Use of GPI-anchored proteins to study biomolecular interactions by surface plasmon resonance

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Abstract Surface plasmon resonance is a powerful tool to examine the kinetics of cell surface receptor-ligand interactions and requires only small amounts of protein. For these studies, one component is required in highly purified form to be coupled to the biosensor surface. The second component does not need to be purified. The human high affinity receptor for immunoglobulin G, FcγRI, presents a problem as the receptor itself cannot readily be produced in large amounts for purification and, as there are eight potential ligands for the receptor (human IgG1–4 and mouse IgG1, 2a, 2b and 3), it is difficult to immobilise the ligand. Using a previously established method for generating GPI-anchored proteins, we have produced and captured a soluble version of FcγRI and shown that it retains its affinity for human IgG1 and specificity for the different IgG subclasses. In addition, we also produced and captured a GPI-anchored version of the cell adhesion molecule CD2. This system circumvents the need for extensive receptor purification and is very rapid as solubilised receptors can be transferred from the cell surface to the sensor chip in 2 h. This system may be generally applicable for biosensor studies to other type I membrane proteins, and/or naturally occurring GPI-anchored proteins, especially where the interaction between a ligand and a panel of variant receptors is to be studied.

Key words: Surface plasmon resonance; High affinity IgG receptor; Glycosyl phosphatidyl inositol protein

1. Introduction

The study of protein-protein interactions by surface plasmon resonance (SPR) has become widespread in recent years. However, the study of receptor-ligand interactions by SPR requires the production of a soluble form of the receptor. Several groups have used a variety of different methods of producing soluble receptors to study the interaction with their ligand [1–4]. In these cases, careful modification of the proteins was required followed by over-production and purification, prior to analysis by SPR.

We are interested in the interaction between IgG and its high affinity receptor, FcγRI. FcγRI is a type I membrane protein expressed on macrophages, monocytes and dendritic cells where it plays a pivotal role linking the cellular and humoral arms of the immune system (for reviews see [5–7]). Binding of IgG to FcγRI triggers a variety of biological responses [8–10]. The extracellular ligand binding domain of FcγRI comprises three V-like IgSF domains, EC1, EC2 and EC3 [11,12]. We have shown that both EC2 and EC3 are required for high affinity binding of IgG, and that amino acids in the second EC domain are important for ligand contact (Harrison and Allen, submitted). To further characterise this interaction between FcγRI and IgG, we have decided to study the interactions between FcγRI and IgG using SPR. Our initial attempts to produce a soluble version of the receptor where the second amino acid of the predicted transmembrane domain [11] was replaced by a stop codon were unsuccessful: when the cDNA for the truncated receptor was expressed in COS cells no soluble FcγRI could be harvested as the protein appeared to be targeted for degradation. Similar results have been found independently in another laboratory (P.M. Hogarth, personal communication). Therefore, we have assessed the feasibility of using glycosyl phosphatidyl inositol (GPI)-anchored proteins to produce a soluble protein suitable for plasmon resonance studies.

GPI proteins are attached to the extracellular surface of the cell membrane via a phosphatidyl inositol linkage. This glycosidic bond can be enzymatically hydrolysed using phosphatidyl inositol phospholipase C (PI-PLC), liberating the protein from the cell surface, to produce a soluble protein [13,14]. FcγRI was expressed as a GPI-anchored protein, using a cassette method for generating GPI-anchored versions of type I membrane proteins [15]. The soluble form of FcγRI produced in this way could be captured by specific monoclonal antibodies. The captured FcγRI could bind IgG subclasses with the same specificity as FcγRI expressed on the surface of monocytes/macrophages. This methodology was also successfully applied to CD2, a T-cell-specific adhesion molecule. It is envisaged that this technique could be used for the study of a variety of type I membrane proteins with their ligands.

2. Materials and methods

2.1. Transient expression

COS-7 cells (obtained from B. Seed) were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin. COS cells were plated at a density of approximately 4×10^7 cells per 100 mm plate 1 day before transfection. The SV40-based expression vector CDM [16] was used for the transient expression for all clones in COS cells using the DEAE-dextran method [11]. cDNAs for FcγRI-GPI (and FcγRI mutants) and CD2-GPI were available in CDM [9]. Soluble protein was harvested at 2 or 3 days post transfection, when surface expression is maximal.

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Abbreviations: SPR, surface plasmon resonance; mAb, monoclonal antibody; FcγRI, high affinity IgG receptor; PI-PLC, phosphatidyl inositol phospholipase C; GPI, glycosyl phosphatidyl inositol

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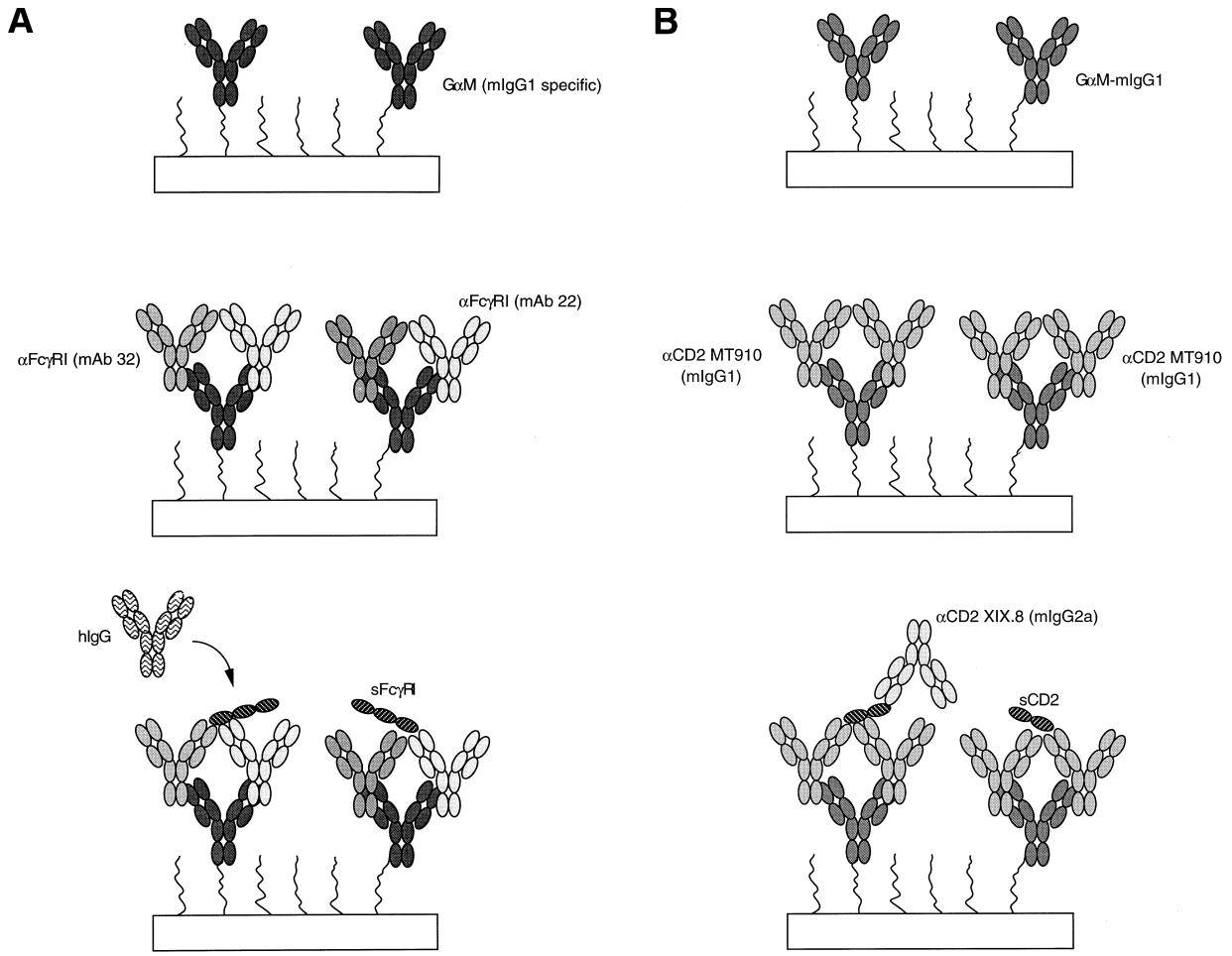

Fig. 1. Schematic representation of the ELISA-like system to capture soluble FcγRI-GPI. GαM IgG1 (Fc-specific) was covalently immobilised to the CM-5 sensor chip surface. A: The immobilised GαM IgG1 (Fc-specific) then captures anti-FcγRI mAbs, 22 and 32, which in turn capture FcγRI-GPI. The captured FcγRI-GPI can be recognised by other anti-FcγRI mAbs or bind IgG. B: The immobilised GαM IgG1 (Fc-specific) then captures the anti-CD2 mAb, MT910, which in turn captures CD2-GPI. The captured CD2-GPI can be recognised by a second anti-CD2 mAb.

2.2. Production of soluble form of GPI-anchored proteins

COS cells transfected with FcγRI-GPI, CD2-GPI or vector only were removed from plates using 1 mM EDTA/PBS, washed once with HBS (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA), resuspended in 1 ml of HBS with 5 units of PI-PLC (Oxford Glycosystems), and incubated at 37°C with gentle rotation, for 90 min. Cells were then spun out at 1500×*g* and the protein stored at −70°C or used immediately.

2.3. Antibodies

Human and mouse IgG subclasses, and goat anti-mouse (GαM) IgG1 (Fc-specific) were from Sigma. Anti-FcγRI monoclonal antibodies 22 and 32, both mIgG1 subclass, and monoclonal 197, a mouse IgG2a, were a gift from Medarex, anti-CD2 mAb MT910 (mIgG1 subclass) was from Dako Ltd, and anti-CD2 mAb XIX.8 (mIgG2a subclass) was from Sera-Lab Ltd.

2.4. Immobilisation of GαM IgG1 on biosensor CM-5 chip

5 μg of GαM IgG1 in a final volume of 100 μl of 10 mM sodium acetate buffer (pH 4.8) was injected over the surface of a CM-5 sensor chip (Pharmacia Biosensor), and chemically immobilised using NHS and EDC in accordance with the manufacturers' instructions. All injections were 10 μl unless otherwise stated. The flow rate was set at 10 μl/min for all experiments unless otherwise stated. The sensor chip was regenerated with 100 mM glycine buffer, pH 2.5.

2.5. Addition of mAbs to GαM IgG1 to the CM-5 sensor chip to capture GPI proteins

Following direct coupling of GαM IgG1 to the CM-5 sensor chip, mAbs were used to capture the GPI proteins. To capture FcγRI-GPI, both mAbs 22 (710 ng) and 32 (366 ng) were injected (10 μl injection) over the CM-5/GαM IgG1 surface. To capture CD2-GPI, mAb MT910 (155 ng) was injected (10 μl injection) over the CM-5/GαM IgG1 surface. Following binding of GPI-anchored proteins, a second mAb was used to confirm the identity of the bound protein. To identify bound FcγRI-GPI, 10 μl mAb 197 (200 ng) was injected. To identify bound CD2-GPI, 10 μl mAb XIX.8 (100 ng) was injected.

2.6. Binding of GPI proteins

10 μl of the FcγRI-GPI protein was injected over the CM-5/GαM IgG1 surface, that had already captured mAbs 22 and 32. The captured FcγRI-GPI protein was used for subsequent experiments to determine the affinity for human IgG1 and the specificity for various IgG subclasses. For the affinity measurements, the flow rate was reduced to 5 μl/min although some of the measurements were also repeated at a flow rate of 20 μl/min (data not shown) to ensure that the kinetic parameters were unaffected by flow rate. The kinetics of association and dissociation of human IgG for FcγRI was measured over four different concentrations of the ligand (Table 1). The kinetics of the interaction were analysed using BIA evaluation 2.1 software and calculated for the different concentrations of ligand. All calculations were performed after subtraction from a reference flow cell (blank) where the primary antibody (GαM IgG1) was not coupled to the chip but was otherwise treated in an identical way to the experimental cell. This blank was used to account for bulk refractive index changes and non-specific binding. For the CD2-GPI studies a 10 μl injection was also used for the capture of CD2-GPI protein.

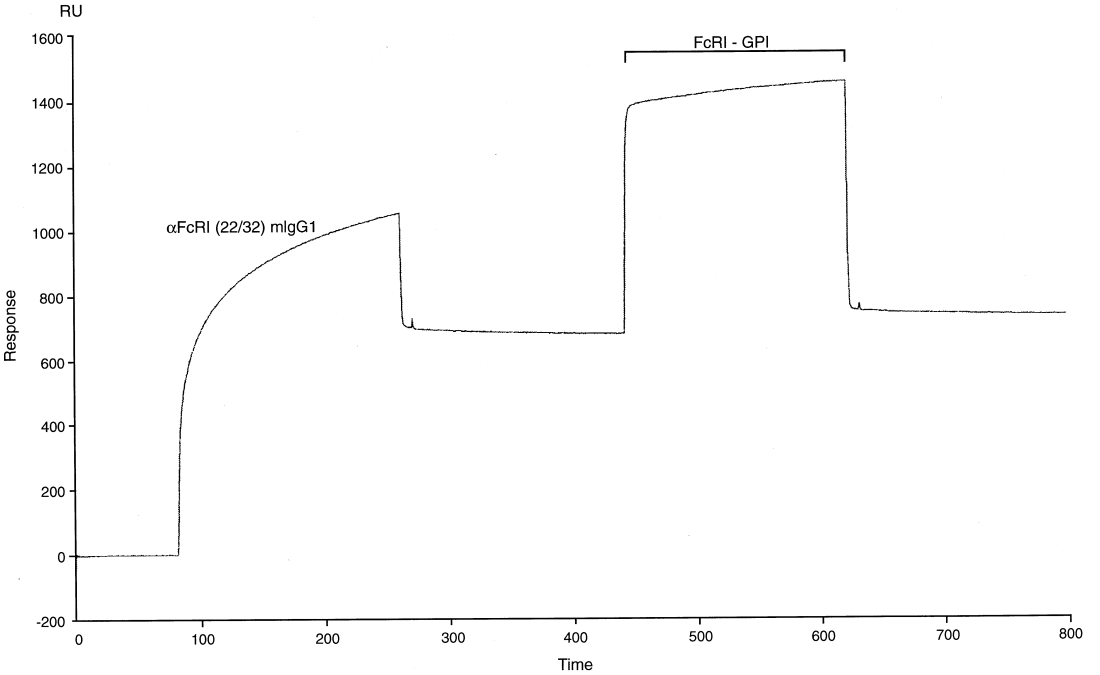

Fig. 2. Sensorgram showing the binding of various proteins to the GαM IgG1 (Fc-specific) sensor chip surface. Anti-FcγRI(22/32) mIgG1: injection of anti-FcγRI mAbs 22 (mIgG1) and 32 (mIgG1); FcγRI-GPI: injection of FcγRI-GPI.

3. Results

3.1. Production and capture of soluble FcγRI-GPI protein

We have previously described a method for the production of GPI-anchored version of type I transmembrane proteins, using as examples a low affinity adhesion molecule CD2 and a high affinity receptor molecule, FcγRI [15]. To generate soluble forms of these molecules, COS cells transiently expressing either CD2-GPI or FcγRI-GPI were treated with PI-PLC. The supernatant from the treated cells was then injected directly, without further purification, across the appropriately prepared CM-5 sensor chip surface on a BIAcore 2000 (BIACORE, Stevenage, UK).

To detect the presence of the soluble GPI proteins in the supernatant of PI-PLC-treated cells, we devised an 'ELISA-like' sandwich assay system on the sensor surface (see Fig. 1A). GαM IgG1 (Fc-specific) was chemically immobilised on the sensor surface. Once covalently coupled, this antibody was used to capture monoclonal antibodies of the mIgG1 subclass to identify specific GPI proteins. This approach has two advantages compared to coupling the mAbs directly to the sensor surface. First, because the GαM IgG1 is specific for the Fc region of the mAbs, the Fab region of these antibodies will be correctly orientated for binding of their target protein. Second, the non-covalently captured mAbs can be stripped from the covalently coupled GαM IgG1 at the end of an experiment, and the regenerated surface used to capture other mAbs to detect other target proteins. Thus, only one flow cell of a sensor chip is required to detect a variety of different proteins.

To detect soluble FcγRI-GPI, two anti-FcγRI mAbs (22 and 32) which use non-overlapping epitopes and do not affect IgG binding [17] were captured onto the GαM IgG1 sensor chip (Fig. 1A). Both anti-FcγRI mAbs (22 and 32) were used together based upon the observation that FcγRI is immunoprecipitated much more efficiently with both than either alone. As shown in Fig. 2, the injection of 22/32 results in 693 RU of binding. Injection of PI-PLC-treated supernatant from FcγRI-GPI expressing COS cells resulted in a further 66 RU of binding. In contrast, injection of supernatant from PI-PLC-treated mock-transfected or CD2-GPI-transfected cells yielded none (data not shown). Thus, PI-PLC-solubilised FcγRI-GPI can be readily captured by this system. The identity of the captured protein as FcγRI could be confirmed when the mAb 197 (mIgG2a subclass) was injected (Fig. 2). Similar results were used when the mAb 10.1 [18] was used (data not shown). However, since this mAb displaces IgG binding to FcγRI, we used the detection system employing both 22 and 32 in all subsequent experiments.

Table 1
Kinetic analysis of hIgG1 binding FcγRI-GPI

Flow rate (μl/min)	hIgG1 conc (μM)	k_{ass} (10^4 M^{-1} s^{-1})	k_{diss} (10^{-3} s^{-1})	K_D (10^{-7} M)
5	6.67	1.97	2.00	1.01
5	4	1.24	2.18	1.75
5	2.67	2.1	2.22	1.05
5	1.33	1.97	2.23	1.13

Mean k_{ass}: $1.82\times10^4\pm0.39\times10^4$ M^{-1} s^{-1}; mean k_{diss}: $2.16\times10^{-3}\pm0.11\times10^{-3}$ s^{-1}; mean K_D: $1.24\times10^{-7}\pm0.35\times10^{-7}$ M.

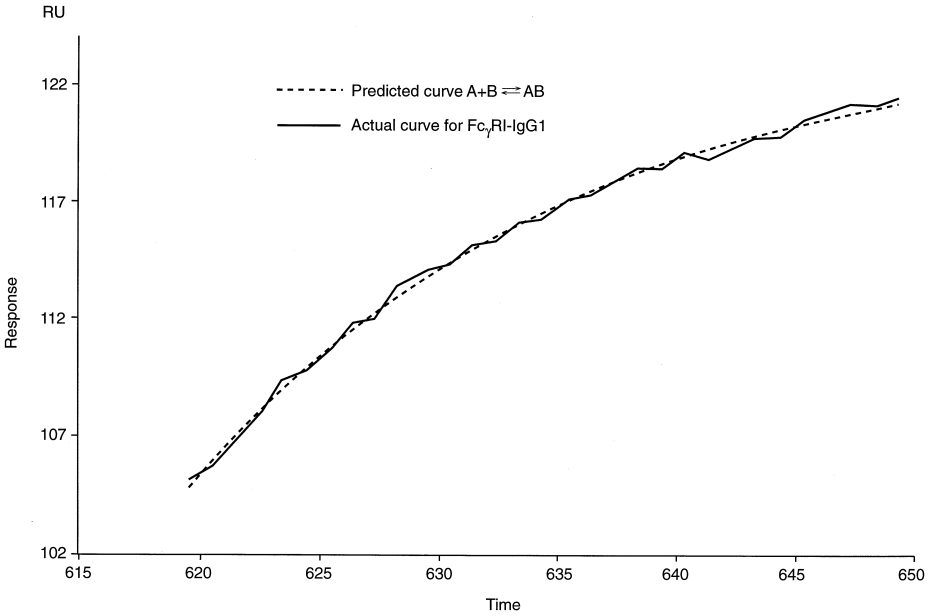

Fig. 3. BIA evaluation 2.1 curve fit for the association of FcγRI for human IgG1. This curve shows the area of the sensorgram used to calculate the k_{ass}. The dashed line shows the ideal fit for the simple association model A+B⇄AB. The rate constants were obtained using linear regression analysis.

3.2. *Captured FcγRI retains its affinity for human IgG1 and its specificity for human and mouse immunoglobulin subclasses*

Having shown that FcγRI could be captured using mAbs 22 and 32, and bind polyclonal human IgG, we wished to see if it retained its kinetic properties for the binding of human IgG1, and specificity for different human and mouse IgG subclasses. For the measurements of the kinetics of the interaction of FcγRI with ligand, the sensorgram was subjected to the BIA evaluation 2.1 software package. The observed rate of association matched that predicted for a simple A+B⇄AB association model within a χ^2 of 0.08 (Fig. 3). Direct measurements of the k_{ass} and k_{diss} were consistent over a range of concentrations of the ligand (human IgG1) (Table 1) and flow rates. The average k_{ass} was $1.82\times10^4 \pm 0.39\times10^4$ M^{-1} s^{-1} and k_{diss} $2.16\times10^{-3} \pm 0.11\times10^{-3}$ s^{-1}. The overall affinity was calculated from these kinetics and gave an average K_D of

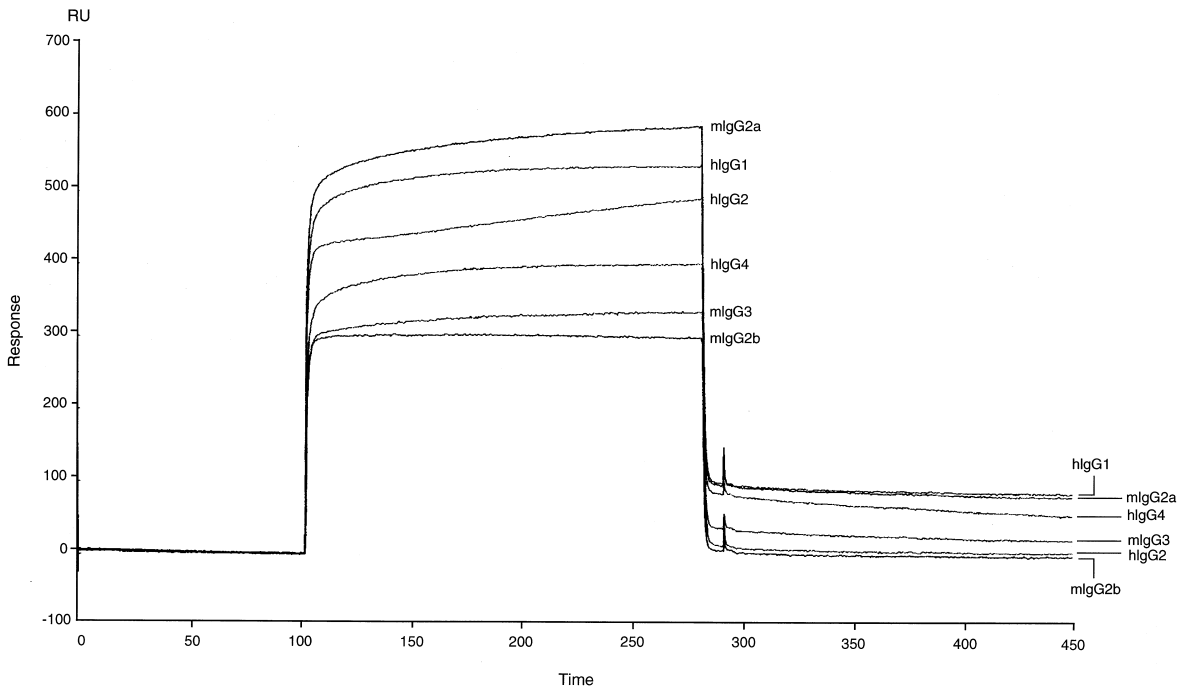

Fig. 4. Sensorgram showing the binding of human and mouse IgG subclasses to captured FcγRI-GPI. hIgG1, hIgG4, mIgG2a and mIgG3 bind, but hIgG2 and mIgG2b do not bind. The trace for human IgG2 displays mass transport effects.

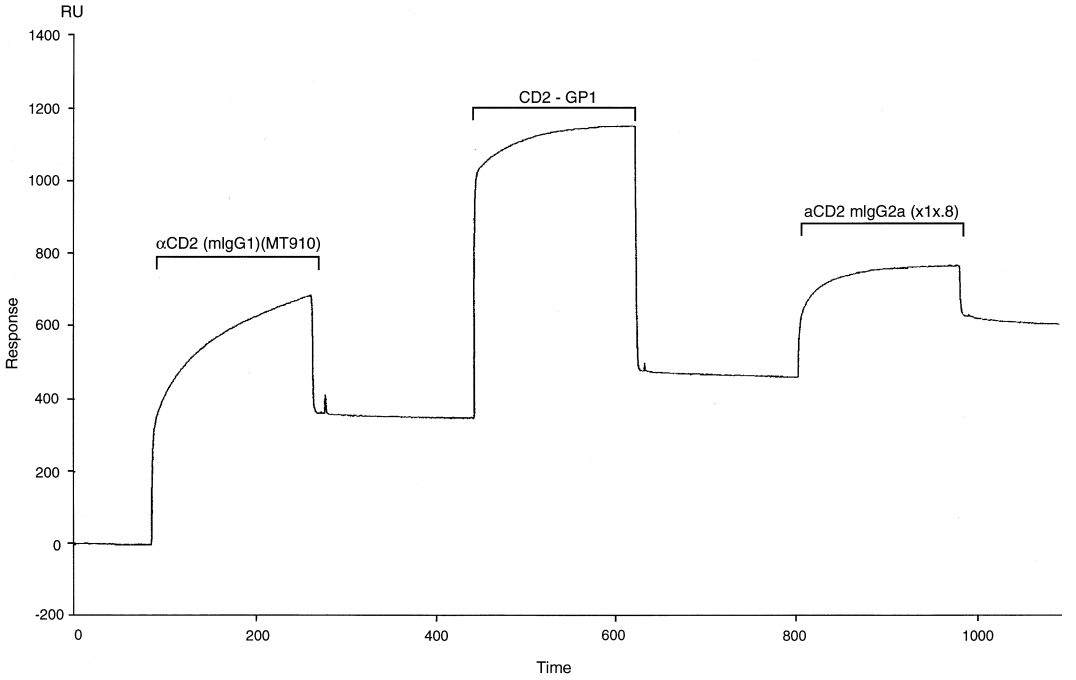

Fig. 5. Sensorgram showing the binding of various proteins to the GαM IgG1 (Fc-specific) sensor chip surface. αCD2 (mIgG1) MT910: injection of anti-CD2 mAb MT910 (mIgG1); CD2-GPI: injection of CD2-GPI; aCD2mIgG2a (x1x.8): injection of mAb XIX.8 (mIgG2a).

$0.123\times10^{-8}\pm0.035\times10^{-8}$ M, a result comparable to that observed for FcγRI when expressed on the surface of macrophages and macrophage-derived cell lines [19–21]. It is slightly higher than the value for FcγRI expressed on the surface of COS cells [11].

FcγRI endogenously expressed on the surface of macrophages or monocytes, or heterogeneously expressed on the surface of COS cells will bind human IgG1, IgG3 and IgG4 and mouse IgG2a but not human IgG2 or mouse IgG1 or IgG2b. As shown in Fig. 4, captured FcγRI-GPI specifically bound human IgG1, hIgG3 and hIgG4 and also mouse IgG2a (Table 2). The captured receptor did not bind human IgG2 or mIgG2b. In the absence of captured FcγRI-GPI no binding of any IgG subclasses is observed (data not shown). mIgG1 could not be tested as it is bound directly by the immobilised GαM IgG1-specific antibody. Attempts to block all the mIgG1 binding sites with an excess of mIgG1 were not successful, so we cannot at present confirm that mIgG1 is not recognised.

3.3. Production and capture of soluble CD2-GPI protein for SPR analysis

To identify CD2-GPI, the mAb MT910 (mouse IgG1 subclass) was used as shown in Fig. 1B. As shown in Fig. 5, injection of PI-PLC-treated supernatant from COS cells expressing CD2-GPI resulted in 354 RU binding, whereas injection of supernatant from PI-PLC-treated mock-transfected or FcγRI-GPI-transfected cells yielded none.

To confirm that the captured moiety was CD2, a different anti-CD2 mAb, XIX.8 (which is of the mIgG2a subclass and is not be recognised by the immobilised GαM IgG1-specific antibody) was injected. This resulted in 155 RU of binding. In contrast, injection of mAb XIX.8 in the absence of a prior injection of CD2-GPI gave no increase in RU. This shows that the mAb XIX.8 binds a different non-overlapping epitope from MT910.

4. Discussion

We have shown that soluble protein produced by PI-PLC treatment of GPI-anchored receptors can be detected by an 'ELISA-like' capture system on a BIAcore sensor surface and that the captured proteins can be used to study receptor-ligand interactions. By immobilising a GαM IgG1 Fc-specific antibody, and using that to capture monoclonal antibodies of the mouse IgG1 subclass, we were able to make a reusable detection system able to recognise different GPI-anchored proteins. The first protein captured was solubilised FcγRI-GPI. The proteins identity was verified by the binding of another FcγRI-specific mAb, 197. Moreover, the captured receptor retained the ability to bind human IgG1 with similar affinity to that measured for the receptor expressed on the cell surface. More importantly, its specificity for six different IgG

Table 2
IgG subtypes bound (in resonance units) to soluble FcγRI

Immunoglobulin	RUs bound
hIgG1	82
hIgG2	0.1
mIgG3	4
hIgG4	61
mIgG2a	79
mIgG2b	−3.0

At time point 400 s.
The relative amount of each of the IgG subtypes bound to FcγRI was calculated by measuring the resonance units after the injection (400 s) of each of the various IgG subtypes and subtracting the pre-injection value at 95 s; see Fig. 4.

subclasses. The second protein captured was solubilised CD2-GPI. Its identity was verified by the binding of another CD2-specific mAb, XIX.8.

The use of GPI-anchored proteins, coupled with the 'ELISA-like' system described here, offers several advantages when compared to other systems used to generate soluble proteins for analysis by SPR. The major advantage is that the protein does not require purification prior to use. This is due to the fact that the GPI protein is, in effect, affinity purified when injected over the sensor surface by the appropriately captured mAbs. A second advantage is that the protein can be easily pre-concentrated whilst still attached to the cell surface. Usually a total of about 10^6 cells are harvested from four 100 mm dishes and the cells concentrated into 0.5 ml before being subjected to PI-PLC treatment. Another major advantage is speed; the protein can be transferred from the cell surface to the sensor surface in approximately 2 h. Since the sensor chip surface can be rapidly regenerated, this means that many different receptors can be assayed using the same chip. This is especially useful if the interaction between a ligand and a panel of variant receptors is to be assayed. Finally, the conversion of type I membrane receptors into GPI-anchored proteins requires little genetic manipulation [14,15].

The vector described here for expressing GPI proteins can be efficiently transfected into COS cells and results in very high levels of cell surface expression; the transfection efficiency of FcγRI-GPI is in excess of 30% in COS cells [15] and 10^6–10^7 copies of FcγRI are expressed per COS cell [11]. Thus, although endogenous GPI proteins will be released following PI-PLC digestion, along with the protein of interest (e.g. FcγRI-GPI), the extracellular region of the heterologously expressed GPI-anchored protein is the predominant protein.

We intend to explore the use of this system for obtaining kinetic data for the interactions between FcγRI and IgG. We also wish to extend the scope of this system for more detailed epitope mapping applications and explore the possibility of studying the interactions between IgG and a panel of FcγRI variants.

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