plated for 120 min, stained for Tac (D) or α_{IIb} (E). (C) and (F) are the merged images of (A) + (B) and (D) + (E) respectively. Arrows show areas of co-localization (yellow); arrowheads point to examples of focal adhesion complexes; bar, 10 μ m.

The nature of tetraspanin interactions with other proteins is currently unknown. We suggest that the association of CD9 with $\alpha_{IIb}\beta_3$ was stabilized by hydrophobic interactions. We observed that this complex was stable in Brij-35 but not TX-100 (Figure 1). Although TX-100 and Brij-35 are both members of the poly(oxyethylene) family of detergents, TX-100 is markedly more hydrophobic than Brij-35 (HLB, 13.5 versus 16.9 [30]). Furthermore, when detergents of the Brij series having an HLB similar to TX-100 were used to solubilize platelets, no co-precipitation of CD9 and $\alpha_{IIb}\beta_3$ was seen (Figure 3). As the sole difference between Brij-35 ($C_{12}E_{23}$) and $C_{12}E_9$ or $C_{12}E_{10}$ is in the number of their poly(oxyethylene) moieties and hence their relative hydrophobicity (HLB 16.9, 14.1 and 13.6 respectively), this has supported the conclusion that the increased hydrophobicity of TX-100 was responsible for its capacity to dissociate the CD9- $\alpha_{IIb}\beta_3$ complex.

Tetraspanins are the first integrin-associated proteins that may interact with integrin via the transmembrane domain. Several other proteins have been reported to associate with β_1 or β_3 integrins: alpha-actinin [36], talin [37], CD47 [38], β_3 endonexin [39], p125^{FAK} [40] and other kinases [41,42]. In all cases except CD47 the proteins were intracellular; CD47 interacts with $\alpha_v\beta_3$ integrin via its extracellular domain [43]. Although we cannot rule out the possibility that CD9 interacts via its cytoplasmic or extracellular domains, both the hydrophobic nature of the

interaction and the relative extent of the CD9 transmembrane domains [9,10] suggest that the transmembrane region plays a major role.

Anti-CD9 mAbs co-precipitated several unidentified proteins, both surface and intracellular (Figure 1 and Table 1). Tetraspanins have shown a propensity to be involved in membrane protein complexes. For example, in B-cells, the tetraspanin CD81 (TAPA-1) is part of the membrane protein complex (together with CD19 and CD21) involved in the primary immune response [44]. The tetraspanins CD81 and CD82 were found to co-precipitate with CD4 in T-cells and to be essential for T-cell maturation [45]. The urothelial membrane tetraspanins, uroplakin Ia and uroplakin Ib, are part of a protein complex that forms 16 nm membrane particles that participate in stabilizing the apical surface of mammalian urothelium [46]. Analogously, CD9 and $\alpha_{IIb}\beta_3$ may also be part of a large multimeric membrane-protein structure.

The relationship between CD9 and $\alpha_{IIb}\beta_3$ appears to be dynamic, with co-localization in the cell periphery at the initial stages of adhesion and spreading. At later stages of cell spreading and migration, co-localization remains at the leading edge of lamellipodia, but not in focal adhesions (Figure 5C). In filopodia, CD9 seems to occupy the distal portion, whereas $\alpha_{IIb}\beta_3$ is found more proximally to the cell (Figures 5A and 5B). Similar to our observations in transfected CHO cells, electron microscope

Figure 6 Transfected CD9 and $\alpha_{IIb}\beta_3$ co-cluster with microbeads coated with anti- $\alpha_{IIb}\beta_3$ or anti-CD9 mAbs

Microbeads (DynaP-450) were coated with either anti-CD9 or anti-Tac mAbs and incubated at 37 °C with A5 cells transfected, as described in the Experimental section, for 120 min in BSA-coated wells. Cells were adhered to fibrinogen-coated coverslips for 30 min and processed for immunofluorescence. Antibody-coated beads used are shown on the left: (A) and (B) anti-CD9; (C) and (D) anti-Tac. Antigen stain is shown at the top: (A) and (C) anti- $\alpha_{IIb}\beta_3$; (B) anti-CD9; (D) anti-Tac. All cells shown had focal adhesion complexes on a basal focal plane and were photographed in a more apical focal plane, to visualize antigen localization around the beads. Bead diameter, 4.5 μ m.

studies of platelets spreading on fibrinogen confirm the presence of CD9 in the platelet-membrane periphery and lamellipodia [13]. In Schwann cells, CD9 mRNA expression is regulated by axonal contact in culture and *in vivo* [47]. In *Drosophila*, a tetraspanin encoded by the *late bloomer (lbl)* gene and expressed on the leading edge of motor neurons and lamellipodia-like growth cones was essential for synapse formation [48]. Thus the absence of CD9– $\alpha_{IIb}\beta_3$ co-localization in the anchored areas (presence of focal adhesions) of the cell, as observed by us (Figure 5), together with its presence in the migrating portion (lamellipodia) of the cell, is in agreement with tetraspanin behaviour in neural growth cones.

The tetraspanin family possesses a diversity of members and tissue distribution that is similar to that of the integrin family and can thus modulate integrin function. The precise nature of this interaction and the role of CD9 and other tetraspanins in integrin function warrants further study.

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REFERENCES

- Hynes, R. O. (1992) *Cell* **69**, 11–25
- Ginsberg, M. H., Du, X. and Plow, E. F. (1992) *Curr. Opin. Cell Biol.* **4**, 766–771
- Williams, M. J., Du, X., Loftus, J. C. and Ginsberg, M. H. (1995) *Semin. Cell Biol.* **6**, 305–314
- O'Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Loftus, J. C., Shattil, S. J. and Ginsberg, M. H. (1994) *J. Cell Biol.* **124**, 1047–1059
- Hughes, P. E., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J. A., Shattil, S. J. and Ginsberg, M. H. (1996) *J. Biol. Chem.* **271**, 6571–6574
- Shattil, S. J., Ginsberg, M. H. and Brugge, J. S. (1994) *Curr. Opin. Cell Biol.* **6**, 695–704
- Clark, E. A. and Brugge, J. S. (1995) *Science* **268**, 233–239
- Schwartz, M. A., Schaller, M. D. and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* **11**, 549–599
- Jennings, L. K., Crossno, J. T. J., Fox, C. F., White, M. M. and Green, C. A. (1994) *Ann. N. Y. Acad. Sci.* **714**, 175–184

- 10 Boucheix, C., Benoit, P., Frachet, P., Billard, M., Worthington, R. E., Gagnon, J. and Uzan, G. (1991) *J. Biol. Chem.* **266**, 117–122
- 11 Shattil, S. J., Hoxie, J. A., Cunningham, M. and Brass, L. F. (1985) *J. Biol. Chem.* **260**, 11107–11114
- 12 Hato, T., Ikeda, K., Yasukawa, M., Watanabe, A. and Kobayashi, Y. (1988) *Blood* **72**, 224–229
- 13 Cramer, E. M., Berger, G. and Berndt, M. C. (1994) *Blood* **84**, 1722–1730
- 14 Boucheix, C., Soria, C., Mirshahi, M., Soria, J., Perrot, J. Y., Fournier, N., Billard, M. and Rosenfeld, C. (1983) *FEBS Lett.* **161**, 289–295
- 15 Worthington, R. E., Carroll, R. C. and Boucheix, C. (1990) *Br. J. Haematol.* **74**, 216–222
- 16 Slupsky, J. R., Seehafer, J. G., Tang, S. C., Masellis-Smith, A. and Shaw, A. R. (1989) *J. Biol. Chem.* **264**, 12289–12293
- 17 Shadle, P. J., Ginsberg, M. H., Plow, E. F. and Barondes, S. H. (1984) *J. Cell Biol.* **99**, 2056–2060
- 18 Du, X., Fox, J. E. and Pei, S. (1996) *J. Biol. Chem.* **271**, 7362–7367
- 19 Loftus, J. C., O'Toole, T. E., Plow, E. F., Glass, A., Frelinger, A. L. and Ginsberg, M. H. (1990) *Science* **249**, 915–918
- 20 Wu, C., Keivens, V. M., O'Toole, T. E., McDonald, J. A. and Ginsberg, M. H. (1995) *Cell* **83**, 715–724
- 21 Du, X., Saido, T. C., Tsubuki, S., Indig, F. E., Williams, M. J. and Ginsberg, M. H. (1995) *J. Biol. Chem.* **270**, 26146–26151
- 22 O'Toole, T. E., Ylanne, J. and Culley, B. M. (1995) *J. Biol. Chem.* **270**, 8553–8558
- 23 Chen, Y. P., O'Toole, T. E., Shipley, T., Forsyth, J., LaFlamme, S. E., Yamada, K. M., Shattil, S. J. and Ginsberg, M. H. (1994) *J. Biol. Chem.* **269**, 18307–18310
- 24 LaFlamme, S. E., Akiyama, S. K. and Yamada, K. M. (1992) *J. Cell Biol.* **117**, 437–447
- 25 Aruffo, A. and Seed, B. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8573–8577
- 26 Ginsberg, M. H., Frelinger, A. L., Lam, S. C., Forsyth, J., McMillan, R., Plow, E. F. and Shattil, S. J. (1990) *Blood* **76**, 2017–2023
- 27 Forsyth, J., Plow, E. F. and Ginsberg, M. H. (1992) *Methods Enzymol.* **215**, 311–316
- 28 Lewis, J. M. and Schwartz, M. A. (1995) *Mol. Biol. Cell* **6**, 151–160
- 29 Jennings, L. K. and Phillips, D. R. (1982) *J. Biol. Chem.* **257**, 10458–10466
- 30 Jones, M. N. and Chapman, D. (1995) *Micelles, Monolayers and Biomembranes*, Wiley-Liss, New York
- 31 Wetlaufer, D. B. and Koenigbauer, M. R. (1986) *J. Chromatogr.* **359**, 55–60
- 32 Hjelmeland, L. M., Nebert, D. W. and Osborne, Jr., J. C. (1983) *Anal. Biochem.* **130**, 72–82
- 33 Rubinstein, E., Le Naour, F., Billard, M., Prenant, M. and Boucheix, C. (1994) *Eur. J. Immunol.* **24**, 3005–3013
- 34 Nakamura, K., Iwamoto, R. and Mekada, E. (1995) *J. Cell Biol.* **129**, 1691–1705
- 35 Berditchevski, F., Bazzoni, G. and Hemler, M. E. (1995) *J. Biol. Chem.* **270**, 17784–17790
- 36 Horwitz, A., Duggan, K., Buck, C., Beckerle, M. C. and Burridge, K. (1986) *Nature (London)* **320**, 531–533
- 37 Otey, C. A., Pavalko, F. M. and Burridge, K. (1990) *J. Cell Biol.* **111**, 721–729
- 38 Brown, E., Hooper, L., Ho, T. and Gresham, H. (1990) *J. Cell Biol.* **111**, 2785–2794
- 39 Shattil, S. J., O'Toole, T., Eigenthaler, M., Thon, V., Williams, M., Babior, B. M. and Ginsberg, M. H. (1995) *J. Cell Biol.* **131**, 807–816
- 40 Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5192–5196
- 41 Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C. and Dedhar, S. (1996) *Nature (London)* **379**, 91–96
- 42 Dorahy, D. J., Berndt, M. C. and Burns, G. F. (1995) *Biochem. J.* **309**, 481–490
- 43 Blystone, S. D., Lindberg, F. P., LaFlamme, S. E. and Brown, E. J. (1995) *J. Cell Biol.* **130**, 745–754
- 44 Fearon, D. T. and Carter, R. H. (1995) *Annu. Rev. Immunol.* **13**, 127–149
- 45 Boismenu, R., Rhein, M., Fischer, W. H. and Havran, W. L. (1996) *Science* **271**, 198–200
- 46 Wu, X. R., Medina, J. J. and Sun, T. T. (1995) *J. Biol. Chem.* **270**, 29752–29759
- 47 Banerjee, S. A. and Patterson, P. H. (1995) *Mol. Cell. Neurosci.* **6**, 462–473
- 48 Kopczynski, C. C., Davis, G. W. and Goodman, C. S. (1996) *Science* **271**, 1867–1870