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Ligand

The invention relates to a ligand that binds a receptor expressed by blood platelets to inhibit or promote platelet aggregation during thrombus formation.

The coagulation of blood is a complex and multi-step process that results in the formation of a thrombus. Coagulation resulting from a cut or contusion is initiated by platelets that bind to and are activated by collagen in the blood vessel endothelium. The activated platelets then release a number of substances that enhance further platelet activation and platelet recruitment to the wound site. Platelets are anuclear, disc shaped and contain, amongst other things, RNA, lysosomes which contain acid hydrolases; dense bodies containing ADP, ATP, serotonin and calcium; alpha granules containing fibrinogen, factor V, vitronectin and von Willebrand factor which are released from the platelet upon activation and play important roles in thrombus formation and inflammation.

There are a number of known activators of platelets. These include collagen when bound by the platelet receptor GPVI; thrombin through cleavage of the extracellular domain of PAR 1 and PAR 4; and convulxin which binds GPVI. ADP is also a platelet activator. There are also known inhibitors of platelet activation, for example, prostacyclin, nitric oxide, clotting factors II, IX, X, XI and XII and nucleotidases that hydrolyse ADP. There are also a number of drugs that inhibit platelet activation, for example aspirin, non-steroidal anti-inflammatory drugs that inhibit prostaglandin synthesis, abciximab which inhibits the activation of fibrinogen receptors and quinidine which is a calcium channel inhibitor.

Platelets are associated with a number of diseases and conditions that result from platelet dysfunction. For example, conditions such as thrombocytopenia result in coagulation problems and result from low circulating platelet numbers. Other diseases that result from low platelet numbers include Gaucher's disease and aplastic anaemia. Some conditions can result from low platelet numbers or dysfunctional platelets, for example HELLP syndrome and haemolytic-uremic syndrome. Conversely, conditions can result from elevated platelet numbers, for example thrombocytosis. Further platelet pathologies result in abnormal adhesion or aggregation; for example Von Willebrand disease or Glanzmann's disorder. A further class of disease can result from altered

platelet metabolism, for example in the case of decreased cyclooxygenase activity which can be congenital.

Clearly, there is a need to provide therapies that will ameliorate the effects of these diseases and in some cases the provision of specific therapeutic agents that will cure or decrease disease symptoms.

A number of anti-thrombotic agents are known in the art. For example, US7, 018, 985B1 discloses compounds that are dinucleotide polyphosphates that are selective for $P2_T$ receptors expressed by platelets. Antibodies and peptides that bind to and inhibit the activity of proteins expressed by platelets are also known. For example, WO2005/056575 discloses $\alpha_{IIb}\beta_3$ specific antibodies and peptides that inhibit platelet aggregation. US 2006/0088531 disclose human antibodies and single chain antibody fragments (scFv) to GPVI and their inhibitory activity with respect to collagen activated platelet activation. Similarly, EP1647596 describes monoclonal antibodies that bind to and inhibit GPVI mediated activation of platelets. There is also disclosure of antibodies and antibody fragments in Cauwenberghs et al (Arteriosder Thromb Vasc Biol. 2000 May; 20(5) 1347-53) which describes antibodies that interfere with the binding of glycoprotein Ib with von Willebrand factor induced platelet agglutination. Similarly, Kageyama et al (Br J Pharmacol. 1997 Sep: 122 (1):165-71) describes a monoclonal antibody directed to von Willebrand factor that binds to and interferes with the binding to glycoprotein Ib. It is apparent that there are a number of molecules that purport to be anti-thrombotic agents and have use in the treatment of diseases or conditions that result in or from thrombus formation.

The present disclosure relates to ligands that bind to a platelet expressed receptor to inhibit platelet activation.

The human *G6B* gene is located in the MHC Class III region and encodes a member of the Immunoglobulin (Ig) superfamily (Ribas, G et al J Immunol 1999 63(1): 278-87). The gene has a number of splice forms that translate into both cell surface bound and secreted isoforms (De Vet et al J Biol Chem 2001 276(45):42070-6). The two principal cell surface isoforms (G6B-A and G6B-B) have the same single N terminal Ig-like domain but differ in their C terminal cytoplasmic tails. The G6B-A form contains a proline rich region suggesting a signalling function for this protein. In contrast G6B-B has previously been shown to have two immunoreceptor tyrosine-based inhibitory motifs (ITIM) within its cytoplasmic tail and to associate with SHP-1 and SHP-2 protein-tyrosine

phosphatases. Moebius et al (Mol Cell Proteomics 2005 4(11):1754-61) describes a proteomic study of platelet membrane proteins of which *G6B-A* is shown to be expressed by platelets. However, no physiological function has been assigned to *G6B*.

This disclosure relates to G6B which is expressed on the surface of resting platelets and that crosslinking G6B with, for example an antibody, has a significant inhibitory effect on platelet aggregation and activation. The disclosure shows activation of platelets with agonists. After crosslinking of G6B with antisera, G6B inhibits a final platelet activation pathway potentially through the G6B-B ITIM. It is possible that either one or both of the ITIMs prevents clustering of Alpha2 Beta3 integrin which is necessary for final platelet aggregation. In light of these observations G6B represents a potentially novel anti thrombotic drug target.

We disclose ligands useful in the treatment of diseases and conditions that either inhibit thrombus formation or promote the formation of a thrombus.

According to an aspect of the invention there is provided a ligand wherein the ligand binds to and modulates the activity of a G6B polypeptide for use as a pharmaceutical

In a preferred embodiment of the invention the ligand inhibits the platelet activation.

According to an aspect of the invention there is provided a ligand wherein the ligand binds to and modulates the activity of a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f or 4g;

ii) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet;

iii) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented by Figures 5a, 5b, 5c, 5d, 5e, 5f or 5g.

According to an aspect of the invention there is provided a ligand wherein the ligand binds to and modulates the activity of a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f or 4g ;

ii) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented by Figures 5a, 5b, 5c, 5d, 5e, 5f or 5g.

iii) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet for use as a pharmaceutical

Hybridization of a nucleic acid molecule occurs when two complementary nucleic acid molecules undergo an amount of hydrogen bonding to each other. The stringency of hybridization can vary according to the environmental conditions surrounding the nucleic acids, the nature of the hybridization method, and the composition and length of the nucleic acid molecules used. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001); and Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes Part I, Chapter 2 (Elsevier, New York, 1993). The T_m is the temperature at which 50% of a given strand of a nucleic acid molecule is hybridized to its complementary strand. The following is an exemplary set of hybridization conditions and is not limiting:

Very High Stringency (allows sequences that share at least 90% identity to hybridize)

Hybridization:	5x SSC at 65°C for 16 hours
Wash twice:	2x SSC at room temperature (RT) for 15 minutes each
Wash twice:	0.5x SSC at 65°C for 20 minutes each

High Stringency (allows sequences that share at least 80% identity to hybridize)

Hybridization:	5x-6x SSC at 65°C-70°C for 16-20 hours
Wash twice:	2x SSC at RT for 5-20 minutes each
Wash twice:	1x SSC at 55°C-70°C for 30 minutes each

Low Stringency (allows sequences that share at least 50% identity to hybridize)

Hybridization:	6x SSC at RT to 55°C for 16-20 hours
Wash at least twice:	2x-3x SSC at RT to 55°C for 20-30 minutes each.

According to a further aspect of the invention there is provided a ligand wherein the ligand binds to and modulates the activity of a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g, or a variant polypeptide wherein said variant polypeptide comprises an amino acid sequence that is modified by addition, deletion or substitution of at least one amino acid residue with reference to the amino acid sequences presented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g and which retains the activity associated with a G6B polypeptide.

According to a further aspect of the invention there is provided a ligand wherein the ligand binds to and modulates the activity of a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g, or a variant polypeptide wherein said variant polypeptide comprises an amino acid sequence that is modified by addition, deletion or substitution of at least one amino acid residue with reference to the amino acid sequences presented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g and which retains the activity associated with a G6B polypeptide for use as a pharmaceutical

A variant polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations that may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and aspartic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan. Most highly preferred are variants that retain or enhance the same biological function and activity as the reference polypeptide from which it varies.

In addition, the invention features polypeptide sequences having at least 75% identity with the polypeptide sequences as herein disclosed, or fragments and functionally equivalent polypeptides thereof. In one embodiment, the polypeptides have at least 85% identity, more preferably at least 90% identity, even more preferably at least 95% identity, still more preferably at least 97% identity, and most preferably at least 99% identity with the amino acid sequences illustrated herein.

In a preferred embodiment of the invention said ligand is an antibody, or an active binding fragment of an antibody. Preferably said antibody, or binding fragment, is a

monoclonal antibody.

Antibodies or immunoglobulins (Ig) are a class of structurally related proteins consisting of two pairs of polypeptide chains, one pair of light (L) (low molecular weight) chain (κ or λ), and one pair of heavy (H) chains (γ, α, μ, δ and ε), all four linked together by disulphide bonds. Both H and L chains have regions that contribute to the binding of antigen and that are highly variable from one Ig molecule to another. In addition, H and L chains contain regions that are non-variable or constant. The L chains consist of two domains. The carboxy-terminal domain is essentially identical among L chains of a given type and is referred to as the "constant" (C) region. The amino terminal domain varies from L chain to L chain and contributes to the binding site of the antibody. Because of its variability, it is referred to as the "variable" (V) region. The variable region contains complementarity determining regions or CDR's which form an antigen binding pocket. The binding pockets comprise H and L variable regions which contribute to antigen recognition.

Various fragments of antibodies are known in the art, i.e., Fab, Fab_2, $F(ab')_2$, Fv, Fc, Fd, scFvs, etc. A Fab fragment is a multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, covalently coupled together and capable of specifically binding to an antigen. Fab fragments are generated via proteolytic cleavage (with, for example, papain) of an intact immunoglobulin molecule. A Fab_2 fragment comprises two joined Fab fragments. When these two fragments are joined by the immunoglobulin hinge region, a $F(ab')_2$ fragment results. An Fv fragment is multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region covalently coupled together and capable of specifically binding to an antigen. A fragment could also be a single chain polypeptide containing only one light chain variable region, or a fragment thereof that contains the three CDRs of the light chain variable region, without an associated heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multi specific antibodies formed from antibody fragments, this has for example been described in US patent No 6,248,516. Fv fragments or single region (domain) fragments are typically generated by expression in host cell lines of the relevant identified regions. These and other immunoglobulin or antibody fragments are within the scope of the invention and are described in standard immunology textbooks such as Paul, *Fundamental*

Immunology or Janeway et al. *Immunobiology* (cited above). Molecular biology now allows direct synthesis (via expression in cells or chemically) of these fragments, as well as synthesis of combinations thereof. A fragment of an antibody or immunoglobulin can also have bispecific function as described above.

Domain antibodies are the smallest binding part of an antibody (approximately 13kDa). Examples of this technology is disclosed in US6, 248, 516, US6, 291, 158, US6,127, 197 and EP0368684 which are all incorporated by reference in their entirety.

In a preferred embodiment of the invention said antibody fragment is selected from the group consisting of: Fab; Fab_2; $F(ab')_2$; Fv; Fc; Fd; single chain antibody variable region fragment; a domain fragment.

In a preferred embodiment of the invention said antibody fragment is a single chain antibody variable region fragment.

More preferably still said antibody fragment binds a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g.

In a further preferred embodiment of the invention said antibody, or binding fragment thereof, is a chimeric antibody. In an alternative preferred embodiment of the invention said antibody, or binding fragment thereof, is a humanised antibody.

A chimeric antibody is produced by recombinant methods to contain the variable region of an antibody with an invariant or constant region of a human antibody. A humanised antibody is produced by recombinant methods to combine the CDR's of an antibody with both the constant regions and the framework regions from the variable regions of a human antibody.

Antibodies from non-human animals provoke an immune response to the foreign antibody and its removal from the circulation. Both chimeric and humanised antibodies have reduced antigenicity when injected to a human subject because there is a reduced amount of rodent (i.e. foreign) antibody within the recombinant hybrid antibody, while the human antibody regions do not elicit an immune response. This results in a weaker immune response and a decrease in the clearance of the antibody. This is clearly desirable when using therapeutic antibodies in the treatment of human diseases. Humanised antibodies are designed to have less "foreign" antibody regions and are

therefore thought to be less immunogenic than chimeric antibodies.

In a preferred embodiment of the invention said antibody or antibody fragment is a human antibody.

Human antibodies are distinct from humanised or chimeric antibodies in so far as the antibodies do not contain any rodent sequences. Fully human antibodies can be isolated from a number of sources. For example, some human antibodies can be obtained from immune donors using either EBV transformation of B- cells or by PCR cloning and phage display. Alternatively, fully human antibodies can be isolated from synthetic phage libraries which include synthetic human antibody variable regions. The synthetic antibodies are selected against antigen and have the properties of naturally occurring human antibodies. Human antibodies also include human antibodies prepared in, for example, transgenic mice. Transgenic mice have been created that include human immunoglobulin germ-line gene segments. When immunised with antigen the mice make human antibodies that can be isolated or used to make hybridomas' that produce human antibodies.

In an alternative preferred embodiment of the invention said ligand is a peptide wherein said peptide binds a polypeptide as disclosed herein. Preferably said peptide is a modified peptide.

It will be apparent to one skilled in the art that peptides are susceptible to modifications such as acetylation and/or amidation. Alternatively or preferably, said modification includes the use of modified amino acids in the production of peptides according to the invention. It will be apparent that modified amino acids include, by way of example and not by way of limitation, 4-hydroxyproline, 5-hydroxylysine, N^6-acetyllysine, N^6-methyllysine, N^6,N^6-dimethyllysine, N^6,N^6,N^6-trimethyllysine, cyclohexyalanine, D-amino acids, ornithine. Other modifications include amino acids with a C_2, C_3 or C_4 alkyl R group optionally substituted by 1, 2 or 3 substituents selected from halo (eg F, Br, I), hydroxy or C_1-C_4 alkoxy. Alternatively, peptides could be modified by cyclisation. Cyclisation is known in the art, (see Scott et al Chem Biol (2001), 8:801-815; Gellerman et al J. Peptide Res (2001), 57: 277-291; Dutta et al J. Peptide Res (2000), 8: 398-412; Ngoka and Gross J Amer Soc Mass Spec (1999), 10:360-363.

In a further alternative embodiment of the invention said ligand is an aptamer.

Nucleic acids have both linear sequence structure and a three dimensional structure which in part is determined by the linear sequence and also the environment in which these molecules are located. Conventional therapeutic molecules are small molecules, for example, peptides, polypeptides, or antibodies, which bind target molecules to produce an agonistic or antagonistic effect. It has become apparent that nucleic acid molecules also have potential with respect to providing agents with the requisite binding properties which may have therapeutic utility. These nucleic acid molecules are typically referred to as aptamers. Aptamers are small, usually stablised, nucleic acid molecules which comprise a binding domain for a target molecule. A screening method to identify aptamers is described in US 5,270,163 which is incorporated by reference. Aptamers are typically oligonucleotides which may be single stranded oligodeoxynucleotides, oligoribonucleotides, or modified oligodeoxynucleotide or oligoribonucleotides.

The term "modified" encompasses nucleotides with a covalently modified base and/or sugar. For example, modified nucleotides include nucleotides having sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleotides may also include 2' substituted sugars such as 2'-O-methyl-; 2-O-alkyl; 2-O-allyl; 2'-S-alkyl; 2'-S-allyl; 2'- fluoro-; 2'-halo or 2;azido-ribose, carbocyclic sugar analogues a-anomeric sugars; epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, and sedoheptulose.

Modified nucleotides are known in the art and include by example and not by way of limitation; alkylated purines and/or pyrimidines; acylated purines and/or pyrimidines; or other heterocycles. These classes of pyrimidines and purines are known in the art and include, pseudoisocytosine; N4, N4-ethanocytosine; 8-hydroxy-N6-methyladenine; 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil; 5-fluorouracil; 5-bromouracil; 5-carboxymethylaminomethyl-2-thiouracil; 5-carboxymethylaminomethyl uracil; dihydrouracil; inosine; N6-isopentyl-adenine; l-methyladenine; 1-methylpseudouracil; 1-methylguanine; 2,2-dimethylguanine; 2-methyladenine; 2-methylguanine; 3-methylcytosine; 5-methylcytosine; N6-methyladenine; 7-methylguanine; 5-methylaminomethyl uracil; 5-methoxy amino methyl-2-thiouracil; β-D-mannosylqueosine; 5-methoxycarbonylmethyluracil; 5-methoxyuracil; 2 methylthio-N6-isopentenyladenine; uracil-5-oxyacetic acid methyl ester; psuedouracil; 2-thiocytosine; 5-methyl-2 thiouracil, 2-thiouracil; 4-thiouracil; 5-methyluracil; N-uracil-5-oxyacetic acid methylester; uracil 5—

oxyacetic acid; queosine; 2-thiocytosine; 5-propyluracil; 5-propylcytosine; 5-ethyluracil; 5-ethylcytosine; 5-butyluracil; 5-pentyluracil; 5-pentylcytosine; and 2,6,-diaminopurine; methylpsuedouracil; 1-methylguanine; 1-methylcytosine.

The aptamers of the invention are synthesized using conventional phosphodiester linked nucleotides and synthesized using standard solid or solution phase synthesis techniques which are known in the art. Linkages between nucleotides may use alternative linking molecules. For example, linking groups of the formula P(O)S, (thioate); P(S)S, (dithioate); P(O)NR'2; P(O)R'; P(O)OR6; CO; or CONR'2 wherein R is H (or a salt) or alkyl (1-12C) and R6 is alkyl (1-9C) is joined to adjacent nucleotides through –O- or –S-. The binding of aptamers to a target polypeptide is readily tested by assays hereindisclosed.

According to an aspect of the invention there is provided a composition comprising a ligand according to the invention.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising at least one ligand according to the invention.

When administered, the ligands/pharmaceutical compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers and optionally other therapeutic agents, such as additional anti-thrombotic agents.

The ligands/pharmaceutical compositions of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, for example directly into a joint, intraocular, subcutaneous, or transdermal.

The ligands/compositions of the invention are administered in effective amounts. An "effective amount" is that amount of a ligand/composition that alone, or together with further doses, produces the desired response. In the case of treating a particular disease the desired response is inhibiting the progression of the disease. This may involve slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by

routine methods.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

The ligands/pharmaceutical compositions used in the foregoing methods of treatment preferably are sterile and contain an effective amount of ligand for producing the desired response in a unit of weight or volume suitable for administration to a patient.

The doses of ligand administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired period of treatment. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localised delivery route) may be employed to the extent that patient tolerance permits.

When administered, the ligands/compositions of the invention are applied in therapeutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention.

Ligands/compositions may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt. The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation of ligands which is preferably isotonic with the blood of the recipient. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables.

Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising a ligand according to the invention and at least one further anti-thrombotic agent.

In a preferred embodiment of the invention said further anti-thrombotic agent is selected from the group consisting of: Aspirin (acetylsalicylic acid), Ticlid (ticlodipine), Plavix (clopidogrel), Pletal (cilostazol), Persantine (dipyridamole), Anturane (sulfinpyrazone), and 3 intravenous agents: Rheopro (abciximab), Integrilin (eptifibatide), Aggrastat (tirofiban), heparin and warfarin.

In an alternative preferred embodiment of the invention said further anti-thrombotic agent is a second antibody.

In a preferred embodiment of the invention the second antibody binds and modulates the activity of a second platelet polypeptide wherein the second platelet polypeptide is not G6B.

According to a further aspect of the invention there is provided a screening method for the identification of ligands which bind a polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f or 4g;

b) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g.

c) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet; comprising the steps of

i) forming a preparation comprising the polypeptide and a ligand to be tested;

ii) testing the binding of said ligand for said polypeptide; and

iii) optionally testing the activity of the ligand with respect to the activation of platelets.

In a preferred method of the invention said ligand is an antagonist.

In an alternative method of the invention said ligand is an agonist.

In a preferred method of the invention said polypeptide comprises an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g.

In a further method of the invention said method comprises transfecting a cell with a vector that includes a nucleic acid molecule that is adapted to be operably linked to an expression control sequence selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f or 4g;

ii) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g;

ii) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet and contacting the cell expressing said nucleic acid molecule with an agent to be tested.

Typically, adaptations include the provision of transcription control sequences (promoter sequences) which mediate cell/tissue specific expression. These promoter sequences may be cell/tissue specific, inducible or constitutive.

"Promoter" is an art recognised term and, for the sake of clarity, includes the following features which are provided by example only. Enhancer elements are *cis* acting nucleic acid sequences often found 5' to the transcription initiation site of a gene (enhancers can also be found 3' to a gene sequence or even located in intronic sequences). Enhancers function to increase the rate of transcription of the gene to which the enhancer is linked. Enhancer activity is responsive to *trans* acting transcription factors (polypeptides) which have been shown to bind specifically to enhancer elements. The binding/activity of transcription factors (please see Eukaryotic Transcription Factors, by David S Latchman, Academic Press Ltd, San Diego) is responsive to a number of physiological/environmental cues which include intermediary metabolites and environmental effectors.

Promoter elements also include so called TATA box and RNA polymerase initiation selection sequences which function to select a site of transcription initiation. These sequences also bind polypeptides which function, *inter alia,* to facilitate transcription initiation selection by RNA polymerase.

Adaptations also include the provision of selectable markers and autonomous replication

sequences which facilitate the maintenance of said vector in either the eukaryotic cell or prokaryotic host. Vectors which are maintained autonomously are referred to as episomal vectors. Episomal vectors are desirable since these molecules can incorporate large DNA fragments (30-50kb DNA). Episomal vectors of this type are described in WO98/07876.

Adaptations which facilitate the expression of vector encoded genes include the provision of transcription termination/polyadenylation sequences. This also includes the provision of internal ribosome entry sites (IRES) which function to maximise expression of vector encoded genes arranged in bi-cistronic or multi-cistronic expression cassettes.

There is a significant amount of published literature with respect to expression vector construction and recombinant DNA techniques in general. Please see, Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical Approach Vol III IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

In a further preferred method of the invention said cell is part of a non-human transgenic animal and said animal is administered said ligand to test for agonistic or antagonistic activity.

In a preferred method of the invention said ligand is an antibody, or a binding fragment thereof, as hereindescribed.

In an alternative preferred method of the invention said ligand is a peptide, or modified peptide as hereindescribed.

In a further alternative method of the invention said ligand is an aptamer as hereindescribed.

According to a further aspect of the invention there is provide a non-human transgenic animal wherein said animal is modified to include a nucleic acid molecule selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f or 4g;

ii) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g;

ii) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet.

According to a further aspect of the invention there is provided a method to determine the ability of a molecule to associate with a polypeptide comprising the steps of:

i) providing computational means to perform a fitting operation between said molecule and a polypeptide defined by the amino acid sequence in Figure 5a, 5b, 5c, 5d, 5e, 5f or 5g; ; and

ii) analysing the results of said fitting operation to quantify the association between the molecule and the polypeptide.

In a preferred method of the invention said molecule is further tested for the inhibitory activity with respect to platelet activation.

The rational design of binding entities for proteins is known in the art and there are a large number of computer programs that can be utilised in the modelling of 3-dimensional protein structures to determine the binding of chemical entities to functional regions of proteins and also to determine the effects of mutation on protein structure. This may be applied to binding entities and also to the binding sites for such entities. The computational design of proteins and/or protein ligands demands various computational analyses which are necessary to determine whether a molecule is sufficiently similar to the target protein or polypeptide. Such analyses may be carried out in current software applications, such as the Molecular Similarity application of QUANTA (Molecular Simulations Inc., Waltham, Mass.) version 3.3, and as described in the accompanying User's Guide, Volume 3 pages. 134-135. The Molecular Similarity application permits comparisons between different structures, different conformations of the same structure, and different parts of the same structure. Each structure is identified by a name. One structure is identified as the target (i.e., the fixed structure); all remaining structures are working structures (i.e., moving structures). When a rigid fitting method is used, the working structure is translated and rotated to obtain an optimum fit with the target structure.

The person skilled in the art may use one of several methods to screen chemical entities or fragments for their ability to associate with a target. The screening process may begin by visual inspection of the target on the computer screen, generated from a

machine-readable storage medium. Selected fragments or chemical entities may then be positioned in a variety of orientations, or docked, within that binding pocket.

Useful programs to aid the person skilled in the art in connecting the individual chemical entities or fragments include: CAVEAT (P. A. Bartlett et al, "CAVEAT: A Program to Facilitate the Structure-Derived Design of Biologically Active Molecules". In Molecular Recognition in Chemical and Biological Problems", Special Pub., Royal Chem. Soc., 78, pp. 182-196 (1989)). CAVEAT is available from the University of California, Berkeley, California. 3D Database systems such as MACCS-3D (MDL Information Systems, San Leandro, California). This is reviewed in Y. C. Martin, "3D Database Searching in Drug Design", J. Med. Chem., 35, pp. 2145-2154 (1992); and HOOK (available from Molecular Simulations, Burlington, Mass.). These citations are incorporated by reference.

Once the molecule has been optimally selected or designed, as described above, substitutions may then be made in some of its atoms or side groups in order to improve or modify its binding properties. Generally, initial substitutions are conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. The computational analysis and design of molecules, as well as software and computer systems therefore are described in US Patent No 5,978,740 which is included herein by reference.

In a further preferred method of the invention said molecule is modified to alter its binding affinity and/or specificity for said polypeptide.

According to a further aspect of the invention there is provided a method of treatment of an animal comprising administering a ligand or composition according to the invention to said animal in need of treatment from a thrombotic disease or a condition that may result in thrombus formation.

In a preferred method of the invention said animal is a human.

Ligands and compositions according to the invention are useful in situations where thrombosis might be expected eg, after vascular engraftment, endarterectomy or balloon catheterization. Ligands and compositions may also be useful in coronary heart disease, peripheral vascular disease, cerebrovascular disease, prevention of transient ischemic

attacks, stroke, myocardial infarction, angina, coronary artery stent closure, coronary artery angioplasty, and atherectomy.

According to a further aspect of the invention there is provided a polypeptide comprising an amino acid sequence or part thereof, selected from the group consisting of:

i) GRLRSLDSGIRRLE; or

ii) CKGRHEDESRTVLH.

In a preferred embodiment of the invention said polypeptide is 9-30 amino acids in length.

In a preferred embodiment of the invention said polypeptide is 9-18 amino acids in length; preferably 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 amino acids in length.

According to a further aspect of the invention there is provided a composition comprising a polypeptide according to the invention and optionally a carrier or adjuvant.

An adjuvant is a substance or procedure that augments specific immune responses to antigens by modulating the activity of immune cells. Examples of adjuvants include, by example only, Freunds adjuvant, muramyl dipeptides, liposomes. A carrier is an immunogenic molecule which, when bound to a second molecule, augments immune responses to the latter.

In a further aspect of the invention there is provided a method for preparing a hybridoma cell-line producing monoclonal antibodies according to the invention comprising the steps of:

i) immunising an immunocompetent mammal with an immunogen comprising at least one polypeptide according to the invention;

ii) fusing lymphocytes of the immunised immunocompetent mammal with myeloma cells to form hybridoma cells;

iii) screening monoclonal antibodies produced by the hybridoma cells of step (ii) for binding activity to the amino acid sequences of (i);

iv) culturing the hybridoma cells to proliferate and/or to secrete said monoclonal antibody; and

v) recovering the monoclonal antibody from the culture supernatant.

Preferably, the said immunocompetent mammal is a mouse. Alternatively, said immunocompetent mammal is a rat.

The production of monoclonal antibodies using hybridoma cells is well-known in the art. The methods used to produce monoclonal antibodies are disclosed by Kohler and Milstein in Nature 256, 495-497 (1975) and also by Donillard and Hoffman, "Basic Facts about Hybridomas" in Compendium of Immunology V.II ed. by Schwartz, 1981, which are incorporated by reference.

According to a further aspect of the invention there is provided a hybridoma cell-line formed by the method of the invention.

According to a further aspect of the invention there is provided a hybridoma cell-line that produces monoclonal antibodies that specifically bind the polypeptide according to the invention.

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

An embodiment of the invention will now be described by example only and with reference to the following figures:

Figure 1: G6B is expressed on the surface of platelets. A: Relative expression of the G6B splice forms G6B-A (panel A), G6B-B (panel B), G6B-D (panel C) and G6B-E

(panel 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 is representative of three individual experiments. Panel E: Western blot analysis of G6B expression in (1) Whole Buffy coat, (2) $CD4^+$ T cells, (3) $CD8^+$ T cells, (4) $CD14^+$ Monocytes, (5) $CD16^+$ Granulocytes, (6) $CD19^+$ B cells, (7) Platelet Rich Plasma. 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 to lanes 1-6. Gel was visualised with a G6B mAb and detected with a goat anti-mouse HRP conjugate as described. Size standards are indicated to the right of the gel. Panels F, G: Flow cytometry of platelets showing surface expression, using affinity purified G6B mAb (solid line) and isotype control (dotted line) and platelets alone (Dashed line). Panel F shows expression on washed resting platelets gated on the CD41 positive population and Panel G shows ADP activated platelets gated on the CD62-P positive population.

Figure 2: G6B cross-linking inhibits platelet aggregation in a Ca2+ independent manner. (A) SHP-1 co-immunoprecipitates with G6B in platelets after stimulation with G6B anti-sera. G6B was co-immunoprecipitated with SHP-1 and is tyrosine phosphorylated after incubation of platelets with G6B anti-sera (lane 2). G6B was absent when platelets were incubated with pre-immune sera (lane 3), or nothing (lane 1). SHP-1 co-immunoprecipitation was equivalent for all conditions. 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 and the Red line represents agonist and pre-immune sera. 1 corresponds to the addition of 5 µl of either the Pre-immune sera or the anti-G6B polyclonal; 2 corresponds to addition of either CRP-XL (final concentration 0.1 µg/ml) or ADP (final concentration 5µM). 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 pre-immune sera. The number of repeats for each condition is indicated in the brackets. (E) Calcium flux in the presence of ADP and G6B polyclonal (Blue) or Pre-immune sera (Red). The arrows indicate the addition of the anti-sera (1) and agonist (2). (F) As for panel B but utilising 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 brackets.

Figure 3 is the nucleotide sequence of genomic G6B;

Figure 4a is the nucleotide sequence of G6B transcript variant 1 (G6B isoform G6B-A);
Figure 4b is the nucleotide sequence of G6B transcript variant 2 (G6B isoform G6B-B);
Figure 4c is the nucleotide sequence of G6B transcript variant 3 (G6B isoform G6B-C);
Figure 4d is the nucleotide sequence of G6B transcript variant 4 (G6B isoform G6B-D);
Figure 4e is the nucleotide sequence of G6B transcript variant 5 (G6B isoform G6B-E);
Figure 4f is the nucleotide sequence of G6B transcript variant 6 (G6B isoform G6B-F);
Figure 4g is the nucleotide sequence of G6B transcript variant 7 (G6B isoform G6B-G);

Figure 5a is the amino acid sequences of G6B isoform G6B-A; Figure 5b is the amino acid sequences of G6B isoform G6B-B; Figure 5c is the amino acid sequences of G6B isoform G6B-C; Figure 5d is the amino acid sequences of G6B isoform G6B-D; Figure 5e is the amino acid sequences of G6B isoform G6B-E; Figure 5f is the amino acid sequences of G6B isoform G6B-F; Figure 5g is the amino acid sequences of G6B isoform G6B-G;

Figure 6: Characterisation of anti-G6B polyclonal and monoclonal antibodies. The specificity of the G6B polyclonal anti-sera was evaluated by Western blot analysis (A). Cell lysate of T7-tagged G6B transfected cells was probed using either an anti-T7 HPR-conjugated monoclonal antibody (lane 1), G6B polyclonal (lane 2), or pre-immune sera (lane3). Hybridomas raised against G6B were screened against soluble G6B-Fc fusion proteins by Western blot analysis (B). Clones 8a5, 2h3 and sub-clone 3.2 all produced a band of the correct size (45 kDa) expected for the G6B-Fc fusion. Flow cytometry of G6B transfected cells evaluating the specificity of polyclonal (C) and monoclonal antibodies (D). In each case, the Dotted line shows untransfected cells, the Dashed line shows G6B-transfected cells incubated with isotype control/pre-immune sera, Dotted and Dashed line shows non-G6B transfected cells incubated with the polyclonal and monoclonal antibodies, while the Solid line shows G6B-transfected cell incubated with the anti-G6B polyclonal and monoclonal reagents;

Figure 7: Confocal microscopy of platelets showing surface expression, using purified anti-G6B mAb (Green, panel A) and isotype control (green, panel B). Both panels are counter stained with the platelet marker CD41 (red).

Figure 8 G6B polyclonal anti-sera attenuates platelet aggregation in a dose dependent manner. The dose response curve of G6B anti-sera effect on ADP-induced platelet aggregation was produced by 2-fold serial dilutions of G6B anti-sera in HBS, expressed as a function of percentage maximum inhibition. Graph shows two repeats of two individual donors. Error bars show standard deviation; and

Table 1 summarises PCR primers used in real time PCR analysis of G6B expression.

Materials and Methods

RNA Extractions & QPCR

Total RNA from isolated cells was extracted using an RNEasy kit (Qiagen, Crawley, UK). Total RNA from spleen was purchased from Stratagene (Stratagene Inc, La Jolla, CA). cDNA was synthesised from approximately 1 µg of RNA using an Oligo (dT) primed reverse transcription system (Promega, Southampton, UK). Splice form-specific primers were designed for G6B-A, G6B-B, G6B-D and G6B-E (Table 1) and β2-microglobulin was used as an internal control as previously described[13].

Generation and characterisation of G6B monoclonal and polyclonal antibodies

Polyclonal antibodies against recombinant G6B expressed in E coli were raised in rabbits (Eurogentec, Liege, Belgium). Monoclonal antibodies specific to the G6B peptides GRLRSLDSGIRRLE or CKGRHEDESRTVLH were raised in mice (Moravian Biotech, Brno, Czech Republic) and purified on a HiTrap protein G column (Amersham, Buckinghamshire, UK). Specificity of anti-G6B reagents was determined by Western blot (Figure 6A and B) and flow cytometry (Figure 6 C and D) analysis using epitope tagged G6B transfected cell lines and soluble Fc fusion proteins.

Cell Separations

PBMC were prepared from whole blood collected in 4% sodium citrate by centrifuging over Ficol. $CD4^+$, $CD8^+$, $CD14^+$, $CD16^+$ and $CD19^+$ cells were isolated by positive selection using specific magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Platelet rich plasma (PRP) was collected as previously described[2] and $CD45^+$ cells depleted using 3 rounds of $CD45^+$ Dynabead selection (Invitrogen, Paisley, UK).

Confocal Microscopy

To evaluate G6B expression on the surface of platelets, washed platelets were stained with anti-G6B monoclonal antibody for 1 hour on ice followed by an anti-mouse FITC conjugate (Jackson ImmunoResearch, Bar Harbor, ME) as shown in Figure 7. As a platelet counter-stain, an anti-CD41-PE conjugate (BD Biosciences, San Jose, CA) was used. As isotype control platelets were incubated with mouse IgG1k (Sigma-Aldrich, Poole, UK) and an anti-mouse FITC conjugate. Platelets were viewed on a Zeiss LSM510 META Confocal Microscope using a "Plan-Apochromat" 100x/1.40 Oil DIC objective lens and LSM5 image capture software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were processed using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

QPCR

QPCR reactions were performed using the SensiMix DNA kit (Quantace Ltd, Finchley, UK) 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 two standard deviations above the median expression level across all cell types tested.

Western blotting

Approximately $1x10^5$ cells and $1x10^7$ platelets were lysed and separated on a 15% SDS PAGE gel, transferred and visualised using the purified mouse anti-G6B monoclonal detected with a goat anti-mouse HRP conjugate (Sigma-Aldrich, Poole, UK) as described previously[2].

Flow cytometry

Resting or activated platelets were incubated with an anti-G6B monoclonal antibody or appropriate isotype control (mouse IgG1κ) for 1 hour followed by an anti-mouse FITC conjugate (Jackson ImmunoResearch, Bar Harbor, ME). Resting and activated platelets were counter stained with an anti-CD41-PE conjugate (BD Biosciences, San Jose, CA) or an anti-CD62-PE monoclonal antibody, respectively. G6B expression was then measured on a FACSCalibur flow cytometer (Becton Dickinson) and analysed using the EXPO32 ADC analysis software (Beckman Coulter, High Wycombe, UK).

Immunoprecipitations

Co-immunoprecpitations were performed essentially as described by Senis et al[6]. Briefly, platelet lysates were co-immunoprecipitated using a rabbit anti-human SHP-1 polyclonal antibody (Upstate Biotechnology, NY) after incubation with G6B anti-sera, pre-immune sera or buffer alone. After Western blotting the membrane was incubated with an anti-phophotyrosine monoclonal antibody (Upstate Biotechnology, NY), stripped and re-probed with an anti-SHP-1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or the anti-G6B monoclonal antibody.

Platelet function studies

Platelet aggregation studies were performed as previously described[7]. Platelets were incubated with 5 µl of G6B polyclonal anti-sera or pre-immune serum for 30 seconds before addition of either ADP (final concentration 5 µM), Collagen Related Peptide (CRP-XL) (final concentration 0.1 µg/ml) or Ionomycin (400 nM). The end point aggregation was expressed as a function of percentage total aggregation with agonist alone. Statistical significance was determined using an unpaired two tailed t-test. Ca^{2+} flux was measured using a Cairn Research Spectrophotometer as described by Melendez et al[8] following the same activation procedures as for the aggregometry.

EXAMPLES

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 1 B). No significant expression was observed in any of the other leukocyte samples analysed. The other cell surface isoform G6B-A, as well as the two secreted isoforms G6B-D and G6B-E, were also expressed in platelets (Figure 1A, C, D). The presence of the G6B isoforms in spleen is most likely due to the presence of platelets as the RNA used was total spleen which will contain platelets.

G6B protein expression was examined in peripheral blood leukocyte preparations using a mAb specific to G6B. In agreement with the mRNA data, the greatest expression was observed in platelets (Figure 1E, lane 7). 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 either $CD4^+$ or $CD8^+$ T cells, (Figure 1E, lanes 2 and 3), or in $CD19^+$ B cells (Figure 1E, lane 6). $CD14^+$ monocytes (Figure 1E, lane 4) and $CD16^+$ neutrophils (Figure 1E, lane 5) contain a band of ~20 kDa, which may be immature forms of G6B-D and G6B-E. However, this does not correlate with the mRNA data. Consistent with this observation, in a recent study Macaulay et al[9] demonstrated G6B expression in the megakaryocyte, a platelet precursor.

G6B expression on the surface of platelets was assessed by flow cytometry and confocal microscopy (Figure 1 F and G, Figure 7). G6B expression is detected on resting CD41+ platelets (Figure 1F, Figure 7) and is increased approximately two fold following activation by ADP (Figure 1G).

Cross-linking G6B inhibits platelet function

G6B has previously been shown to associate with SHP-1 in an over-expressed cell line model[2]. This association is important for the inhibitory signalling expected 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). Washed platelets were incubated with buffer alone (lane 1), G6B anti-sera (lane 2) or pre-immune sera (lane 3) and co-immunoprecipitated with an anti-SHP-1 antibody. Association of phosphorylated G6B with SHP-1 was only observed after incubation with the G6B polyclonal anti-sera (Figure 2A, lane 2).

To determine whether signalling 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 prior to activating platelets with the agonist ADP led to a significant reduction in platelet aggregation in a dose dependent manner (Figure 2 C and D, Figure 8), with the end point aggregation being reduced from 100% to 22% ($p<0.0001$). Similarly, cross-linking of G6B prior to activation with CRP-XL led to a 2 fold reduction in platelet aggregation ($p< 0.0001$) (Figure 2 B and 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. Pre-incubation with G6B anti-sera had no effect on Ca^{2+} flux following treatment with agonist (Figure 2 E). 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 the calcium ionophore, ionomycin[10]. Normal aggregation is observed when pre-immune sera is used. However, in the presence of the G6B polyclonal antibody a 2 fold (p=0.0007) inhibition of aggregation is observed (Figure 2 F and H). Figure 2 G shows the corresponding calcium flux in platelets after ionomycin incubation. Again, there is no significant difference between G6B anti-sera and pre-immune sera, suggesting that G6B has an inhibitory effect downstream from initial platelet activation and mobilisation of intracellular calcium stores.

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 these particular cell populations might be good targets for the expression of G6B's protein ligand.

Prior to this study the only described ITIM containing proteins expressed in platelets are Platelet/Endothelial Cellular Adhesion Molecule 1 (PECAM-1) and Trem-Like Transcript 1(TLT-1). However, they appear quite different to G6B in that they either have a distribution across other tissue types as in the case of PECAM-1[11] or they function in an activatory rather than an inhibitory fashion as in the case of TLT-1[12]. In light of these observations G6B represents a novel inhibitory molecule found on the surface of platelets and could be a potential anti-thrombotic drug target.

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Claims

1. A ligand wherein the ligand binds to and modulates the activity of a G6B polypeptide for use as a pharmaceutical.

2. A ligand according to claim 1 wherein the ligand binds to and modulates the activity of a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f, or 4g;

ii) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g;

iii) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet.

3. A ligand according to claim 1 wherein the ligand binds to and modulates the activity of a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g, or a variant polypeptide wherein said variant polypeptide comprises an amino acid sequence that is modified by addition, deletion or substitution of at least one amino acid residue with reference to the amino acid sequences presented in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g and which retains the activity associated with a G6B polypeptide.

4. A ligand according to any of claims 1-3 wherein said ligand is an antibody, or an active binding fragment of an antibody.

5. A ligand according to claim 4 wherein said antibody, is a monoclonal antibody.

6. A ligand according to claim 4 wherein said antibody fragment is selected from the group consisting of: Fab; Fab_2; $F(ab')_2$; Fv; Fc; Fd; single chain antibody variable region fragment; a domain fragment.

7. A ligand according to claim 6 wherein said antibody fragment is a single chain antibody variable region fragment.

8. A ligand according to any of claims 4-7 wherein said antibody fragment binds a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g.

9. A ligand according to any of claims 4-8 wherein said antibody, or binding fragment thereof, is a chimeric antibody.

10. A ligand according to any of claims 4-8 wherein said antibody, or binding fragment thereof, is a humanised antibody

11. A ligand according to any of claims 4-8 wherein said antibody or antibody fragment is a human antibody.

12. A ligand according to any of claims 1-3 wherein said ligand is a peptide.

13. A ligand according to claim 12 wherein said peptide is a modified peptide.

14. A ligand according to any of claims 1-3 wherein said ligand is an aptamer.

15. A pharmaceutical composition comprising at least one ligand according to any of claims 1-14.

16. A pharmaceutical composition comprising a ligand according to any of claims 1-14 and at least one further anti-thrombotic agent.

17. A composition according to claim 16 wherein said further anti-thrombotic agent is selected from the group consisting of: Aspirin (acetylsalicylic acid), Ticlid (ticlodipine), Plavix (clopidogrel), Pletal (cilostazol), Persantine (dipyridamole), Anturane (sulfinpyrazone), and 3 intravenous agents: Rheopro (abciximab), Integrilin (eptifibatide), Aggrastat (tirofiban), heparin and warfarin.

18. A screening method for the identification of ligands which bind a polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f, or 4g;

b) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g;

c) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet; comprising the steps of

i) forming a preparation comprising the polypeptide and a ligand to be tested;

ii) testing the binding of said ligand for said polypeptide; and

iii) optionally testing the activity of the ligand with respect to the activation of platelets.

19. A method according to claim 18 wherein said ligand is an antagonist.

20. A method according to claim 18 wherein said ligand is an agonist.

21. A method according to any of claims 18-20 wherein said polypeptide comprises an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g.

22. A method according to any of claims 18-21 wherein said method comprises transfecting a cell with a vector that includes a nucleic acid molecule that is adapted to be operably linked to an expression control sequence selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f, or 4g;

ii) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g;

iii) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet and contacting the cell expressing said nucleic acid molecule with an agent to be tested.

23. A method according to claim 22 wherein said cell is part of a non-human transgenic animal and said animal is administered said ligand to test for agonistic or antagonistic activity.

24. A method according to any of claims 18-23 wherein said ligand is an antibody, or a binding fragment thereof.

25. A method according to any of claims 18-23 wherein said ligand is a peptide, or modified peptide.

26. A method according to any of claims 18-23 wherein said ligand is an aptamer.

27. A non-human transgenic animal wherein said animal is modified to include a nucleic acid molecule selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 3, 4a, 4b, 4c, 4d, 4e, 4f, or 4g;

ii) a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g;

iii) a nucleic acid molecule which hybridises under stringent hybridisation conditions to a nucleic acid molecule as defined in (i) above and which encodes a polypeptide expressed in a platelet.

28. A method to determine the ability of a molecule to associate with a polypeptide comprising the steps of:

i) providing computational means to perform a fitting operation between said molecule and a polypeptide defined by the amino acid sequence in Figure 5a, 5b, 5c, 5d, 5e, 5f, or 5g; and

ii) analysing the results of said fitting operation to quantify the association between the molecule and the polypeptide.

29. A method according to claim 28 wherein said molecule is further tested for the inhibitory activity with respect to platelet activation.

30. A method according to claim 28 or 29 wherein said molecule is modified to alter its binding affinity and/or specificity for said polypeptide.

31. A method of treatment of an animal comprising administering a ligand or composition according to any of claims 1-17 to said animal in need of treatment from a thrombotic disease or a condition that may result in thrombus formation.

32. A method according to claim 31 wherein said animal is a human.

33. A composition comprising a ligand according to any of claims 1-14.

34. A polypeptide comprising an amino acid sequence or part thereof, selected from the group consisting of:

i) CRLRSLDSGIRRLE; or

ii) CKGRHEDESRTVLH.

35. A polypeptide according to claim 34 wherein said polypeptide is 9-30 amino acids in length.

36. A polypeptide according to claim 34 or 35 wherein said polypeptide is 9-18 amino acids in length.

37. A polypeptide according to claim 36 wherein said polypeptide is preferably 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 amino acids in length.

38. A composition comprising a polypeptide according to any of claims 34-37 and optionally a carrier or adjuvant.

39. A method for preparing a hybridoma cell-line producing monoclonal antibodies comprising the steps of:

i) immunising an immunocompetent mammal with an immunogen comprising at least one polypeptide according to any of claims 34-37;

ii) fusing lymphocytes of the immunised immunocompetent mammal with myeloma cells to form hybridoma cells;

iii) screening monoclonal antibodies produced by the hybridoma cells of step (ii) for binding activity to the polypeptide of (i);

iv) culturing the hybridoma cells to proliferate and/or to secrete said monoclonal antibody; and

v) recovering the monoclonal antibody from the culture supernatant.

40. A hybridoma cell-line formed by the method according to claim 39 or 40.

41. A hybridoma cell-line that produces a monoclonal antibody that specifically bind the polypeptide according to any of claims 34-37.

42. A monoclonal antibody or binding fragment thereof that binds a polypeptide according to any of claims 34-37.

43. A monoclonal antibody according to claim 42 wherein the antibody fragment is selected from the group consisting of: Fab; Fab_2; $F(ab')_2$; Fv; Fc; Fd; single chain antibody variable region fragment; a domain fragment.

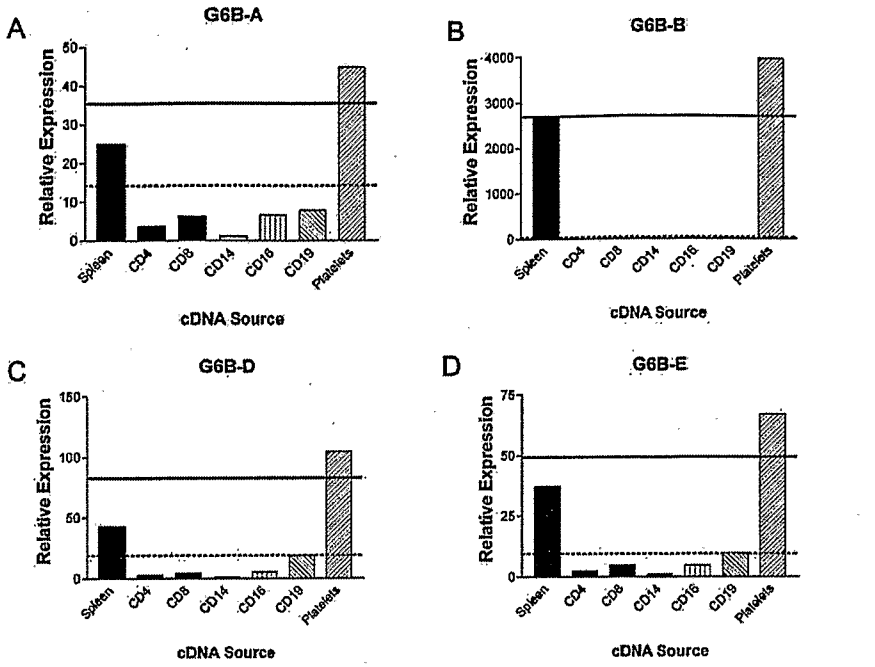
44. A monoclonal antibody according to claim 43 wherein said antibody fragment is a single chain antibody variable region fragment.

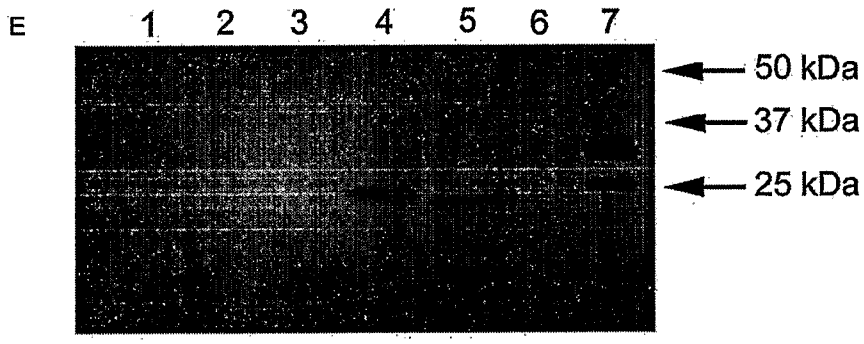
45. A monoclonal antibody according to any of claims 42-44 wherein said antibody, or binding fragment thereof, is a chimeric antibody.

46. A monoclonal antibody according to any of claims 42-44 wherein said antibody, or binding fragment thereof, is a humanised antibody.

47. A monoclonal antibody according to any of claims 42-44 wherein said antibody or antibody fragment is a human antibody.

Figure 1


A
G6B-A
Relative Expression
0
10
20
30
40
50
Spleen
CD4
CD8
CD14
CD16
CD19
Platelets
cDNA Source
B
G6B-B
Relative Expression
0
1000
2000
3000
4000
Spleen
CD4
CD8
CD14
CD16
CD19
Platelets
cDNA Source
C
G6B-D
Relative Expression
0
50
100
150
Spleen
CD4
CD8
CD14
CD16
CD19
Platelets
cDNA Source
D
G6B-E
Relative Expression
0
25
50
75
Spleen
CD4
CD8
CD14
CD16
CD19
Platelets
cDNA Source


E
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3
4
5
6
7
50 kDa
37 kDa
25 kDa

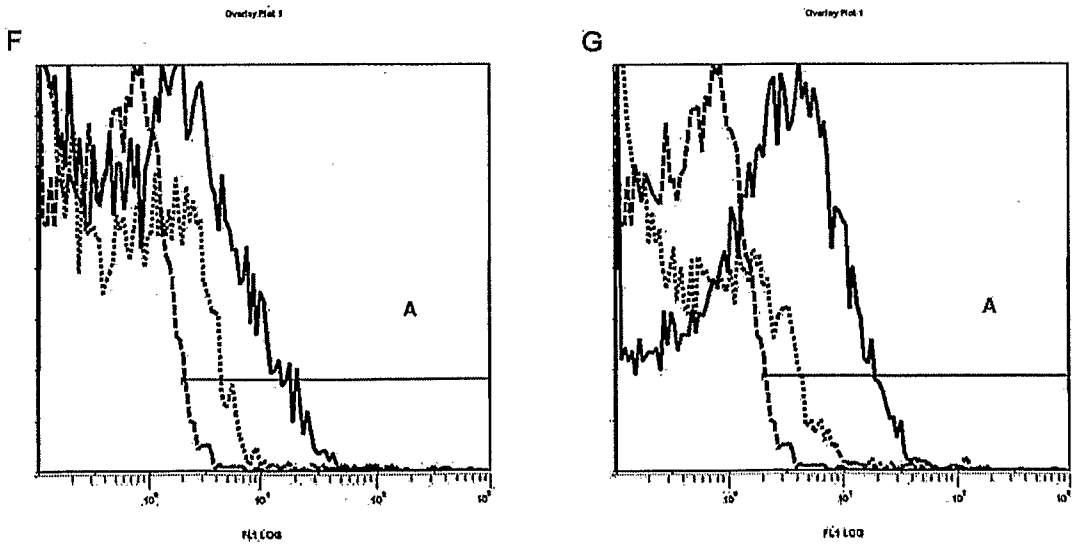

F
A
G
A

Figure 2

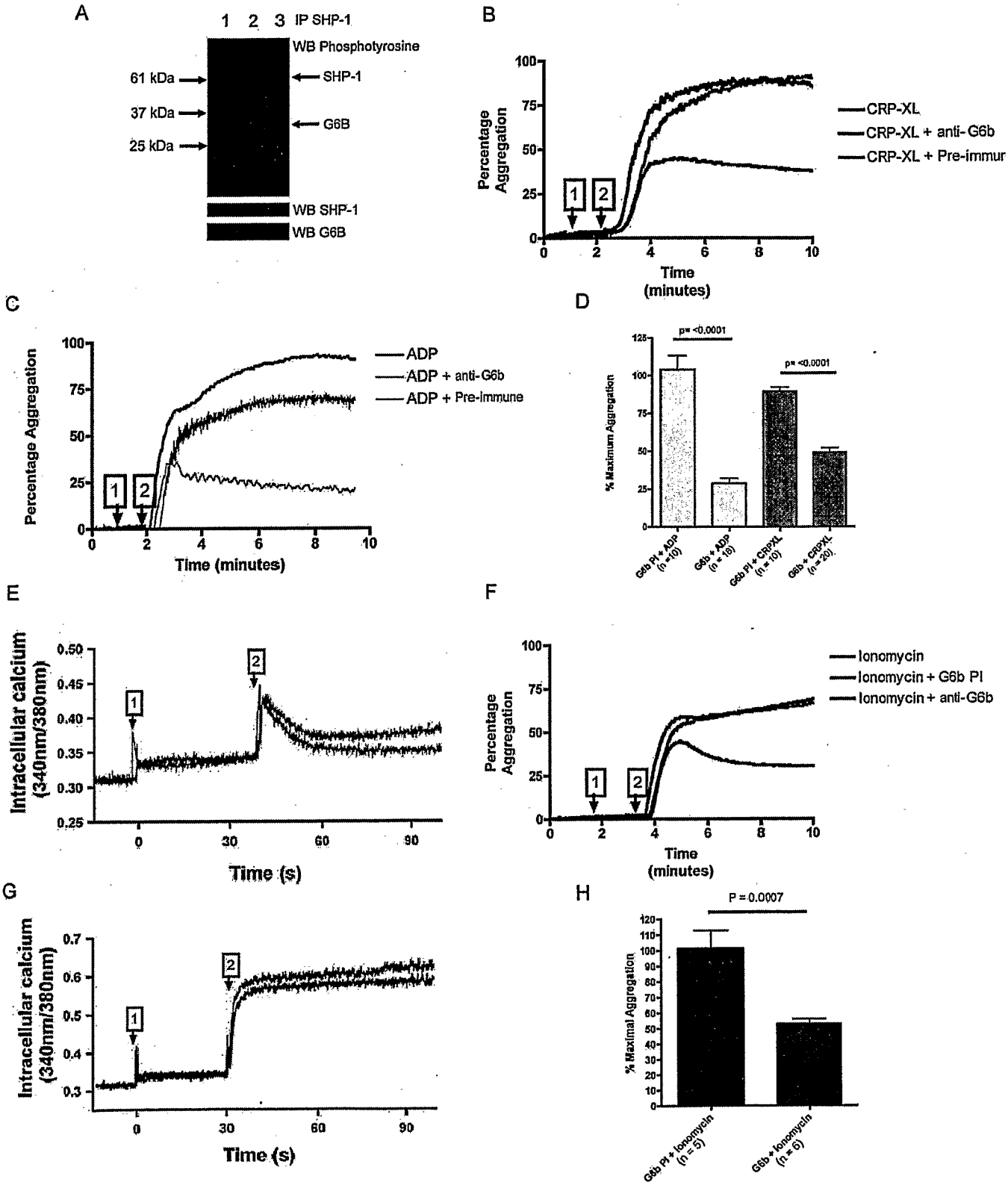

A
1 2 3 IP SHP-1
WB Phosphotyrosine
61 kDa
37 kDa
25 kDa
SHP-1
G6B
WB SHP-1
WB G6B
B
Percentage Aggregation
Time (minutes)
CRP-XL
CRP-XL + anti-G6b
CRP-XL + Pre-immur
C
Percentage Aggregation
Time (minutes)
ADP
ADP + anti-G6b
ADP + Pre-immune
D
% Maximum Aggregation
p= <0.0001
p= <0.0001
G6b PI + ADP (n =10)
G6b + ADP (n = 16)
G6b PI + CRPXL (n = 10)
G6b + CRPXL (n = 20)
E
Intracellular calcium (340nm/380nm)
Time (s)
F
Percentage Aggregation
Time (minutes)
Ionomycin
Ionomycin + G6b PI
Ionomycin + anti-G6b
G
Intracellular calcium (340nm/380nm)
Time (s)
H
P = 0.0007
% Maximal Aggregation
G6b PI + Ionomycin (n = 5)
G6b + Ionomycin (n = 6)

Figure 3 Genomic sequence of G6B
From 24998 - 27095

24901 ggtgccttcc tcttccaggt gaggaaccaa ggaggggaga gggattatgg ggagctggga
24961 tcccctagct tgggaggttt ggagtaaggg cttcccttca aactacaact gcatagatgg
25021 tggaggcatc agcagggtcc gctgtggaca gccggcgggg cctgctgagg gctagatggt
25081 ccagatccgc atagagcagg ctctggagga ggaaggggag gaagacaaga tggtctccac
25141 agtcttcctt ggggttcagg cctttttttt tttggtccat tttcctcaga gattctggct
25201 atccccttcc catccccatg cccttaccgg ttcctggtcc aggtcccctg gaatcttggg
25261 ctcttcctcc tttactggcc tctggggctc ggttttcaca agtggagcta tgtgggggag
25321 acagagctga aggatggaga gaaggtgacc aacgtgtctg ctcccagcaa gggctcagac
25381 tcaggaattg aggggtgggg agtgggcatg taggagaaag aaaatggggt ggaattagtc
25441 tcaccaaatc tagggagtgg tcgaatcggt tgcgggggca ggcgcctgta agggacatat
25501 gagtctttga gaaccaccag tcttgtctca ctggttctcc tgggactcgc cacgccaagg
25561 acagcccagc aacttgaagg ctcagaccgg aaacaactta gactctaccc caccgacagt
25621 ccagcccagg ctctaactct gctgcacttc cagctcatct gctctggtgg ggctctactc
25681 accagccagc ggcctcgctc ccacctttga cctctcccat tcttcgccaa gccaggacca
25741 gagctctagg tctagctctt tcttttgcga aggttctggt cctgcccctt ccacctctgg
25801 tcactcccca tttaacgagg ccgggtccct cctgctcacc tgtgcagcca ccagaccagg
25861 cccaaagctc ccagtccgag caccaaccca gcgcccagca gcgggatcag gagctgggga
25921 tacacggacc ctgggggaaa ggcgtggcgg ggagggatgc tgaggtcagg ggtttgacga
25981 gggacagcca gcagtcccgc tcaagaaccc ccagccaagg ccctcttccc tcctccgggt
26041 atgtgtcgga gttaagtacc cttgtgcgca caggcctgca cctaccatgg gtaggcccgg
26101 gggccttgca ataggtcctg tcccccagca cgtgaagcac tgtacggctc tcgtcctcgt
26161 ggcggccctt gcagaaaaaa gtgcccgagt ctcccgcgct caagaggagc tccagccgcc
26221 ggataccaga gtccagggag cgtaggcggc cgacgaaagg ctggagggga ggcacggtgg
26281 gggtcccgct cgaagaggcc cacaggatcg gtcggcgtcc tttggacagg cccttgcagg
26341 ccgggaagct gggtgcccag acccagcgga tgggatgaga gactcctccg caggagagat
26401 tcacccggtc cccagggcgg ccgtccagag aagctaggag aggccggagg ctgtggttag
26461 ggcacgtcca cagggcaaga cgaccctcca tcccgtctcc tctttctctt ctctgttctg
26521 cactccagcc atcctggatc ttcccacaca caacccaccc tactaggcgg cccttatctt
26581 gcttcagccc ctctgcgtct atcacaactc tcccagggat cgcttacccc cagggttccc
26641 ttgggccctc gagagcagca gcggtagcag ctgcagaaac acagccatgg ttaggatgtg
26701 gtgaggagaa gctgcgagcg aatcagcggc gtggggccgg ttgggggggg accggaggga
26761 agttaggcgt ggggagaacg gaggggctgg ggggctccga taagagaggg ggcggggcca
26821 ccagctgccc agatcccttt gggaatcctc gcaggcaaac tcggagtctt caaatggttc
26881 tcacttaaaa tgtgtgctcg ggccatttac acaaatgctt tggcctgctt aactttttcag
26941 gtgcgcctgt aatcccagct acttaggagg ctgaggaggg aggatcgctt gagctcagga
27001 ggtcgaggtt gcagtgaact gtgatcaggc cattgcactc cagcctgggc gacagagcca
27061 gccagaccct gtctcaaaaa caacaacaac aaaaactcct gttttgtagg ggattgtgat

Figure 4a

>gi|19913372|ref|NM_025260.2| Homo sapiens chromosome 6 open reading frame 25 (C6orf25), transcript variant 1, mRNA

ATGGCTGTGTTTCTGCAGCTGCTACCGCTGCTGCTCTCGAGGGCCCAAGGGAACCCTGGGGCTTCTCTGG
ACGGCCGCCCTGGGGACCGGGTGAATCTCTCCTGCGGAGGAGTCTCTCATCCCATCCGCTGGGTCTGGGC
ACCCAGCTTCCCGGCCTGCAAGGGCCTGTCCAAAGGACGCCGACCGATCCTGTGGGCCTCTTCGAGCGGG
ACCCCCACCGTGCCTCCCCTCCAGCCTTTCGTCGGCCGCCTACGCTCCCTGGACTCTGGTATCCGGCGGC
TGGAGCTCCTCTTGAGCGCGGGAGACTCGGGCACTTTTTTCTGCAAGGGCCGCCACGAGGACGAGAGCCG
TACAGTGCTTCACGTGCTGGGGGACAGGACCTATTGCAAGGCCCCCGGGCCTACCCATGGGTCCGTGTAT
CCCCAGCTCCTGATCCCGCTGCTGGGCGCTGGGTTGGTGCTCGGACTGGGAGCTTTGGGCCTGGTCTGGT
GGCTGCACAGGCGCCTGCCCCCGCAACCGATTCGACCACTCCCTAGATTTGCTCTGTCCCCCCCACATAG
CTCCACTTGTGAAAACCGAGCCCCAGAGGCCAGTAAAGGAGGAAGAGCCCAAGATTCCAGGGGACCTGGA
CCAGGAACCGAGCCTGCTCTATGCGGATCTGGACCATCTAGCCCTCAGCAGGCCCCGCCGGCTGTCCACA
GCGGACCCTGCTGATGCCTCCACCATCTATGCAGTTGTAGTTTGA

Figure 4b

>gi|19913374|ref|NM_138272.1| Homo sapiens chromosome 6 open reading frame 25 (C6orf25), transcript variant 2, mRNA

ATGGCTGTGTTTCTGCAGCTGCTACCGCTGCTGCTCTCGAGGGCCCAAGGGAACCCTGGGGCTTCTCTGG
ACGGCCGCCCTGGGGACCGGGTGAATCTCTCCTGCGGAGGAGTCTCTCATCCCATCCGCTGGGTCTGGGC
ACCCAGCTTCCCGGCCTGCAAGGGCCTGTCCAAAGGACGCCGACCGATCCTGTGGGCCTCTTCGAGCGGG
ACCCCCACCGTGCCTCCCCTCCAGCCTTTCGTCGGCCGCCTACGCTCCCTGGACTCTGGTATCCGGCGGC
TGGAGCTCCTCTTGAGCGCGGGAGACTCGGGCACTTTTTTCTGCAAGGGCCGCCACGAGGACGAGAGCCG
TACAGTGCTTCACGTGCTGGGGGACAGGACCTATTGCAAGGCCCCCGGGCCTACCCATGGGTCCGTGTAT
CCCCAGCTCCTGATCCCGCTGCTGGGCGCTGGGTTGGTGCTCGGACTGGGAGCTTTGGGCCTGGTCTGGT
GGCTGCACAGGCGCCTGCCCCCGCAACCGATTCGACCACTCCCTAGATTTGCTCCACTTGTGAAAACCGA
GCCCCAGAGGCCAGTAAAGGAGGAAGAGCCCAAGATTCCAGGGGACCTGGACCAGGAACCGAGCCTGCTC
TATGCGGATCTGGACCATCTAGCCCTCAGCAGGCCCCGCCGGCTGTCCACAGCGGACCCTGCTGATGCCT
CCACCATCTATGCAGTTGTAGTTTGA

Figure 4c

>gi|19913376|ref|NM_138273.1| Homo sapiens chromosome 6 open reading frame 25 (C6orf25), transcript variant 3, mRNA

ATGGCTGTGTTTCTGCAGCTGCTACCGCTGCTGCTCTCGAGGGCCCAAGGGAACCCTGGGGCTTCTCTGG
ACGGCCGCCCTGGGGACCGGGTGAATCTCTCCTGCGGAGGAGTCTCTCATCCCATCCGCTGGGTCTGGGC
ACCCAGCTTCCCGGCCTGCAAGGGCCTGTCCAAAGGACGCCGACCGATCCTGTGGGCCTCTTCGAGCGGG
ACCCCCACCGTGCCTCCCCTCCAGCCTTTCGTCGGCCGCCTACGCTCCCTGGACTCTGGTATCCGGCGGC
TGGAGCTCCTCTTGAGCGCGGGAGACTCGGGCACTTTTTTCTGCAAGGGCCGCCACGAGGACGAGAGCCG
TACAGTGCTTCACGTGCTGGGGGACAGGACCTATTGCAAGGCCCCCGGGCCTACCCATGGCGCCTGCCCC
CGCAACCGATTCGACCACTCCCTAGATTTGCTCTGTCCCCCCCACATAGCTCCACTTGTGAAAACCGAGC
CCCAGAGGCCAGTAAAGGAGGAAGAGCCCAAGATTCCAGGGGACCTGGACCAGGAACCGAGCCTGCTCTA
TGCGGATCTGGACCATCTAGCCCTCAGCAGGCCCCGCCGGCTGTCCACAGCGGACCCTGCTGATGCCTCC
ACCATCTATGCAGTTGTAGTTTGA

Figure 4d

>gi|19913378|ref|NM_138274.1| Homo sapiens chromosome 6 open reading frame 25 (C6orf25), transcript variant 4, mRNA

ATGGCTGTGTTTCTGCAGCTGCTACCGCTGCTGCTCTCGAGGGCCCAAGGGAACCCTGGGGCTTCTCTGG
ACGGCCGCCCTGGGGACCGGGTGAATCTCTCCTGCGGAGGAGTCTCTCATCCCATCCGCTGGGTCTGGGC
ACCCAGCTTCCCGGCCTGCAAGGGCCTGTCCAAAGGACGCCGACCGATCCTGTGGGCCTCTTCGAGCGGG
ACCCCCACCGTGCCTCCCCTCCAGCCTTTCGTCGGCCGCCTACGCTCCCTGGACTCTGGTATCCGGCGGC
TGGAGCTCCTCTTGAGCGCGGGAGACTCGGGCACTTTTTTCTGCAAGGGCCGCCACGAGGACGAGAGCCG
TACAGTGCTTCACGTGCTGGGGGACAGGACCTATTGCAAGGCCCCCGGGCCTACCCATGCTCTGTCCCCC
CCACATAGCTCCACTTGTGAAAACCGAGCCCCAGAGGCCAGTAAAGGAGGAAGAGCCCAAGATTCCAGGG
GACCTGGACCAGGAACCGAGCCTGCTCTATGCGGATCTGGACCATCTAGCCCTCAGCAGGCCCCGCCGGC
TGTCCACAGCGGACCCTGCTGATGCCTCCACCATCTATGCAGTTGTAGTTTGA

Figure 4e

>gi|19913380|ref|NM_138275.1| Homo sapiens chromosome 6 open reading frame 25 (C6orf25), transcript variant 5, mRNA

ATGGCTGTGTTTCTGCAGCTGCTACCGCTGCTGCTCTCGAGGGCCCAAGGGAACCCTGGGGCTTCTCTGG
ACGGCCGCCCTGGGGACCGGGTGAATCTCTCCTGCGGAGGAGTCTCTCATCCCATCCGCTGGGTCTGGGC
ACCCAGCTTCCCGGCCTGCAAGGGCCTGTCCAAAGGACGCCGACCGATCCTGTGGGCCTCTTCGAGCGGG
ACCCCCACCGTGCCTCCCCTCCAGCCTTTCGTCGGCCGCCTACGCTCCCTGGACTCTGGTATCCGGCGGC
TGGAGCTCCTCTTGAGCGCGGGAGACTCGGGCACTTTTTTCTGCAAGGGCCGCCACGAGGACGAGAGCCG
TACAGTGCTTCACGTGCTGGGGGACAGGACCTATTGCAAGGCCCCCGGGCCTACCCATGCTCCACTTGTG
AAAACCGAGCCCCAGAGGCCAGTAAAGGAGGAAGAGCCCAAGATTCCAGGGGACCTGGACCAGGAACCGA
GCCTGCTCTATGCGGATCTGGACCATCTAGCCCTCAGCAGGCCCCGCCGGCTGTCCACAGCGGACCCTGC
TGATGCCTCCACCATCTATGCAGTTGTAGTTTGA

Figure 4f

>gi|19913382|ref|NM_138276.1| Homo sapiens chromosome 6 open reading frame 25 (C6orf25), transcript variant 6, mRNA

ATGGCTGTGTTTCTGCAGCTGCTACCGCTGCTGCTCTCGAGGGCCCAAGGGAACCCTGGGGCTTCTCTGG
ACGGCCGCCCTGGGGACCGGGTGAATCTCTCCTGCGGAGGAGTCTCTCATCCCATCCGCTGGGTCTGGGC
ACCCAGCTTCCCGGCCTGCAAGGGCCTGTCCAAAGGACGCCGACCGATCCTGTGGGCCTCTTCGAGCGGG
ACCCCCACCGTGCCTCCCCTCCAGCCTTTCGTCGGCCGCCTACGCTCCCTGGACTCTGGTATCCGGCGGC
TGGAGCTCCTCTTGAGCGCGGGAGACTCGGGCACTTTTTTCTGCAAGGGCCGCCACGAGGACGAGAGCCG
TACAGTGCTTCACGTGCTGGGGGACAGGACCTATTGCAAGGCCCCCGGGCCTACCCATGGGTCCGTGTAT
CCCCAGCTCCTGATCCCGCTGCTGGGCGCTGGGTTGGTGCTCGGACTGGGAGCTTTGGGCCTGGTCTGGT
GGCTGCACAGGCGCCTGCCCCCGCAACCGATTCGACCACTCCCTAGATTTGGTGAGACTAATTCCACCCC
ATTTTCTTTCTCCTACATGCCCACTCCCCACCCCTCAATTCCTGAGTCTGAGCCCTTGCTGGGAGCAGAC
ACGTTGGTCACCTTCTCTCCATCCTTCAGCTCTGTCCCCCCCACATAGCTCCACTTGTGAAAACCGAGCC
CCAGAGGCCAGTAAAGGAGGAAGAGCCCAAGATTCCAGGGGACCTGGACCAGGAACCGAGCCTGCTCTAT
GCGGATCTGGACCATCTAGCCCTCAGCAGGCCCCGCCGGCTGTCCACAGCGGACCCTGCTGATGCCTCCA
CCATCTATGCAGTTGTAGTTTGA

Figure 4g

>gi|19913384|ref|NM_138277.1| Homo sapiens chromosome 6 open reading frame 25 (C6orf25), transcript variant 7, mRNA

ATGGCTGTGTTTCTGCAGCTGCTACCGCTGCTGCTCTCGAGGGCCCAAGGGAACCCTGGGGCTTCTCTGG
ACGGCCGCCCTGGGGACCGGGTGAATCTCTCCTGCGGAGGAGTCTCTCATCCCATCCGCTGGGTCTGGGC
ACCCAGCTTCCCGGCCTGCAAGGGCCTGTCCAAAGGACGCCGACCGATCCTGTGGGCCTCTTCGAGCGGG
ACCCCCACCGTGCCTCCCCTCCAGCCTTTCGTCGGCCGCCTACGCTCCCTGGACTCTGGTATCCGGCGGC
TGGAGCTCCTCTTGAGCGCGGGAGACTCGGGCACTTTTTTCTGCAAGGGCCGCCACGAGGACGAGAGCCG
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CCCCAGCTCCTGATCCCGCTGCTGGGCGCTGGGTTGGTGCTCGGACTGGGAGCTTTGGGCCTGGTCTGGT
GGCTGCACAGGCGCCTGCCCCCGCAACCGATTCGACCACTCCCTAGATTTGCTCTGTCCCCCCCACATAG
CTCCACTTGTGAAAACCGAGCCCCAGAGGCCAGTAAAGGAGGAAGAGCCCAAGATTCCAGGGGACCTGGA
CCAGGAACCGGTAAGGGCATGGGGATGGGAAGGGGATAGCCAGAATCTCTGAGGAAAATGGACCAAAAAA
AAAAAGGCCTGAACCCCAAGGAAGACTGTGGAGACCATCTTGTCTTCCTCCCCTTCCTCCTCCAGAGCCT
GCTCTATGCGGATCTGGACCATCTAGCCCTCAGCAGGCCCCGCCGGCTGTCCACAGCGGACCCTGCTGAT
GCCTCCACCATCTATGCAGTTGTAGTTTGA

Figure 5a

>gi|19913373|ref|NP_079536.2| G6B protein isoform G6b-A precursor [Homo sapiens]
MAVFLQLLPLLLSRAQGNPGASLDGRPGDRVNLSCGGVSHPIRWVWAPSFPACKGLSKGRRPILWASSSG
TPTVPPLQPFVGRLRSLDSGIRRLELLLSAGDSGTFFCKGRHEDESRTVLHVLGDRTYCKAPGPTHGSVY
PQLLIPLLGAGLVLGLGALGLVWWLHRRLPPQPIRPLPRFALSPPHSSTCENRAPEASKGGRAQDSRGPG
PGTEPALCGSGPSSPQQAPPAVHSGPC

Figure 5b

>gi|19913375|ref|NP_612116.1| G6B protein isoform G6b-B precursor [Homo sapiens]
MAVFLQLLPLLLSRAQGNPGASLDGRPGDRVNLSCGGVSHPIRWVWAPSFPACKGLSKGRRPILWASSSG
TPTVPPLQPFVGRLRSLDSGIRRLELLLSAGDSGTFFCKGRHEDESRTVLHVLGDRTYCKAPGPTHGSVY
PQLLIPLLGAGLVLGLGALGLVWWLHRRLPPQPIRPLPRFAPLVKTEPQRPVKEEEPKIPGDLDQEPSLL
YADLDHLALSRPRRLSTADPADASTIYAVVV

Figure 5c

>gi|19913377|ref|NP_612117.1| G6B protein isoform G6b-C precursor [Homo sapiens]
MAVFLQLLPLLLSRAQGNPGASLDGRPGDRVNLSCGGVSHPIRWVWAPSFPACKGLSKGRRPILWASSSG
TPTVPPLQPFVGRLRSLDSGIRRLELLLSAGDSGTFFCKGRHEDESRTVLHVLGDRTYCKAPGPTHGACP
RNRFDHSLDLLCPPHIAPLVKTEPQRPVKEEEPKIPGDLDQEPSLLYADLDHLALSRPRRLSTADPADAS
TIYAVVV

Figure 5d

>gi|19913379|ref|NP_612118.1| G6B protein isoform G6b-D precursor [Homo sapiens]
MAVFLQLLPLLLSRAQGNPGASLDGRPGDRVNLSCGGVSHPIRWVWAPSFPACKGLSKGRRPILWASSSG
TPTVPPLQPFVGRLRSLDSGIRRLELLLSAGDSGTFFCKGRHEDESRTVLHVLGDRTYCKAPGPTHALSP
PHSSTCENRAPEASKGGRAQDSRGPGPGTEPALCGSGPSSPQQAPPAVHSGPC

Figure 5e

>gi|19913381|ref|NP_612119.1| G6B protein isoform G6b-E precursor [Homo sapiens]
MAVFLQLLPLLLSRAQGNPGASLDGRPGDRVNLSCGGVSHPIRWVWAPSFPACKGLSKGRRPILWASSSG
TPTVPPLQPFVGRLRSLDSGIRRLELLLSAGDSGTFFCKGRHEDESRTVLHVLGDRTYCKAPGPTHAPLV
KTEPQRPVKEEEPKIPGDLDQEPSLLYADLDHLALSRPRRLSTADPADASTIYAVVV

Figure 5f

>gi|19913383|ref|NP_612120.1| G6B protein isoform G6b-F precursor [Homo sapiens]
MAVFLQLLPLLLSRAQGNPGASLDGRPGDRVNLSCGGVSHPIRWVWAPSFPACKGLSKGRRPILWASSSG
TPTVPPLQPFVGRLRSLDSGIRRLELLLSAGDSGTFFCKGRHEDESRTVLHVLGDRTYCKAPGPTHGSVY
PQLLIPLLGAGLVLGLGALGLVWWLHRRLPPQPIRPLPRFGETNSTPFSFSYMPTPHPSIPESEPLLGAD
TLVTFSPSFSSVPPT

Figure 5g

>gi|19913385|ref|NP_612121.1| G6B protein isoform G6b-G precursor [Homo sapiens]
MAVFLQLLPLLLSRAQGNPGASLDGRPGDRVNLSCGGVSHPIRWVWAPSFPACKGLSKGRRPILWASSSG
TPTVPPLQPFVGRLRSLDSGIRRLELLLSAGDSGTFFCKGRHEDESRTVLHVLGDRTYCKAPGPTHGSVY
PQLLIPLLGAGLVLGLGALGLVWWLHRRLPPQPIRPLPRFALSPPHSSTCENRAPEASKGGRAQDSRGPG
PGTGKGMGMGRG

Figure 6

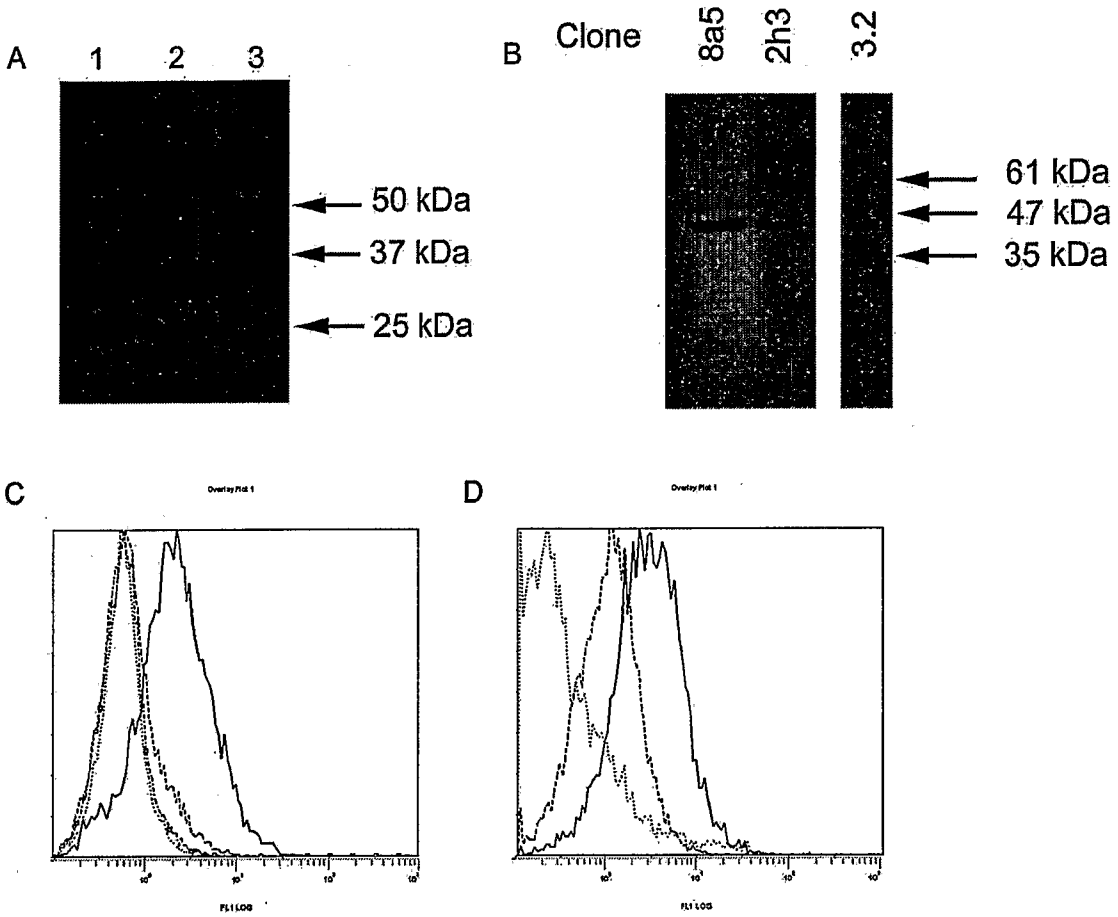

A
1
2
3
50 kDa
37 kDa
25 kDa
B
Clone
8a5
2h3
3.2
61 kDa
47 kDa
35 kDa
C
D

Figure 7

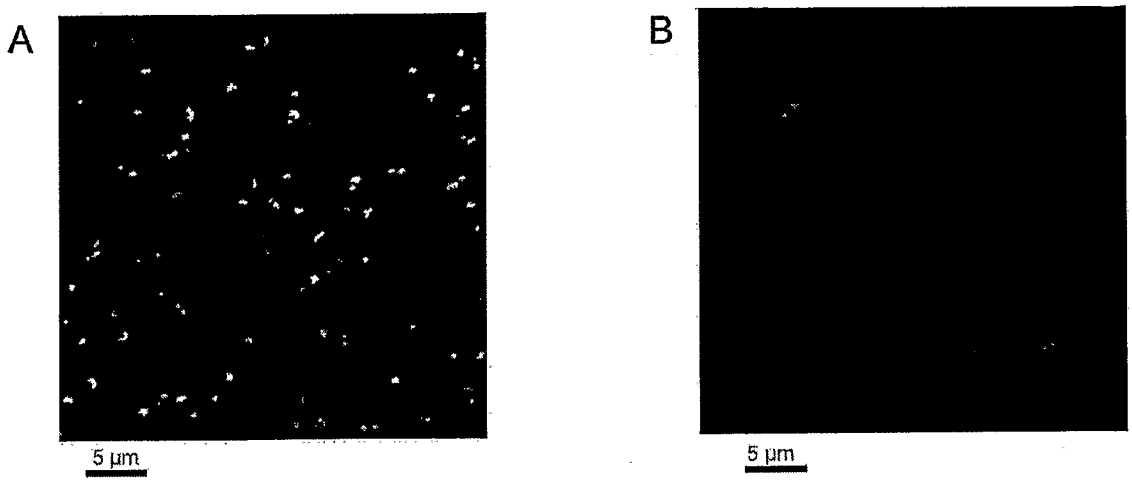


Figure 8

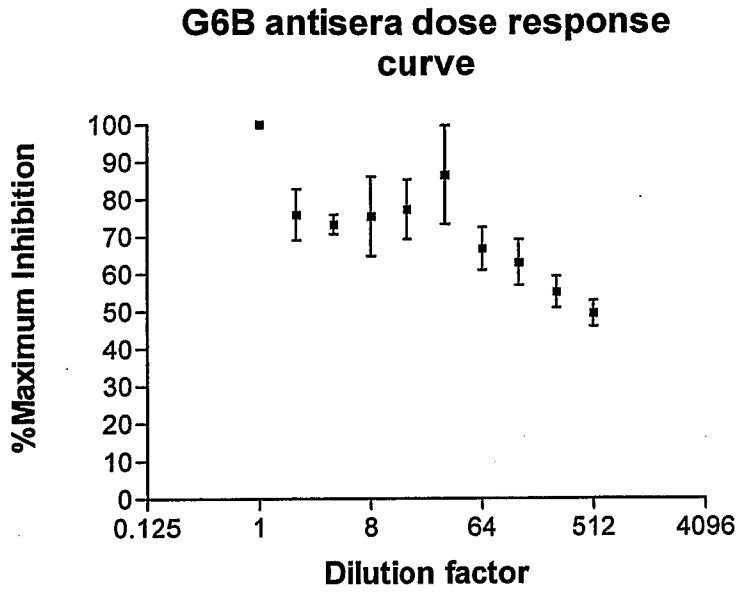

G6B antisera dose response curve
%Maximum Inhibition
100
90
80
70
60
50
40
30
20
10
0
0.125
1
8
64
512
4096
Dilution factor

Table 1: Real Time PCR Primers

Splice Form	Forward Primer	Reverse Primer
G6B – A	ACC CAT GGG TCC GTG TAT CC	GGA GCT ATG TGG GGG GGA CAG A
G6B - B	ACC CAT GGG TCC GTG TAT CC	CGG TTT TCA CAA GTG GAG CAA ATC
G6B - D	T ACC CAT GCT CTG TCC CCC	GGGAGGTTTGGAGTAAGGGC
G6B - E	T ACC CAT GCT CCA CTT GTG	GGGAGGTTTGGAGTAAGGGC