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Interaction between GPIbα and FcγIIA Receptor in Human Platelets

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Glycoprotein (GP) Ib (α and β) in platelets forms a noncovalent hetero-oligomeric complex with GPIX and GPV and serves as a receptor for von Willebrand factor (vWF), which mediates the initial adhesion of platelets to the subendothelium after vascular damage and also plays a role in thrombin-induced platelet activation. We investigated the interaction between GPIbα and FcγIIA receptor using a yeast two-hybrid system and mutagenesis, and we identified residues R542G543R544 in GPIbα and D298D299D300 in FcγIIA receptor as the primary interaction sites. These results further confirmed the interaction between GPIbα and FcγIIA receptor and support the hypothesis that the signal transduction of GPIb-IX-V that leads to platelet activation may be partially mediated through FcγIIA receptor. © 1999 Academic Press

The glycoprotein (GP) Ib-IX-V complex consists of four single-transmembrane proteins including GPIbα, GPIbβ, GPIX and GPV at a ratio of 2:2:2:1. GPIbα and GPIbβ are linked by a disulfide bond. GPIb-IX-V is constitutively expressed on the platelet surface and initiates primary bridging between platelets via von Willebrand factor (vWF) under high shear stress and adhesion of circulating platelets to vWR at the site of vascular injury, leading to secondary platelet adhesion, secretion and aggregation. The extracellular domain of GPIbα contains the binding sites for vWF, thrombin and possibly P-selectin (see review Ref. 1). Ligand binding to GPIb-IX-V triggers transmembrane signaling events including tyrosine phosphorylation of multiple platelet proteins, activation of protein kinase C (PKC) and phosphoinositol 3-kinase (PI3-kinase) (2, 3), elevation of cytosolic Ca^{2+} (4) and Ca^{2+}-dependent activation of αIIbβ3, but the precise molecular signaling events leading to platelet activation remain to be elucidated. It has been reported that GPIbα is physically associated with the platelet cytoskeleton by a direct interaction with actin-binding protein (ABP-280, also known as filamin) (5), and that GPIbα is also associated with an intracellular signaling protein 14-3-3ζ (6, 7). Recently, GPIbα was shown to be co-localized with FcγIIA receptor (FcγRIIA) in the platelet membrane by co-immunoprecipitation and flow cytometric fluorescence energy transfer, suggesting that these receptors may be functionally interactive (8). In the present study, we have used a yeast two-hybrid system and mutagenesis to investigate the regions involved in the interaction between GPIbα and FcγRIIA. Our results provide further support for the functional interplay between GPIbα and FcγRIIA in platelets.

METHODS

Expression vector construction for yeast two-hybrid. Total RNA was extracted from human platelets using RNA Stat-60 (Tel-Test Inc., Friendswood, TX) and subsequently reverse-transcribed into complementary DNA (cDNA), which was used as a template for polymerase chain reaction (PCR) amplification. Numerous specific oligonucleotides were designed to amplify FcγRIIA and GPIbα of varying lengths and regions, and to introduce mutations at different locations, according to published cDNA sequence (9, 10). PCR amplification of cDNA was performed in a Perkin-Elmer 9700 thermal cycler, using denaturation at 94°C for 1 min, annealing at 55°C for 45 s and synthesis for 2 min at 72°C for 30 cycles. PCR products were ligated into plasmid vector pCR2.1 (Invitrogen, Carlsbad, CA) and the specificity of the inserted fragments were confirmed by restriction endonuclease digestion. Then the fragments of FcγRIIA were released from pCR2.1 by EcoRI digestion and recombined into a GAL4-based vector pAS2-1 containing a DNA-binding domain (BD). All GPIbα fragments were released by digestion with BamHI and EcoRV and ligated into vector pACT2 which contains an activation domain (AD, Clontech, Palo Alto, CA). All DNA-BD and -AD constructs were purified by Qiagen (St. Clarita, CA) midi-prep columns, and the orientation and sequence was confirmed by DNA sequencing using an ABI377 auto-sequencer.

Yeast co-transformation and colony-lift filter assay. The yeast strain *Saccharomyces cerevisiae* Y190 was used for co-transformation with a yeast transformation kit from Clontech. The transformants were grown on a triple (-leu/-trp/-his)-selection synthetic dropout (SD) plate containing 10 mM 3-amino-1,2,4-triazole (3-AT, Sigma, St. Louis, MO, to suppress background growth of Y190) at 30°C for 3 days. The colonies were filter-lifted for β-galactosidase activity detection, according to the instructions from Clontech.

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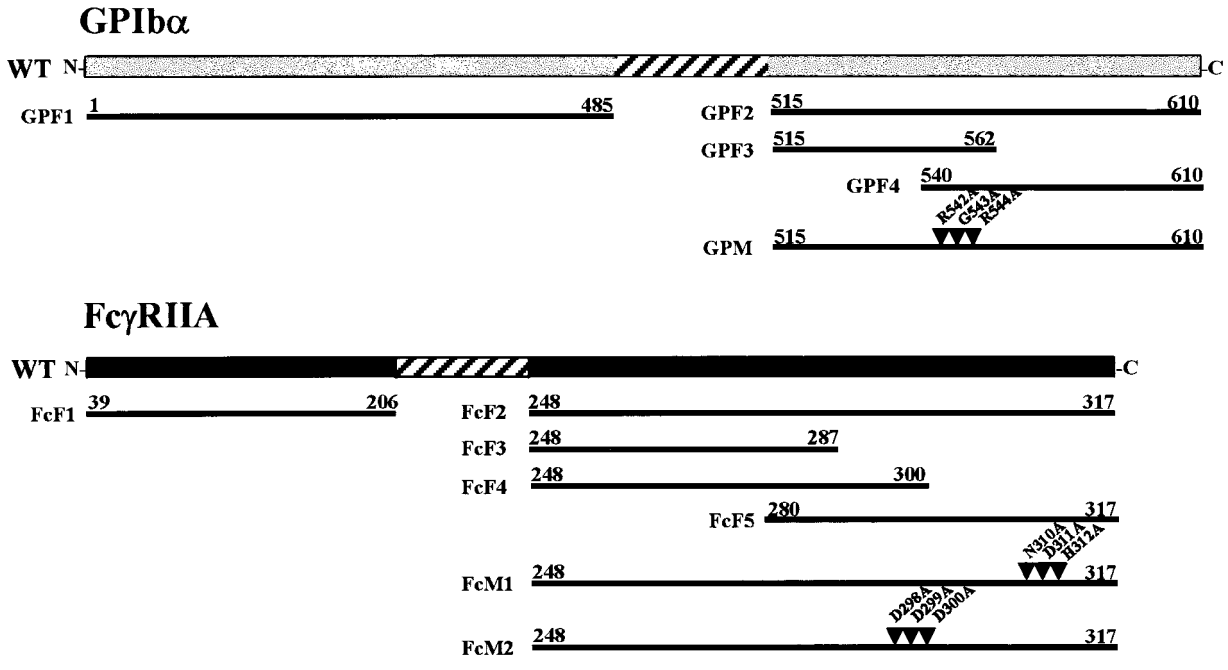

FIG. 1. Construction of truncation fragments and mutants of GPIbα and FcγIIA receptor used in yeast two-hybrid system. The numbering of amino acid residues for GPIbα and FcγRIIA was based on Refs. 9 and 10, respectively. Triple mutation to alanine was derived by PCR-based method.

Briefly, the transformant colonies were lifted onto a nylon filter (Immobilon-Ny+, Millipore, Medford, MA). The yeast were permeabilized by dipping the filter in liquid nitrogen for 10 s followed by thawing at room temperature. The filter was then placed with colony-side up on a Whatman No. 5 filter paper pre-soaked with Z buffer (16.1 g/l $Na_2HPO_4 \cdot 7H_2O$, 5.5 g/l $NaH_2PO_4 \cdot H_2O$, 0.75 g/l KCl, 0.246 g/l $MgSO_4 \cdot 7H_2O$, pH 7.0) plus X-gal (0.33 mg/ml), and incubated at room temperature till the appearance of blue colonies as an indication of positive interaction.

Chemiluminescence liquid assay. Ten yeast colonies were randomly picked and inoculated in -leu/-trp/-his medium and grew at 30°C overnight. Next morning, 2 ml overnight culture was inoculated into 8 ml of fresh YPD medium and grew at 30°C until OD_{600} reached 0.5. After centrifugation and one more wash with Z buffer, the yeast were re-suspended in 200 μl of Z buffer. β-Galactosidase was released by three rounds of freeze/thaw cycle in liquid nitrogen. Twenty-five microliters of yeast lysate supernatant after centrifugation (14,000*g* for 5 min) was mixed with 200 μl reaction buffer (Luminescent β-galactosidase Detection Kit II, Clontech) in a 96-well white plate and incubated at room temperature for 15 min. The luminescence generated was detected in triplet using a Mediators PhL luminescence plate reader (ImmTech, New Windsor, MD).

RESULTS

The yeast two-hybrid system was developed originally by Fields *et al.* (11) to detect specific protein-protein interaction. We were interested in confirming the interaction between GPIbα and FcγRIIA using the system and further map the binding regions. First, we cloned the cDNA fragments for the whole extracellular (minus signal peptide) or intracellular domain of GPIbα and FcγRIIA (Fig. 1) into expression vector pACT-2 or pAS2-1, respectively. Individually expressed fusion proteins after transformation from the recombined GPIbα or FcγRIIA fragments themselves did not cause any transactivation of the reporter gene (lacZ) present in the yeast, as determined by colony-lift filter assay. The interaction between the extracellular or intracellular domains was then tested by pairwise (GPF1/FcF1, GPF2/FcF2) co-transformation, together with pVA3-1/pTD1-1 as a positive control (Clontech). After 3 days of incubation at 30°C followed by colony-lift filter assay, it was found that interaction occurred between the intracellular domain of GPIbα and FcγRIIA but not between the extracellular domains, which were subsequently used as positive and negative controls, respectively. To test the interaction stringency between the intracellular domains, co-transformed *S. cerevisiae* Y190 were grown on triple-selection SD plate, or plus 2.5, 5, 10, 25 or 50 mM 3-AT. Following 3 to 4 days incubation, no large (>1 mm) colonies appeared on the plates containing 25 mM or higher 3-AT. Colony-lift filter assay of the small colonies showed no X-gal staining. These results suggest that the interaction between the intracellular domains of GPIbα and FcγRIIA is relatively weak.

To locate the interaction site in GPIbα, two fragments (GPF3 and GPF4, Fig. 1) were constructed. As shown in Fig. 2A, both fragments retained the interaction property with FcγRIIA, which narrowed down the binding site to the region of amino acids 540–562. By examining the amino acid residues carefully, the residues R542, G543 and R544 were intentionally mutated

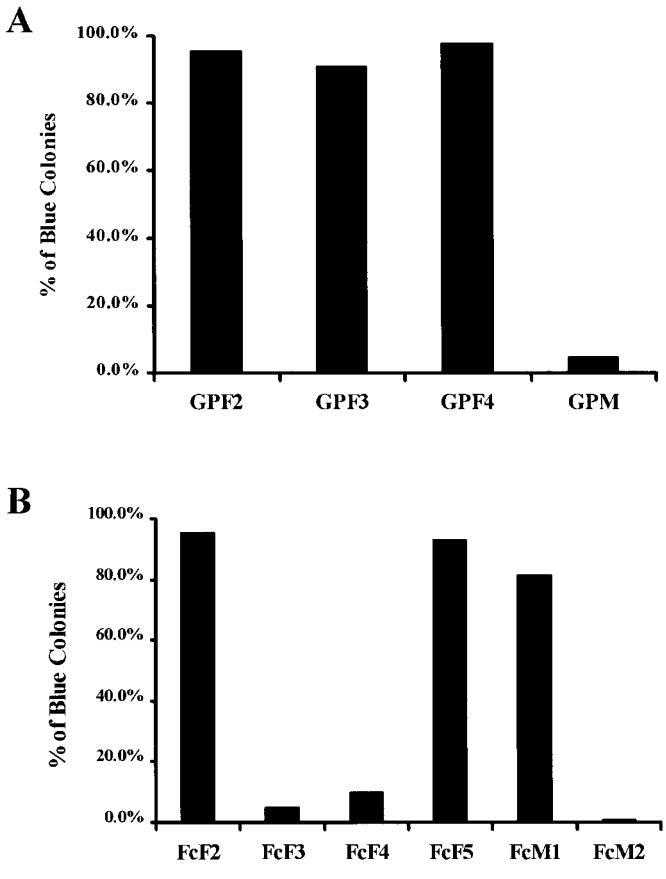

FIG. 2. β-Galactosidase colony-lift filter assay of GPIbα and FcγRIIA interaction in yeast two-hybrid. (A) Co-transformation of GPIbα fragments and mutants with full-length intracellular domain of FcγRIIA (FcF2). (B) Co-transformation of FcγRIIA fragments and mutants with full-length intracellular domain of GPIbα (GPF2). The interaction between the intracellular domains of GPIbα (GPF2) and FcγRIIA (FcF2) was used as a control. Blue colonies were counted in the plate and expressed as the percentage of total colonies. Data presented are the average of at least two independent assays.

to alanine. Repeated colony-lift filter assay showed that positive colonies decreased from 95.6% to 4.6%. To confirm the result, ten colonies were picked randomly for β-galactosidase chemiluminescence quantification, a more sensitive assay. As shown in Fig. 3, β-galactosidase activity in the co-transformed colonies with full intracellular domain of FcγRIIA (FcF2) and mutated intracellular domain of GPIbα (GPM) was decreased to 19.6 ± 2.6% as compared to the wide-type (FcF2/GPF2) activity.

Similarly, three FcγRIIA fragments (FcF3, FcF4 and FcF5, Fig. 1) were constructed in order to identify the GPIbα binding site in FcγRIIA. Since FcF3 and FcF4 lost the interaction capability with GPIbα, and FcF5 retained the interaction site (Fig. 2B), the binding site for GPIbα was assumed to be between residue 301 and the C-terminal end. However, mutation of residues N310, D311 and H312 to alanine (a hydrophilic region) resulted in no loss of interaction with GPIbα. Following the identification of residue R542G543R544 (positively charged) in GPIbα as the interaction site with FcγRIIA, a negatively charged region in FcγRIIA (D298D299D300) were mutated (FcM2, Fig. 1) and tested by filter assay. As shown in Fig. 2B, FcγRIIA intracellular domain bearing the above mutation lost interaction with GPIbα completely. This result was further confirmed by randomly selected colonies for β-galactosidase chemiluminescence quantification. Compared with wide-type, β-galactosidase activity decreased to 15.6 ± 2.2% (Fig. 3).

DISCUSSION

FcγRIIA is the low-affinity receptor for IgG in platelets and contains the immunereceptor tyrosine-containing activation motif (ITAM) (12). Only 2 ITAM-containing proteins (FcγRIIA and Fc receptor γ-chain) have been described in platelets. They can mediate the activation of Syk by engagement of the tandem Src homology 2 (SH2) domains of Syk with their doubly phosphorylated tyrosine residues. Cross-linking of FcγRIIA induces platelet shape change, secretion and aggregation, together with a number of cellular events including tyrosine phosphorylation, activation of PI3-kinase and phospholipase C-γ2 (PLC-γ2) and Ca^{2+} mobilization (13). Recent evidence suggests that conformational change of GPIb-IX-V after ligand binding may bring the subcomponents of the complex as well as FcγRIIA together, resulting in signal transduction (8). The present results not only confirmed the interaction between the GPIbα and FcγRIIA intracellular domains using a yeast two-hybrid system, but also identified residues R542G543R544 in GPIbα and D298D299D300 in FcγRIIA as the primary interaction sites. Evidence to date also shows that the cytoplasmic domain of GPIbα is directly associated with actin binding protein

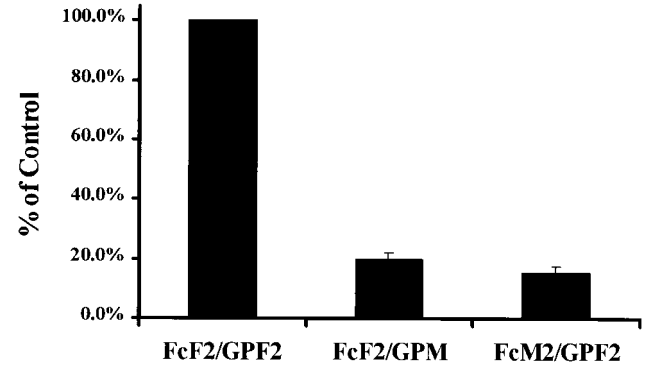

FIG. 3. β-Galactosidase chemiluminescence assay of GPIbα mutant (GPM) and FcγRIIA mutant (FcM2) in yeast two-hybrid system. Background counts from nontransformed *S. cerevisiae* Y190 were subtracted. The interaction between the intracellular domains of GPIbα (GPF2) and FcγRIIA (FcF2) was used as a control. Data are expressed as percentage of control ± SD. $n = 3$.

in the resting platelets (5), which may indirectly couple GPIb-IX-V to the myriad of cytoskeletal-associated signaling molecules such as PI3-kinase, Syk and focal adhesion kinase (Fak) (1). Interestingly, a region between T536 and F568 of GPIbα, where residues R542G543R544 were shown in the present study to bind to FcγRIIA, has been shown to participate in the interaction of GPIb-IX complex with actin-binding protein (14). It remains to be determined whether residues R542G543R544 also serve as part of the binding site for actin-binding protein. 14-3-3ζ is a member of a highly conserved protein family which functions as an accessory or adaptor molecule that facilitates interactions within the signaling complex. GPIb interacts with 14-3-3ζ directly through the extreme C terminal of GPIbα606-610 (SGHSL) (6) and the protein kinase A (PKA) serine phosphorylation site on GPIbβ (7). Most recently, Falati *et al.* demonstrated that GPIb-IX-V couples physically and functionally to the Fc receptor γ-chain, another ITAM-containing protein in platelets (15). The interaction domains of Fcγ chain and GPIb remain to be determined. In summary, the process of GPIb-IX-V-mediated platelet activation may involve multiple signal transducion pathways. Our results also further confirm that part of the signaling transduction is mediated through FcγRIIA in platelet activation.

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