Expression of the Recombinant Soluble Glycoprotein IIb: Lessons Learned and Future Directions

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Abstract: The platelet glycoprotein IIb (GPIIb) is an integral membrane protein, which forms with GPIIIa a calcium-dependent heterodimer. This heterodimer forms in the endoplasmic reticulum (RE) and in the absence of heterodimer formation, both GPIIb and GPIIIa are retained and degraded in the ER. Efforts have been previously made to produce recombinantly the soluble GPIIb, which can be used in the solid phase assays for the antibody identification. However, all previous attempts to produce such a construct have not yield any product. This mini-review describes the previous attempts and the future challenges.

Keywords: GPIIb, Soluble GPIIb, Recombinant GPIIb, Retention signal.

INTRODUCTION

The platelet glycoprotein IIb (GPIIb**)** and IIIa form a calcium-heterodimer (Figure **1**) on the platelet surface that acts as a receptor for fibrinogen and other adhesive proteins [1]. The heterodimer is formed in the endoplasmic reticulum (ER), undergoes N-linked glycosylation, and forms disulfide bonds. The glycoproteins are then transported to the Golgi apparatus through a process of bulk flow where final oligosaccharides processing is completed and cleavage of a single-chain pro-GPIIb into mature GPIIb is finalized comprising a heavy and light chain [2]. Both GPIIb and GPIIIa must be correctly folded and assembled to be successfully expressed as a heterodimer on the platelet cell surface [3]. The production of the recombinant soluble (rs) forms of GPIIb and/or GPIIIa is useful to study the structure and function of the heterodimer and to use them in the solid phase assays to identify the antibodies (Abs) directed against the antigens reside on the heterodimer [4]. The first group investigating the feasibility of producing the rs GPIIIa was Bennett *et al*. (1993) [5]. However, the amount produced was modest, the structural integrity has not been evaluated, and the protein was not demonstrated to be monomeric [6]. Recently, we have demonstrated and for the first time the feasibility of producing the rs GPIIIa in a large scale using the lentiviral transduction strategy. The rs GPIIIa was also suitable for the detection of Abs directed against the human platelet antigen 1a (HPA-1a) and HPA-1b, which reside on GPIIIa [4]. On the other hand, all previous attempts to produce the rs GPIIb were failed. The rs GPIIb is retained in the transfected cells, which was attributed to the presence of retention signal in the extracellular domain of the GPIIb [5]. Better understanding of the dynamics of GPIIb/GPIIIa formation, better vector design, transduction of various normal and cancerous mammalian cells, cultivation of transduced cells under different conditions, and many other factors may contribute in the future to successful production of the soluble form of GPIIb. This mini-review is an attempt to provide some pathways, which might lead in the future to the production of the rs GPIIb.

PHYSIOLOGY OF PLATELET GPIIB/GPIIIA FORMATION

Platelets are the smallest (1-2 μM) blood cells, which are responsible for proper clotting. Glycoprotein IIb/IIIa is one of the platelet surface expressed proteins that plays a major role in the common pathway of platelet aggregation; a cornerstone reaction in hemostasis and thrombosis [7]. GPIIb couples to GPIIIa at a micromolar concentration of Ca^{2+}, expressed on human platelets as a heterodimer in an apparent 1:1 stoichiometry, and accounts for 18% of the platelet plasma membrane protein mass [8]. GPIIb, with a molecular weight of an approximately 136,000, [8] is produced when its DNA blueprint is copied and the code translated into the matching amino acid sequence. The linear chain is then modified into a three dimensional conformation and folded to couple to GPIIIa in the ER. The heterodimer undergoes glycosylation and then transported to the Golgi apparatus, where the cleavage of the GPIIb occurs. The GPIIb is stabilized by chemical forces between the heavy and light chains of the GPIIb and the forces between the GPIIb and GPIIIa. Because the protein

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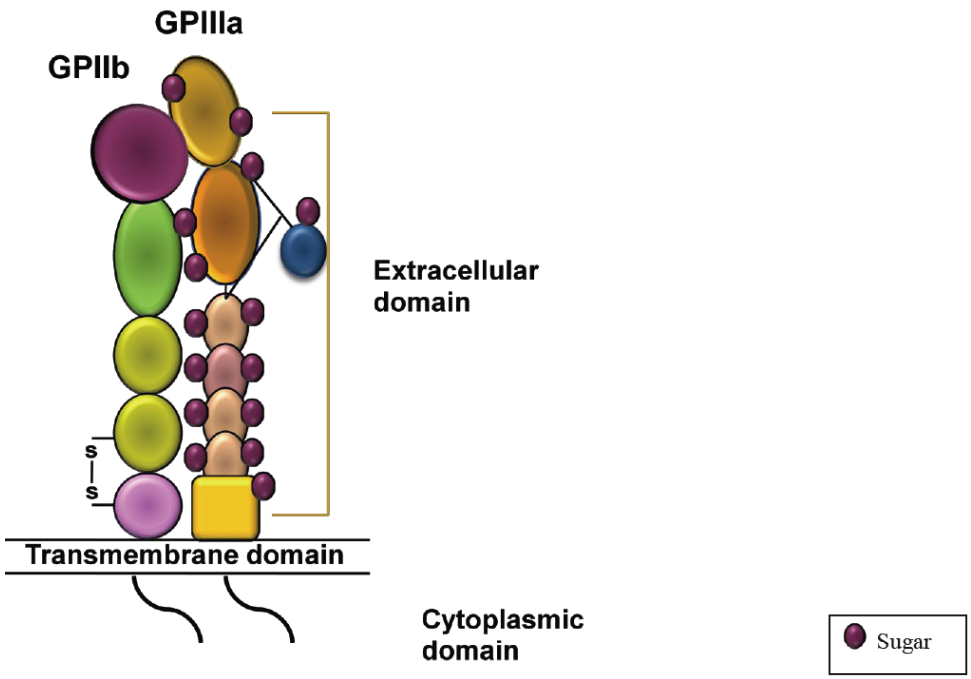

Figure 1: The GPIIb/IIIa integrin heterodimer.

folding problem is still an issue on track for investigation [9], it is also still unclear how the GPIIb/IIIa achieves its native fold.

HUMAN PLATELET ALLOANTIGENS EXPRESSED ON GPIIB: FREQUENCY AND ASSOCIATED DISEASES

To date, 6 human platelet alloantiges (HPAs) have been identified on GPIIb (Table **1**). Notwithstanding immunization against those antigens is infrequent, fetal / neonatal alloimmune thrombocytopenia (FNIAT) and intracranial hemorrhage is usually severe. The epitopes expressed on the GPIIb are the second most favored targets of antibodies detected in alloimmune platelet disorders [10]. HPA-3 system is expressed on GPIIb, and is determined by an isoleucine-serine amino acid substitution at position 843, and is account for approximately 2 percent of FNIAT [11, 12]. Therefore, detection of the HPA-3a alloantibodies is required. However, it has been shown that their detection can been overlooked by the current used assays [13]. The rest of HPA systems on the GPIIb are also important and their corresponding antibodies can cause FNIAT.

Table 1: Human Platelet Allo-Antigens Expressed on Glycoprotein IIb

Antigens	Amino Acid Change	Immune Platelet Disorder
HPA-3a HPA-3b	I843S	FNAIT, PTP, MRP
HPA-9bw	V837M	FNAIT
HPA-20bw	T619M	FNAIT
HPA-22bw	K164T	FNAIT
HPA-24bw	S472N	FNAIT
HPA-27bw	L841M	FNAIT

FNIAT, Neonatal alloimmune thrombocytopenia; PTP, post-transfusion purpura; MRP, multiple-platelet transfusion refractoriness

IMPORTANCE OF RECOMBINANT SOLUBLE PLATELET GLYCOPROTEINS IN THE PLATELET ANTIBODY IDENTIFICATION

Timely and accurate testing for alloantibodies directed against the HPAs residing on GPIIb is vital for clinical diagnosis of FNAIT and to ensure adequate blood supply for patients who need allogeneic blood transfusions. The advantages of using the recombinant soluble platelet glcoproteins (PGPs) are twofold: they can be incorporated in the solid-phase assays such as ELISA and they are single antigens and hence can be used for direct antibody identification [4, 14, 15]. Several PGPs have been recombinantly produced and used in the solid phase assays [4, 16, 17]. However, the attempts to produce the rs GPIIb in 1990s were the 'flash- in-the-pan' that has now fizzled. Production of the rs GPIIb would be very interesting and challenging first to establish a proof of principle for rs GPIIb production, and second to check their suitability for the detection not only HPA-3a alloantibodies but also the

antibodies directed against the rest of antigens residing on GPIIb.

IIB OR NOT IIB: THE CINDERELLA MOMENT (EXPRESSION OF THE RS GPIIB) IS STILL DISTANT

The first study investigating the feasibility of producing the rs GPIIb was Bennett *et al*. 1993 [5]. They found that the transfected COS-1 cells with the plasmid containing the truncated GPIIb gene could not produce the soluble form of GPIIb, and that GPIIb was retained and degraded in the ER. They have concluded that retention of the soluble GPIIb in the ER might be attributed to the presence of a retention signal in the extracellular domain of the GPIIb. The study showed that the soluble GPIIb failed to undergo endoproteolysis cleavage into heavy and light chains, however, the GPIIb chain cleaves when it is expressed as a soluble heterodimer. Because endoproteolysis occurs in the Golgi apparatus, this confirms that the soluble GPIIb is retained in the ER [18]. The chaperone binding protein (BiP) and the intracellular calcium were not responsible for the retention of soluble GPIIb [5].

Cells have normally the Go no Go mechanism that orchestrates the transport of well-folded proteins out of the ER or retention of the malfolded proteins in the ER [19]. Association of the intact extracellular domain of the GPIIb with the intact extracellular domain of the GPIIIa is a prerequisite for their successful transport as a soluble heterdimer to the Golgi bodies. Thus argues the notion that the truncated GPIIb is malfolded. The soluble heterodimer, however, will not be transported to the Golgi apparatus if a part of the light chain of the GPIIb is deleted [20]. Therefore, the presence of the GPIIIa could not mask the defect in the GPIIb. An alternatively spliced truncated form of the GPIIb has been identified in human leukemia, prostate adenocarcinoma, and melanoma cells but not in the platelets or normal prostate epithelial or normal breast epithelial cells [21]. This variant consists of the extracellular domain (heavy chain and part of the light chain) and lacks the transmembrane and cytoplasmic domains [21]. It is still unknown how the cancer cells can produce this truncated variant of the GPIIb and why the normal cells can't express it. Thus, the question of whether the soluble GPIIb monomer can be recombinantly produced is still unanswered.

FUTURE DIRECTIONS

Expression of the rs GPIIb is still challenging. The importance of expression of rs GPIIb would be first a proof-of-concept and second the expectations that this rs GPIIb would be suitable for antibody identification. Therefore, several strategies can still be done in to overcome the rs GPIIb retention by the ER and to ensure its export to the Golgi apparatus. One model is to utilize the lentiviral technology to transduce mammalian cells [22]. We have recently showed the value of using the lentiviral vector system in the expression of large amounts of rs GPIIIa in the human embryonic kidney 293 (HEK) cells [4]. In addition, multiple approaches have been described for better engineering of lentiviral constructs such as using of the spleen focus-forming virus (SFFV) promoter, which enhances the protein production [23], or addition of various DNA elements to the virus [24]. Cotransfection with lentiviral vectors expressing chaperone proteins might lead to the expression of correctly folded soluble GPIIb and its export to the Golgi apparatus. Several studies have proved the impact of different protein tags on the folding, stability, proteolytic resistance, hydrogen bond formation, and solubility of the recombinant protein production [4, 25, 26]. Therefore, it is worthwhile to add different tags while engineering the lentiviral constructs that contain the truncated GPIIb gene. Cultivation of the transduced cells under different culture conditions might affect on the protein production [23]. The alternative spliced truncated GPIIb that has been previously determined in different tumor cells [21] is an example of the special machine the tumor cells have to create a wide spectrum of proteins. This truncated GPIIb could escape the chaperones in the ER and the terminator "non-sense mediate mRNA decay" [27]. Therefore, transduction of highly proliferative tumor cell lines (*e.g.*, K562, prostate cancer cells) with the lentiviral vectors containing the truncated GPIIb might be a possibility that the rs GPIIb would escape the checkpoint in the ER.

CONCLUSIONS

The platelet glycoprotein IIb (GPIIb) is an integral membrane protein, which forms with GPIIIa a calcium-dependent heterodimer [1]. All previous attempts to produce the rs GPIIb form have failed. The importance of expressing the rs GPIIb is that it can be used in the solid phase assays for the antibody identification. Several pathways such as better vector design, transduction of various normal and cancerous mammalian cells, or cultivation of transduced cells under different conditions can be performed to try to overcome the rs GPIIb retention by the ER. Yet, it is still challenging and unresolved question whether the soluble monomer form of the GPIIb can be produced or not and if it would be suitable for the antibody

identification. Decryption the code of rs GPIIb expression needs to be determined.

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