In Vivo Expression of Murine Platelet Glycoprotein Ibα

By Hiroyuki Fujita, Yoshimi Hashimoto, Susan Russell, Barbara Zieger, and Jerry Ware

We have performed a systematic in vivo evaluation of gene expression for the glycoprotein (GP) Ibα subunit of the murine platelet adhesion receptor, GP Ib-IX-V. This study is warranted by in vitro observations of human GP Ibα expression in cells of nonhematopoietic lineage and reports of regulation of the GP Ibα gene by cytokines. However, an in vivo role for a GP Ib-IX-V receptor has not been established beyond that described for normal megakaryocyte/platelet physiology and hemostasis. Our Northern analysis of mouse organs showed high levels of GP Ibα mRNA in bone marrow with a similar expression pattern recapitulated in mice containing a luciferase transgene under control of the murine GP Ibα promoter. Consistently high levels of luciferase activity were observed in the two hematopoietic organs of mice, bone marrow (1,400 relative light units/μg of protein [RLUs]) and spleen (500 RLUs). Reproducible, but low-levels of luciferase activity were observed in heart, aorta, and lung (30 to 60 RLUs). Among circulating blood cells, the luciferase activity was exclusively localized in platelets. No increase in GP Ibα mRNA or luciferase activity was observed after treatment of mice with lipopolysaccharides (LPS) or tumor necrosis factor-α (TNF-α). We conclude the murine GP Ibα promoter supports a high level of gene expression in megakaryocytes and can express heterologous proteins allowing an in vivo manipulation of platelet-specific proteins in the unique environment of a blood platelet.

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PLATELET GLYCOPROTEIN (GP) membrane receptors provide circulating platelets with the ability to recognize distinct adhesive ligands exposed as a result of vascular injury or perturbation to the vascular lining.1 The platelet GP Ib-IX-V receptor complex contributes to this process initiating hemostasis through interactions with the adhesive ligand, von Willebrand factor.2-4 This receptor has a second unrelated role in maintaining circulating platelet morphology, as suggested by the congenital absence of the receptor, the Bernard-Soulier syndrome, and the release of “giant” platelets.1,2 Biochemical characterizations of the GP Ib-IX-V complex have documented the assembly of the complex from four distinct gene products5-7 with the ligand binding properties and linkages to the platelet membrane skeleton contained in the α-subunit of GP Ib (GP Ibα).8-13

While an in vivo role for platelet GP Ib-IX-V in hemostasis is well-documented, the expression of the complex in cells of nonhematopoietic lineage has been a controversial subject. A recent report characterizes an endothelial cell form of the GP Ib-IX-V complex14 contrasted by reports concluding endothelial cells lack the GP Ibα subunit and the ligand binding properties of the entire complex.15,16 Whereas the physiologic relevance, if any, of GP Ibα protein or a GP Ib-IX-V receptor complex on the surface of endothelial cells has not been determined, speculation has been fueled by in vitro assays characterizing the ability of cytokines to increase GP Ibα expression by endothelial cells and the possibility of a GP Ib-IX-V-dependent link between thrombotic events and inflammation.17,18 But again, these results are not without controversy, as others have been unable to reproduce the findings.15 Certainly, a systematic evaluation of GP Ibα in vivo expression would aid in ascertaining the biological sequelae that have been suggested from observations on cultured cells.

We have recently cloned and sequenced mouse genomic DNA containing the homologue to the human platelet GP Ibα gene.19 The mouse gene contains a similar exon/intron arrangement to the human gene and has allowed us an opportunity to perform a systematic evaluation of GP Ibα in vivo gene expression in a murine model system. Here we demonstrate high levels of GP Ibα mRNA in mouse bone marrow with a similar expression pattern obtained in transgenic animals containing a fragment of the mouse GP Ibα gene promoter. Our studies identified low-levels of GP Ibα promoter activity in heart, aorta, and lung, but contradictory to in vitro analyses, no increase in gene expression was observed after treatment of mice with cytokines or lipopolysaccharides (LPS). The presence of cis-acting elements in the murine GP Ibα gene promoter common among other megakaryocytic-specific promoters is discussed along with the physiologic relevance, if any, of low-levels of gene expression observed in some nonhematopoietic organs.

MATERIALS AND METHODS

Source of nucleic acids. The isolation and characterization of a P1 plasmid containing approximately 80-kb pairs of mouse 129/SvJ genomic DNA has been previously described.19 The sequence of 5,371 bp from this clone has been determined and deposited in GenBank (accession no. U91967). The characterized sequence contains the mouse homologue to the human platelet GP Ibα gene and a schematic organization of the gene is presented in Fig 1.

Northern analysis to detect the murine GP Ibα transcript was performed using a 1-kb radiolabeled fragment corresponding to nucleotides 2880-3871 (numbering according to GenBank U91967) and encoding mature mouse GP Ibα polypeptide sequence from residues 57-386.19 Northern analysis to detect the luciferase transcript in transgenic mice was performed by radiolabeling a BamHI restriction fragment (2.6 kb) containing the complete coding sequence for luciferase present in the plasmid, p19/LUC.19 A mouse cDNA fragment encoding intracellular adhesion molecule (ICAM)-1 was obtained from Genome Systems (St Louis, MO) as a deposited clone in the dbEST database and available through the cdNA consortium (accession no.

From the Roen Research Center for Arteriosclerosis and Thrombosis, Division of Experimental Hemostasis and Thrombosis, Departments of Molecular and Experimental Medicine and Vascular Biology, The Scripps Research Institute, La Jolla, CA.

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Address reprint requests to Jerry Ware, PhD, Mail Drop SBR8, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA 92037; e-mail: jware@scripps.edu.

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aa11579). Characterization of the clone showed a 1.2-kb insert with a restriction fragment pattern identical to that for the ICAM-1 sequence and limited sequencing confirmed the presence of a partial cDNA sequence for murine ICAM-1.23 For hybridization studies, the DNA fragments were radiolabeled with [α-32P]deoxyadenosine triphosphate (dATP) using a Prime-It II labelling kit available from Stratagene (La Jolla, CA).

RNA isolation and Northern analysis. Organs were dissected from mice ranging from 6 to 12 weeks of age. Dissected organs were immediately frozen in liquid nitrogen and manually pulverized in a liquid nitrogen-chilled porcelain mortar with a pestle. Immediately after evaporation of the liquid nitrogen, the organ powder was dissolved in 4 mol/L guanidinium isothiocyanate/0.1 mol/L Tris (pH 7.5)/0.5% n-lauroyl sarcosine. A centrifugation (3,000g) was performed to remove large particulate matter and the supernatant was applied to a cesium chloride cushion to isolate total RNA.22 Gel electrophoresis of RNA was performed through denaturing formaldehyde gels containing 1% agarose.22 Transfer to nitrocellulose was performed by capillary action and the filters were hybridized at 42°C in a solution containing 50% formamide/5× Denhardt’s solution/0.75 mol/L NaCl/50 mmol/L NaH2PO4/5 mmol/L EDTA/0.1% sodium dodecyl sulfate (SDS)/100 μg/mL denatured salmon sperm DNA. After overnight hybridization filters were washed three times in 0.3 mol/L sodium chloride/0.03 mol/L sodium citrate/0.1% SDS (10 minutes at room temperature [RT]) and one time in 0.03 mol/L sodium chloride/0.003 mol/L sodium citrate/0.1% SDS (30 minutes at 50°C). The washed nitrocellulose filters were analyzed by autoradiography using Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

Generation of transgenic mice. A 2.6-kb BamHI fragment from the mouse GP Ibα gene containing exon I, portions of the single intron, and approximately 2.4 kb of sequence 5’ to the transcription initiation site was cloned into the vector p19/LUC immediately upstream of the luciferase coding sequence. p19/LUC is a promoterless reporter plasmid used to assay heterologous promoter activity.20 Before injection into mouse zygotes, the mouse promoter/luciferase cassette was removed from the vector and extensively dialyzed against a buffer containing 1 mg/mL denatured salmon sperm DNA. After overnight hybridization filters were washed three times in 0.3 mol/L sodium chloride/0.03 mol/L sodium citrate/0.1% SDS (10 minutes at room temperature [RT]) and one time in 0.03 mol/L sodium chloride/0.003 mol/L sodium citrate/0.1% SDS (30 minutes at 50°C). The washed nitrocellulose filters were analyzed by autoradiography using Kodak X-OMAT film (Eastman-Kodak, Rochester, NY).

RESULTS

We previously reported the sequence of a 2.8-kb BamHI/EcoRI restriction fragment containing a single open reading frame encoding the mouse GP Ibα precursor polypeptide.19 The sequence of a 5′ contiguous 2.6-kb BamHI fragment has also been determined allowing a complete depiction of the murine GP Ibα gene (Fig 1). To examine the in vivo expression of the mouse GP Ibα gene, Northern analysis was performed on RNA isolated from the major murine organs (Fig 2). Using probes from the protein-coding sequence of the murine GP Ibα gene, a single 2.7-kb GP Ibα mRNA was exclusively detected in bone marrow RNA and is consistent with the predicted size of the murine GP Ibα transcript. No hybridization signals were visible in any other organ even after lengthy autoradiographic exposures (2 weeks). The mouse GP Ibα transcript is approximately 300 nucleotides longer than the human mRNA owing to length divergence within the region of the gene encoding the macroglycopeptide domain.19 Thus, within the limits of detection using total RNA isolated from murine organs, GP Ibα gene expression is restricted to bone marrow.

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BamHI

EcoRI

Fig 1. Genomic arrangement of the mouse GP Ibα gene. Restriction enzyme analysis of a P1 plasmid containing a fragment of mouse genomic DNA identified contiguous BamHI and BamHI/EcoRI restriction fragments that were chosen for DNA sequence determination. Based on sequence alignment with the human GP Ibα gene, boundaries for two exons are proposed (boxed regions) with an open reading frame (shaded box) encoding the putative mouse GP Ibα precursor polypeptide.19 A shaded gray region under the schematic representation of the gene identifies the approximate position of a radiolabeled fragment used for Northern analysis (Fig 2). The nucleotide sequence has been deposited in GenBank (accession no. U91967).
of a mouse femur. The size of the RNA is consistent with the predicted
autoradiograph obtained after hybridization and washing docu-
mRNA preparations (lower panel).
standard is shown to the left. After obtaining the autoradiograph, the
rehybridized with a radiolabeled DNA probe from the mouse 18S rRNA gene
nitrocellulose membrane was stripped of radioactivity and rehybrid-
mice and electrophoresed through a 1% agarose/formaldehyde dena-
organisms from two transgenic colonies (Fig 5). In all transgenic
were analyzed, the highest levels of luciferase activity were
be a major hematopoietic organ in the mouse. A
reproducible, but low-level of transgene expression, was
been heart, aorta, and lung even after extensive perfusion of
mice analyzed, the highest levels of luciferase activity were
observed in bone marrow isolated from a femur. Significant
luciferase activity was also observed in spleen, consistent with
mRNA was transferred to a
nucleotides in the RNA prepared from bone marrow of a mouse femur. The size of the RNA is consistent with the predicted
sample size of the transcript encoding mouse GP Ibβ coding sequence.29 A representative photograph of
hybridization and washing documents an RNA species of 2.7 kb in RNA prepared from bone marrow of mouse femur. The size of the RNA is consistent with the predicted
size of the transcript encoding mouse GP Ibβ (Fig 1). No other
released to a nitrocellulose membrane and hybridized with a radiolabeled probe of
murine GP Ibβ: coding sequence.29 A representative photograph of
the autoradiograph obtained after hybridization and washing docu-
mice were responsive to cytokines. As a model of gram-negative
situation was just the opposite in bone marrow with an absence
increase in GP Ibα transcript 24 hours post-LPS treatment (Fig 8A). The
luciferase activity present in whole blood is not apparent if the activity is
normalized to light units per µg of total protein. Experiments
assaying PRP and PPP from different transgenic lines confirmed
luciferase activity in whole blood coincides with the
presence of platelets exhibiting a linear correlation between
luciferase activity and the number of platelets (Fig 6). Thus, the
apparent absence of luciferase activity in whole blood as
presented in Fig 5 reflects the minuscule contribution of platelet
proteins to the complete protein composition of whole blood
rather than an absence of luciferase activity. Indeed, mouse
platelets represent 0.53% of the unit volume of whole blood.30
Having characterized the normal in vivo expression pattern
of the GP Ibα gene and the ability of transgenic colonies
expressing a reporter protein to recapitulate this expression
pattern, we performed experiments to determine whether the
endogenous GP Ibα gene or the GP Ibα/luciferase transgene
were responsive to cytokines. As a model of gram-negative
sepsis, LPS was administered to mice by intraperitoneal injec-
tion (25 mg/kg). The wide range of LPS-induced effects and
toxicity in mice is well-documented and the doses administered
to individual mice were sufficient to achieve maximal levels for
a variety of cytokines within 3 to 6 hours.27,28 Examining
luciferase activity in transgenic mouse organs, no increase in GP
Ibα expression was detected (Fig 7). Assaying for the presence
of the endogenous GP Ibα transcript in mouse organs, no increase in GP
Ibα expression was detected (Fig 8). In fact, the
situation was just the opposite in bone marrow with an absence
of GP Ibα transcript 24 hours post-LPS treatment (Fig 8A). The
induction of an LPS-induced inflammatory state was confirmed
with an increase in ICAM-1 mRNA (Fig 8B). Additionally,
mice were administered recombinant murine TNF-α either

Sequence alignment between the 5’ regions of human and mouse GP Ibα genes is shown defining potential regulatory elements within the 5’ region of the mouse GP Ibα gene
promoter and identifying the intron/exon boundaries of the
mouse gene (Fig 3). Specifically, mouse nucleotides 2,387 to
2,466 (numbering corresponds to GenBank accession no.
U91967) represent exon I with the thymine at nucleotide position 2,387 corresponding to a similar thymine identified as
the transcription initiation site of the human gene.20 Again,
based on sequence alignment, exon II was defined as nucleo-
tides 2,659 to 5,313 and contains an open reading frame
beginning at nucleotide 2,665 extending through nucleotide
4,869.19 Thus, the characterized mouse gene sequence has an
overall genomic arrangement similar to the human GP Ibα gene
with an 80 nucleotide 5’ untranslated exon, a single intron of
192 nucleotides, and a single exon encoding the mouse GP Ibα
polypeptide.24,25

The in vivo activity of a mouse GP Ibα promoter fragment was tested with the generation of transgenic mice expressing the
reporter enzyme luciferase under the control of the 5’ 2.6-kb BamHI fragment depicted in Fig 1. Individual founder mice
were expanded into independent transgenic colonies and the luciferase mRNA transcript was visualized by Northern analysis of
the major murine organs (Fig 4). The expression of the
transgene transcript showed an organ-specific pattern similar to
that obtained by probing for the endogenous GP Ibα transcript,
namely detectable levels of transcript exclusively in bone
marrow RNA preparations.

The expression of the luciferase transgene was further evaluated by examining luciferase activity in organ homoge-

Fig 2. Northern blot analysis of RNA prepared from the major
murine organs. Total RNA was isolated from nine organs of adult
mice and electrophoresed through a 1% agarose/formaldehyde dena-
turing gel. After electrophoresis, the RNA was transferred to a
nitrocellulose membrane and hybridized with a radiolabeled probe of
the murine GP Ibα: coding sequence.29 A representative photograph of
the autoradiograph obtained after hybridization and washing docu-
mments an RNA species of 2.7 kb in RNA prepared from bone marrow
of a mouse femur. The size of the RNA is consistent with the predicted
size of the transcript encoding mouse GP Ibα (Fig 1). No other
hybridizing signals were observed after a lengthy (2 weeks) exposure
to x-ray film. The migrating position of an RNA molecular weight
standard is shown to the left. After obtaining the autoradiograph, the
nitrocellulose membrane was stripped of radioactivity and rehybrid-
with a radiolabeled DNA probe from the mouse 18S rRNA gene
to confirm similar amounts of RNA were loaded from the different
RNA preparations (lower panel).
intraperitoneally (20 µg/kg maximal dose) or intravascular (4 µg/kg maximal dose). At time points up to 24 hours postinjection, organs were assayed for luciferase activity and levels of GP Ibα mRNA were determined by Northern analysis. At all doses and time points examined, administration of TNF-α had no demonstrable effects on GP Ibα gene or luciferase transgene expression (data not shown).

DISCUSSION

Among platelet receptors, the GP Ib-IX-V complex is important because it initiates thrombus formation through a tethering of the circulating platelet to von Willebrand factor, an adhesive ligand of the subendothelial matrix.4 The importance of this receptor-ligand interaction for normal platelet biology is best exemplified by congenital bleeding disorders resulting from the lack of either the receptor or the ligand, the Bernard-Soulier syndrome, and von Willebrand disease, respectively. In addition, the absence of a GP Ib-IX-V complex coincides with the release of giant platelets, leading to speculations that the synthesis and assembly of the complex is directly linked to normal platelet morphology. Thus, the importance of the complex in normal megakaryocyte and platelet physiology is clear and well-documented. Nevertheless, expression of GP Ibα and other subunits of the complex by nonmegakaryocytic cells has been suggested by a number of in vitro observations documenting expression of the individual subunits of the complex in cultured human endothelial and smooth muscle cells.17,18,29-31 Despite these observations on cultured cells, little is known concerning the regulation of GP Ibα expression in vivo, the only corollary is an immunologic identification of GP Ibα in tonsillar endothelium.14,17

The current study examined GP Ibα gene expression to determine in a systematic manner the relative abundance of the GP Ibα transcript among the major organs of the mouse. We conclude the GP Ibα mRNA is a dominant transcript in bone marrow, as identified by Northern analysis of total mouse RNA. No other major organ of the mouse expresses GP Ibα to the same extent. We also used a transgenic model of GP Ibα expression using the highly-sensitive reporter gene product, luciferase, and observed the highest levels of luciferase activity in bone marrow and spleen (Fig 5), both hematopoietic organs in the mouse. Consistent and reproducible luciferase activity was observed in lung, heart, and aorta, yet the gene expression in these satellite organs was, on average, 20 to 50 times lower than the activity present in bone marrow preparations. Northern blots examining GP Ibα transcript levels, luciferase transcript levels, and assays of luciferase protein activity showed that differences in technique sensitivity can bias conclusions on the expression of a particular gene product. In fact, we did not observe GP Ibα or luciferase mRNA in spleen, yet luciferase...
activity assays showed the luciferase transgene was capable of expressing protein to a level nearly one third that of bone marrow.

Our results identifying a low level of in vivo GP Ibα gene activity within some nonhematopoietic organs, namely, heart, aorta, and lung, forces a more compelling question as to whether low levels of gene activity provide previously unrecognized functional properties for GP Ibα or a GP Ib-IX-V complex? No phenotypic abnormalities beyond hemostatic deficiency and giant platelets have been described for patients with the Bernard-Soulier syndrome. Others have suggested from in vitro experiments there exists a GP Ib-IX-V link between the release of cytokines and thrombotic potential.17,18 However, our in vivo results do not support such a link. At LPS doses sufficient to achieve maximal levels for a variety of cytokines,27,28 we observed no increase in GP Ibα gene expression (Figs 7 and 8). Direct administration of murine TNF-α did not increase GP Ibα gene expression. In fact, in the LPS-induced model of gram-negative sepsis, the GP Ibα mRNA was absent 24 hours posttreatment (Fig 8A). This result may reflect the more global changes, such as platelet depletion, which is reported to occur after LPS-induced toxicity in mice.32 Of course, one caveat in our experiment is the use of a mouse model, which may or may not, extrapolate to expression of the human GP Ibα gene.

The expression of GP Ibα mRNA and protein in cells other than megakaryocytes is not without controversy. Some investigators have been unable to document expression of GP Ibα by cultured endothelial cells15 or identify von Willebrand factor binding to endothelial cells dependent on a GP Ib-IX-V receptor complex.16 Reconciling these discrepant results from different laboratories can be difficult. Complicating factors for the interpretation of GP Ibα expression by endothelial cells could include the apparent changes in gene expression occurring during the transition of an endothelial cell from an in vivo to an in vitro state.
in vitro environment, or even interlaboratory variations using similar techniques, but achieving different levels of sensitivity. An alternative explanation might be low levels of transcription for a tissue-specific gene in nonspecific cells. Two of the best documented examples are the presence of clotting cofactor VIII mRNA in lymphocytes and the detection of the Duchenne muscular dystrophy transcripts in nonmuscle cells. The transcription of a cell-specific gene in a variety of other cell types can be accounted for by two different mechanisms. The first is a basal level of transcription that has been referred to as “illegitimate” or “leaky” transcription. The second is expression by a subset of cells within a given tissue and can be particularly confusing if the vascular cells of a tissue or organ are contributing “leaky” activity. Thus, in light of no identified physiologic relevance for GP Ibα gene activity beyond that described for megakaryocytes and platelets, it is conceivable the low level of gene activity observed in some cells and organs may represent “leaky” transcription. Perhaps studies characterizing a targeted disruption of the GP Ibα gene in mice may provide more definitive answers.

While characterizing the endogenous in vivo expression pattern of GP Ibα, this study has identified a murine promoter fragment capable of directing the synthesis of heterologous gene products in megakaryocytes. Thus, the promoter must contain all necessary elements for in vivo expression of the GP Ibα gene. The human GP Ibα promoter has been characterized and contains megakaryocytic-specific cis-acting elements, specifically GATA and Ets motifs, common among megakaryocytic-specific genes. Both GATA and Ets motifs are conserved within the murine GP Ibα promoter along with a positive megakaryocytic regulatory element identified in the rat and human platelet factor 4 promoters (Fig 3). Previous in vitro analysis of the human GP Ibα promoter identified 253 nucleotides 5′ to the transcription initiation site as essential for maximum activity in human erythroleukemia cells. The alignment between the mouse and human GP Ibα sequences displays some highly conserved sequences within this region of both promoters along with conserved sequences in both exon I and the single intron of each gene (Fig 3). Indeed, the identification of conserved elements between the human and mouse promoter sequences may have merit. We have previously shown that the human GP Ibα promoter functions in vivo to express the human GP Ibα polypeptide on the surface of murine transgenic platelets. Thus, conserved mechanisms must exist for megakaryocytic gene expression between the two species. Alignment of genomic sequences extending 5′ to that shown in Fig 3 did not display long stretches of sequence similarity between the human and murine promoters, but without further in vivo tests of promoter activity, it is impossible to suggest that
the relatively short promoter regions displayed in Fig 3 are sufficient for in vivo activity.

Overall, these studies support the long-term objective of manipulating membrane receptors in the unique cellular characteristics of a platelet. The expression of megakaryocytic genes and the identification of promoter fragments supporting expression in a manner that mirrors the endogenous gene product provide crucial information to achieve this objective, namely, the in vivo expression of variant receptors. Such studies should provide new information relevant to the in vivo physiology and pathophysiology of platelet receptor function.

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