Brief report

The novel inhibitory receptor G6B is expressed on the surface of platelets and attenuates platelet function in vitro

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The G6B cell-surface receptor, which contains a single Ig-like domain, has been shown to bind to SHP-1 and SHP-2 after phosphorylation of 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic tail, classifying this protein as a new member of the family of inhibitory receptors. In this study, we demonstrate by real-time polymerase chain reaction (PCR) and Western-blot analysis that G6B is expressed on platelets. Cross-linking of G6B with polyclonal antisera has a significant inhibitory effect on platelet aggregation and activation by agonists such as ADP and collagen-related peptide (CRP). This inhibitory function of G6B appears to operate in a calcium-independent manner. Our results suggest that G6B represents a novel inhibitory receptor found on the surface of platelets and that it could be an antithrombotic drug target. (Blood. 2007;109:4806-4809)

Introduction

The human *G6B* gene is located in the major histocompatibility complex (MHC) class III region and encodes a member of the immunoglobulin (Ig) superfamily.[1] The gene encodes a number of splice forms that are translated into both cell-surface and secreted isoforms.[2] The 2 principle cell-surface isoforms (G6B-A and G6B-B) have the same extracellular N-terminal Ig-like domain but differ in their C-terminal cytoplasmic tails. The G6B-B isoform has previously been shown to have 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within its cytoplasmic tail, suggesting an inhibitory role.[2,3] Furthermore, the extracellular domain of G6B has been shown to bind heparin tightly.[4] In this study, we demonstrate that G6B is expressed on platelets and functions as a negative regulator of platelet function.

Materials and methods

Generation and characterization of G6B antibodies

The generation and characterization (Figures S1-2, available on the *Blood* website; see the Supplemental Materials link at the top of the online article) of polyclonal and monoclonal antibodies against G6B is described in full in Document S1.

QPCR

Quantitative polymerase chain reactions (QPCRs) were performed using the SensiMix DNA kit (Quantace, Finchley, United Kingdom) according to the manufacturer's instructions. Relative expression levels were calculated using the ΔΔCT method.[5] Significant expression was defined as a value greater than 2 standard deviations above the median expression level across all cell types tested.

Western blotting

Approximately 1×10^5 cells and 1×10^7 platelets were lysed and separated on a 15% SDS-PAGE gel, transferred, and detected using the purified mouse anti-G6B monoclonal antibody visualized with a goat anti–mouse HRP conjugate (Sigma-Aldrich, Poole, United Kingdom) as described previously.[2]

Flow cytometry

Washed platelets were prepared as described previously.[6] Resting or activated platelets were incubated with a G6B monoclonal antibody (mAb) or appropriate isotype control (mouse IgG1κ) for 1 hour followed by an antimouse FITC conjugate (Jackson ImmunoResearch, Bar Harbor, ME). Resting and activated platelets were counterstained with an anti-CD41–PE conjugate (BD Biosciences, San Jose, CA) or an anti-CD62–PE mAb, respectively. G6B expression was then measured on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using the EXPO32 ADC analysis software (Beckman Coulter, High Wycombe, United Kingdom).

Immunoprecipitations

Coimmunoprecipitations were performed essentially as described by Senis et al.[7] Briefly, platelet lysates were coimmunoprecipitated using a rabbit anti–human SHP-1 polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) after incubation with G6B antisera, preimmune sera, or buffer alone. After Western blotting the membrane was incubated with an antiphosphotyrosine mAb (Upstate Biotechnology), stripped, and reprobed with an anti–SHP-1 mAb (Santa Cruz Biotechnology, Santa Cruz, CA) or the anti-G6B mAb.

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Platelet function studies

Platelet aggregation studies were performed as previously described.[8] Platelets were incubated with 5 μL of G6B polyclonal antiserum or preimmune serum for 30 seconds before addition of either ADP (final concentration 5 μM), cross-linked collagen-related peptide (CRP-XL; final concentration 0.1 μg/mL), or ionomycin (400 nM). The end point aggregation was expressed as a percentage of total aggregation with agonist alone. Statistical significance was determined using an unpaired 2-tailed *t* test. Ca^{2+} flux was measured under the same conditions using a Cairn Research Spectrophotometer (Faversham, United Kingdom) as described by Melendez et al.[9]

Additional methodology is provided as Document S1.

Results and discussion

G6B is expressed on the surface of platelets

To determine the relative expression profiles of each of the G6B splice forms, QPCR was performed on a panel of RNA samples (Figure 1A-D). Significant expression of the cell-surface isoform G6B-B was detected in platelets and spleen (Figure 1B), though the presence of G6B in spleen is most likely due to platelet contamination of the total spleen mRNA preparation. No significant expression was observed in any of the leukocyte samples analyzed. The other cell-surface isoform G6B-A, as well as the 2 secreted isoforms G6B-D and G6B-E, were also detected in platelets (Figure 1A,C-D).

G6B protein expression was examined in peripheral-blood leukocyte preparations using a G6B-specific mAb (Figure 1E). In agreement with the mRNA data, expression was only observed in platelets. Two bands of 32 kDa and 26 kDa were observed, consistent with the sizes expected for the glycosylated and unglycosylated membrane-bound forms of G6B, respectively.[2] There was no detection of G6B in $CD4^+$ or $CD8^+$ T cells or in $CD19^+$ B cells. An approximately 20-kDa band was detected in $CD14^+$ monocytes and $CD16^+$ neutrophils, which may be immature forms of G6B-D and G6B-E, although this does not correlate with the mRNA data.

G6B expression on the surface of platelets was assessed by flow cytometry and confocal microscopy (Figure 1F-G; Figure S2). G6B expression is detected on resting $CD41^+$ platelets (Figure 1F; Figure S2) and is increased approximately 2-fold following activation by ADP (Figure 1G).

Cross-linking G6B inhibits platelet function

G6B has previously been shown to associate with SHP-1 in a transfected cell-line model.[2] This association is important for the inhibitory signaling predicted for an ITIM-containing molecule. To determine whether G6B cross-linking lead to phosphorylation of the ITIMs in platelets, we looked for association of G6B with SHP-1 after G6B cross-linking (Figure 2A). As shown in Figure 2A, washed platelets were incubated with buffer alone (lane 1), G6B antisera (lane 2), or preimmune sera (lane 3) and coimmunoprecipitated with anti–SHP-1. Association of phosphorylated G6B with SHP-1 was only observed after incubation with the G6B polyclonal antisera (Figure 2A lane 2).

To determine whether signaling through G6B had an effect on platelet function, platelet aggregation was studied following treatment with a number of agonists (Figure 2). Cross-linking of G6B

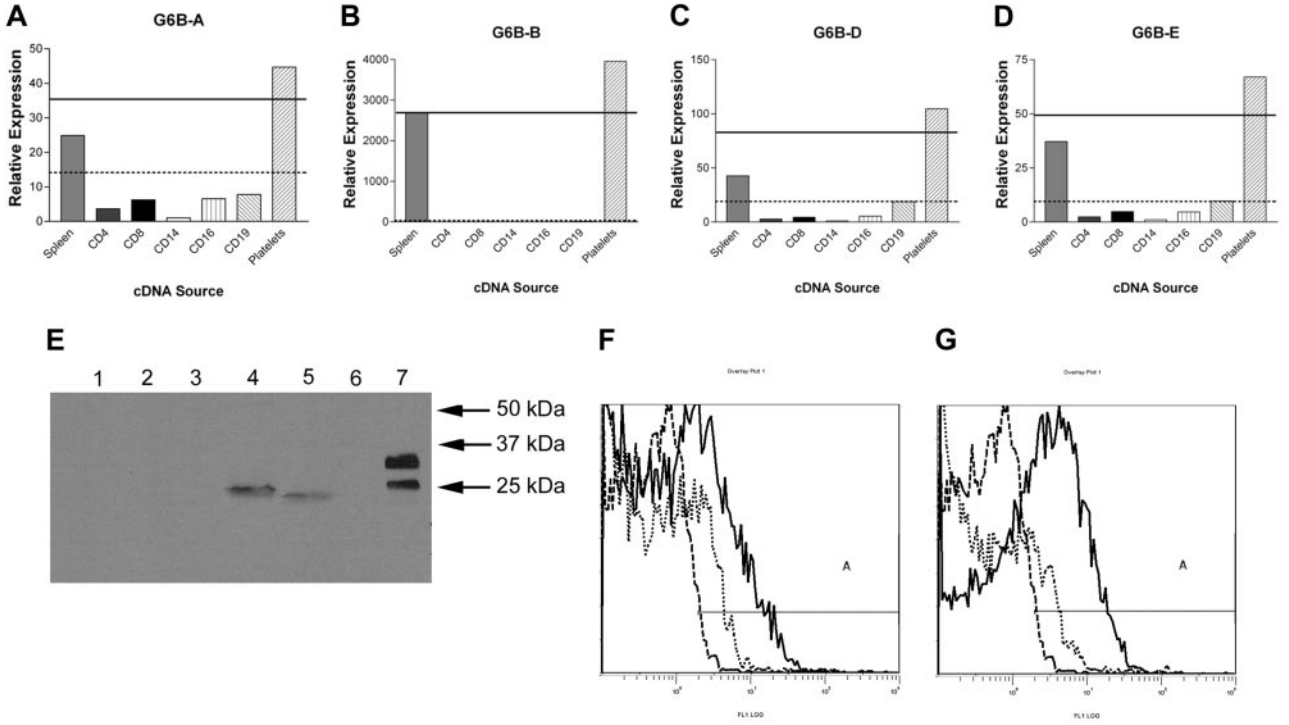

Figure 1. G6B is expressed on the surface of platelets. Relative expression of the G6B splice forms G6B-A (A), G6B-B (B), G6B-D (C), and G6B-E (D) in a range of cell types. In each panel, expression is shown relative to the cell type with the lowest ΔCT value.[5] In each case, the dashed line shows the median value; and the solid black line, 2 standard deviations above the median value. Data are representative of 3 individual experiments. (E) Western-blot analysis of G6B expression in whole buffy coat (lane 1), $CD4^+$ T cells (lane 2), $CD8^+$ T cells (lane 3), $CD14^+$ monocytes (lane 4), $CD16^+$ granulocytes (lane 5), $CD19^+$ B cells (lane 6), and platelet-rich plasma (lane 7). Due to the size differential between nucleated blood cells and platelets and to ensure equivalent protein mass per lane, 100-fold more cells were loaded in lane 7 compared with lanes 1 to 6. Gel was visualized with a G6B mAb and detected with a goat anti–mouse HRP conjugate as described in "Materials and methods." Size standards are indicated to the right of the gel. (F-G) Flow cytometry of platelets showing surface expression using affinity-purified G6B mAb (solid line), isotype control (dotted line), and platelets alone (dashed line). Panel F shows expression on washed resting platelets gated on the $CD41^+$ population and panel G shows ADP-activated platelets gated on the CD62-P^+ population. Region A represents the number of positive cells above unstained platelets.

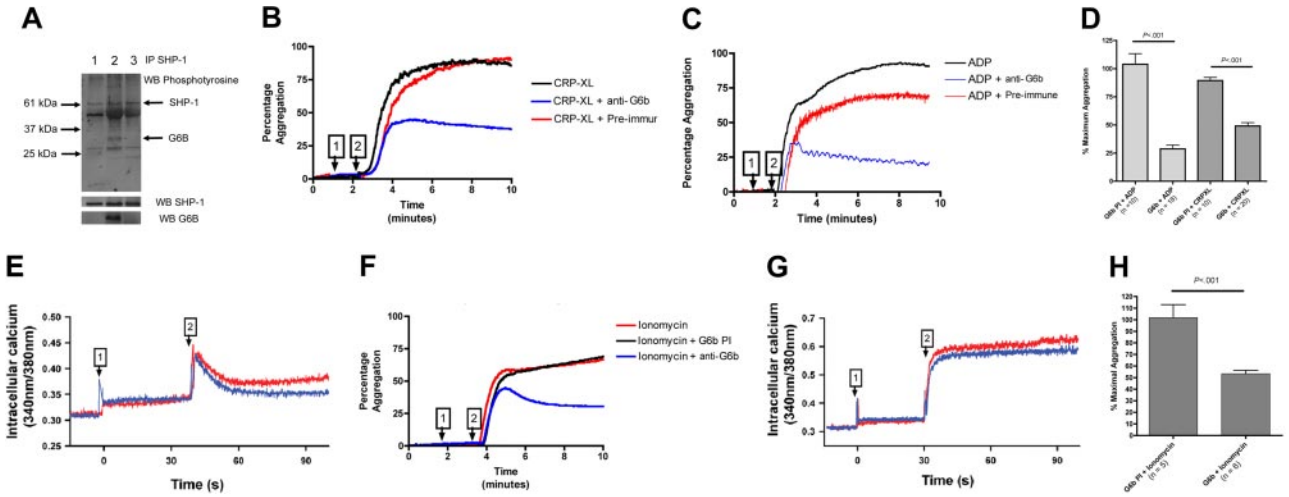

Figure 2. G6B cross-linking inhibits platelet aggregation in a Ca^{2+}-independent manner. (A) SHP-1 coimmunoprecipitates with G6B in platelets after stimulation with G6B antisera. G6B was coimmunoprecipitated (IP) with SHP-1 and is tyrosine phosphorylated after incubation of platelets with G6B antisera (lane 2). G6B was absent when platelets were incubated with preimmune sera (lane 3) or nothing (lane 1). SHP-1 coimmunoprecipitation was equivalent for all conditions. WB indicates Western blot. Platelet aggregation following treatment with CRP-XL (B) or ADP (C). In each panel, the black line represents agonist alone, the blue line represents agonist and the anti-G6B polyclonal antibody, and the red line represents agonist and preimmune sera. 1 corresponds to the addition of 5 μL of either the preimmune sera or the anti-G6B polyclonal antibody; 2 corresponds to addition of either CRP-XL (final concentration 0.1 μg/mL) or ADP (final concentration 5 μM). Traces are representative of at least 3 independent experiments. (D) Graph showing platelet aggregation in response to ADP and CRP-XL as a function of percentage total aggregation in the presence of the G6B polyclonal or the preimmune sera. The number of repeats for each condition is indicated in the parentheses. (E) Calcium flux in the presence of ADP and G6B polyclonal (blue) or preimmune sera (red). The arrows indicate the addition of the antisera (1) and agonist (2). (F) As for panel B but using ionomycin to induce aggregation. (G) The corresponding Ca^{+} trace after incubation with ionomycin. (H) Graph showing platelet aggregation as a function of percentage total aggregation in response to ionomycin. Each experiment was carried out the number of times shown in parentheses. Error bars in panels D and H indicate standard deviation about the mean.

prior to activating platelets with the agonist ADP led to a significant, dose-dependent reduction in platelet aggregation (Figure 2C-D; Figure S3), with the end point aggregation being reduced from 100% to 25% ($P < .001$). Similarly, cross-linking of G6B prior to activation with CRP-XL led to a 2-fold reduction in platelet aggregation ($P < .001$; Figure 2B,D). Thus, G6B shows an inhibitory effect on platelet aggregation in response to both ADP and CRP-XL.

Calcium-flux experiments were performed to mirror the aggregation experiments. Preincubation with G6B antisera had no effect on Ca^{2+} flux following treatment with agonist (Figure 2E). Observed platelet aggregation in the cuvette after the experiment (data not shown) correlated with the previous aggregation data, suggesting a calcium-independent inhibition of platelet aggregation. To investigate this further, platelets were activated with ionomycin, a calcium ionophore.[10] Normal aggregation is observed when preimmune sera is used. However, in the presence of the G6B polyclonal antibody, a 2-fold ($P < .001$) inhibition of aggregation is observed (Figure 2F,H). Figure 2G shows the corresponding calcium flux in platelets after ionomycin incubation. Again, there is no significant difference between G6B antisera and preimmune sera, suggesting that G6B has an inhibitory effect downstream from initial platelet activation and mobilization of intracellular calcium stores.

Recent studies have converged upon G6B as a novel platelet receptor.[6,7,11] In this study, we demonstrate that G6B is expressed on the surface of resting platelets and that cross-linking G6B has a significant inhibitory effect on platelet aggregation and activation. The detection of the secreted G6B isoforms in the $CD14^{+}$ and $CD16^{+}$ cell preparations in the absence of any detectable RNA suggests that these cell populations might be good targets for the expression of G6B's protein ligand.

Until recently, the only described ITIM-containing proteins expressed in platelets were platelet/endothelial cellular adhesion molecule 1 (PECAM-1) and Trem-like transcript 1(TLT-1). However, they differ from G6B in that they either have a distribution across other tissue types (eg, PECAM-1)[12] or exhibit an activatory rather than an inhibitory function (TLT-1).[13] G6B appears to be restricted to the platelet and megakaryocyte lineage and represents a novel inhibitory platelet surface receptor, making it a potential target for novel, platelet-specific antithrombotics.

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Authorship

Contribution: S.A.N., I.C.M., R.A.F., W.H.O., R.D.C., P.A.L., and N.A.W. participated in designing and performing research; E.C.d.V. contributed to new reagents; S.A.N., I.C.M., and R.A.F. analyzed data; and S.A.N., P.A.L., and R.D.C. wrote the paper.

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