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Generation and expression of a minimal hybrid Ig-receptor formed between single domains from proteins L and G

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Abstract

The Ig-binding properties of protein L from *Peptostreptococcus magnus* and protein G from *Streptococcus* have been successfully combined through the construction of a novel hybrid protein, consisting of a single Ig-binding domain from each protein. The biophysical and biochemical properties of this construct have been characterized through equilibrium and pre-equilibrium fluorescence spectroscopy, circular dichroism, isothermal titration calorimetry, affinity chromatography, and conformational stability studies using a chemical denaturant in order to examine the structure and availability of ligand binding sites in each domain. These studies show that despite the small size of the protein (Mw = 16.5 kDa) each domain behaves in an independent manner with respect to the binding characteristics of the same domain in isolation.

Keywords: Ig-receptor; Hybrid; Isothermal titration calorimetry (ITC); Fluorescence; Immunodiffusion; Affinity chromatography; Protein L; Protein G

Several proteins that exhibit non-antigenic binding to Igs have been isolated from the surface of Gram-positive pathogenic bacteria. Protein A from *Staphylococcus aureus* [1], Protein G of group C and G streptococci [2,3] and protein L of *Peptostreptococcus magnus* [4,5] are examples of such proteins. Structural analyzes of these proteins have revealed that although they share certain characteristics; including hydrophobic/charged tail domains anchoring them to cell membranes and C-terminal cell-wall spanning motifs, these proteins contain multiple repeated domains (55–76 amino-acid residues) that are divergent in nature [6–9]. These repeated domains are responsible for binding some plasma proteins including Igs.

Protein A has five such domains (SpA, 58–61 residues each) each consisting of three α-helices [10]. Two of these (helices 1 and 2) interact with the C_H2–C_H3 regions of IgG subclasses 1, 2, and 4. Helices 2 and 3 of SpA can also bind simultaneously (albeit with a lower affinity) to the V_H-Fab region of Igs, provided they contain heavy chains from the V_HIII family [11].

Streptococcal protein G has a broader binding specificity for IgG (including IgG_3) throughout the mammalian species. This protein consists of 2 or 3 (depending upon species) domains (SpG) that are comprised of two pairs of anti-parallel β-sheets connected by a single α-helix. The loop connecting β-strand 3 to the helix interacts with the C_H2–C_H3 region of IgG-Fc. Protein G can also interact via its β-strand 2 with the C_H1 region of Fab derived from 10% to 50% of human IgG [12,13]. However, there is no evidence to date that binding at both sites can occur simultaneously. Nevertheless, both SpA and SpG have two Ig-binding sites per domain and are useful tools for the isolation of a limited number of classes of Igs.

Protein L of *P. magnus* has four/five Ig-binding repeat units depending upon the strain from which it is isolated [6], each consists of 72–76 amino-acid residues that facili-

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tate its binding interaction with the framework regions in the variable domain of Ig light chains [14]. The primary ligands of this molecule are κ light chains of Igs, though some Igs containing λ light chains also show affinity with protein L [5,15]. Thus protein L is able to bind all classes and sub-classes of Ig [4,15]. Recent studies have shown that domains derived from protein L of *P. magnus* strain 3316 have two binding sites for κ-chains [16,17]. One site is centered around residues on β-strand 2 and the other involves residues on β-strand 3. Both sites include additional interactions with residues on the α-helix [16]. Thus PpL acts bivalently in common with SpA and SpG.

At present there is no structural detail of the organization of domains in the native proteins. Various studies suggest that between two and four equivalents of IgG are able to bind simultaneously to one molecule of protein A that contains five sequential IgG-binding domains [18–20] thus suggesting that steric restrictions may prevent saturation of all available sites.

In these studies we have constructed a minimal hybrid Ig-binding protein consisting of a single domain of protein L and a single domain of protein G in order to probe the availability of each binding site to its ligand. This was accomplished by fusing a gene encoding a single light-chain binding domain of protein L from *P. magnus* strain 3316 [21] via a short linker to that encoding a single Fc-binding domain of protein G (SpG C2-1)[2] [8]. We demonstrate that this minimal hybrid protein (LG) not only possesses the folding properties of both of the individual bacterial proteins but that despite its small size, each domain can interact with its target proteins in an independent manner. The PpL domain is able to bind two equivalents of κ light chains and the SpG domain one Fc simultaneously illustrating this protein's potential as an immunological reagent.

Experimental

Materials

Phosphate buffers and other reagents were all of analytical grade and were purchased from BDH. All PCR reagents including *Pfu* DNA polymerase were purchased from Promega, UK.

Construction of the protein LG gene

The genes encoding a single domain Ig-binding domain of protein L (PpL) [21]) and two Ig-binding domains of protein G joined through a short linker region (SpG C2-2) [22] have previously been cloned into the pKK223-3 bacterial expression vector and have been overexpressed in *Escherichia coli* JM103 cells. Through a series of PCR reactions the genes encoding PpL and the second Ig-binding domain of SpG C2-2 were linked giving rise to the protein LG gene, through standard molecular biology protocols. Briefly, an initial PCR reaction cloned the PpL gene from its expression vector with primers L1 (5′-ACA TCA TAA CGG TTC TGG CAA ATA-3′) and L2 (5′-GAT AAC TTC CGG TTT TCC AGC AAA TTT AAT-3′), whilst the SpG domain portion of the LG construct was made in a second PCR using the SpG C2-2 plasmid DNA template with primers G1 (5′-AAA TTT GCT GGA AAA CCG GAA GTT ATC GAC G-3′) and G2 (5′-GAG TTC GGC ATG GGG TCA GGT G-3′). A third PCR using the products from the previous two reactions as template DNA, with primers L1 and G2 yielded the protein LG gene, which was cloned into the pKK223-3 expression vector using SacI and PstI restriction enzymes.

Mutagenesis

The I34W and Y64W mutations were introduced into the protein LG construct by substituting the Wt PpL template used above for I34W PpL and Y64W PpL as appropriate. The deletion of the C-terminal cysteine of protein LG to facilitate solution studies was carried out through two-sided SOEing PCR mutagenesis using primers L1 and G2 as flanking primers with 5′-TCA CTA TTA TTC GGT AAC CGT GAA G-3′ and 5′-GTT ACC GAA TAA TAG TGA CTG CAG C-3′ as mutagenic primers.

The sequence of each of the protein LG gene constructs was confirmed by DNA sequencing (MWG Biotech, Germany).

Protein purification

The purification of protein LG was carried out in a similar manner to that previously described for Wt PpL [21]. Briefly, a 33.5 g bacterial cell pellet from 4 L of *E. coli* culture collected in sonication buffer (20 mM Tris, 1 mM EDTA, 0.1 mM EGTA, and 0.5% Triton X-100 (v/v)) was thawed and resuspended in an additional 50 ml of sonication buffer with 5 mg lysozyme. Broad range protease inhibitors (Sigma) were also added to prevent degradation of extracted proteins as was 125 μM DNAse I (Sigma) to digest DNA that was released by thawing of the bacteria. The re-suspended cells were homogenized by passage through a 10 ml disposable syringe and the resulting cell suspension was then sonicated. Following sonication, the volume was then made up to 125 ml to facilitate centrifugation. Cell debris was removed by centrifugation at 24,000*g* for 20 min at 4 °C to obtain the soluble protein extract. The extract was then heat treated to 70 °C for 40 min, then further buffer (about 10 ml) was added before the precipitated protein was removed by centrifugation at 24,000*g* for 20 min at 4 °C. The clarified supernatant was applied to a

[2] *Abbreviations used:* Ig, immunoglobulin; PpL, a single Ig-binding domain of *Peptostreptococcal* protein L; SpG, a single Ig-binding domain of *Streptococcal* protein G; LG, a hybrid protein expressed from a gene encoding PpL linked to SpG; Hu-Ig, human Ig; Hu-Fc, Fc fragment from a Hu-Ig; Fab, antigen binding fragment from a Hu-Ig; GdnHCl, guanidine hydrochloride.