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Expression of human recombinant β_2-glycoprotein I with anticardiolipin antibody cofactor activity

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To enable the synthesis of β_2-glycoprotein I mutants we have established a stable Chinese hamster ovary cell line that expresses human β_2-glycoprotein I up to 2.9 $\mu g/10^6$ cells/day. Recombinant β_2-glycoprotein I is identical to the purified native protein with respect to cofactor activity revealed in a modified anti-cardiolipin ELISA. Autoimmune type anti-cardiolipin antibody requires recombinant β_2-glycoprotein I in a dose-dependent manner to bind cardiolipin whilst binding of infectious type antibody is inhibited. The purified recombinant β_2-glycoprotein I in serum free medium exists as two oligosaccharide species which upon deglycosylation have identical apparent molecular weight to the deglycosylated native protein.

β_2-glycoprotein I; Anti-cardiolipin antibody; Anti-phospholipid antibody

1. INTRODUCTION

β_2-Glycoprotein I is a highly glycosylated plasma protein comprised of 326 amino acids with an apparent molecular weight of about 50 kDa [1]. The complete nucleotide sequence of the human, rat, bovine and mouse β_2-glycoprotein I has been determined revealing a high degree of homology between species [2–8].

Current evidence suggests that β_2-glycoprotein I may function as a circulating anticoagulant as it inhibits contact activation of the intrinsic blood coagulation pathway [9], ADP-dependent aggregation of platelets [10], and prothrombinase activity of activated platelets [11].

β_2-Glycoprotein I is a cofactor in the recognition of the phospholipid antigen cardiolipin by anticardiolipin antibodies [12,13]. Anticardiolipin antibodies purified from patients with autoimmune disease require β_2-glycoprotein I to bind cardiolipin whilst anti-cardiolipin antibodies from patients with infection can bind cardiolipin without the need for β_2-glycoprotein I [14,15]. The presence of anti-cardiolipin antibodies in patients with autoimmune disease confers an increased risk of thrombo-embolic complications [16]; however the mechanism by which autoantibodies directed against a β_2-glycoprotein I/phospholipid complex predisposes to thrombotic disorders is unknown.

The analysis of mutant forms of β_2-glycoprotein I would delineate the antigenic epitope(s) recognised by autoimmune anti-cardiolipin antibodies and as a first step in that direction, we report the expression in transfected Chinese hamster cells of human recombinant β_2-glycoprotein I.

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2. MATERIALS AND METHODS

The pB2C6 plasmid (6.1 kb) was constructed by inserting the mouse dihydrofolate reductase cDNA from the plasmic pSV2-dhfr (ATCC) into pB21-1 (pCDM8; Invitrogen) containing the full-length human β_2-glycoprotein I cDNA (1.15 kb) which includes 5′ and 3′ untranslated regions and a putative leader sequence [2].

The pSV2-dhfr plasmid was double digested with *Pvu*II and *Bam*HI to produce the dihydrofolate reductase and SV40 promoter cDNA fragment (1.9 kb) which was then repaired to blunt ends. The pB21-1 plasmid was double-digested with *Sca*I and *Stu*I and the resulting large fragment (4.2 kb) ligated to the DHFR/SV40 fragment to form pB2C6 using standard techniques. The ligated DNA was used to transform *E. coli* MC1061 P3.

The Chinese hamster ovary cell dihydrofolate reductase deficient mutant DG44 was propagated in α^- (Gibco) supplemented with 2% (v/v) heat-inactivated and dialysed fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM hypoxanthine, 16 μM thymidine and 2 mM glutamine. Selection and amplification was performed using the same medium without hypoxanthine and thymidine and with the addition of methotrexate (Sigma) at concentrations indicated.

pB2C6 DNA was transfected into DG44 cells (40 $\mu g/10^6$ cells) by the standard calcium phosphate precipitation technique [17]. Cells were grown in selective media for 1 week before addition of methotrexate at 0.1 μM. After 19 days selection, methotrexate-resistant colonies were picked using cloning cylinders into 24-well plates and the culture medium assayed for β_2-glycoprotein I production at confluency by radioimmunassay [14]. β_2-Glycoprotein I producing clones were innoculated into 100 mm^2 petri dishes at the split ratio of 1:9 for a further 25 days at 0.1 μM methotrexate, followed by 19 days at 0.5 μM and finally 13 days at 2.5 μM. High β_2-glycoprotein I secreting clones were weaned into serum free medium (Gibco; CHO-S-SFM) by sequentially decreasing the concentration of added dialysed fetal bovine serum. Cell populations in mid log growth phase were transferred to CHO-

S-SFM with 2% FBS, then 0.5% FBS and finally to CHO-S-SFM alone.

Rabbit anti-β_2-glycoprotein I sera was affinity purified by the application of 45 ml of rabbit sera (immunised against native β_2-glycoprotein I) to an Affigel-10 agarose gel (BioRad) coupled to 2.4 mg of native β_2-glycoprotein I. Affinity purified rabbit anti-β_2-glycoprotein I (1.5 mg) was coupled to 1.5 ml of Affi-prep hydrazide support according to manufacturers instructions (BioRad) and packed into a Pharmacia 5/5 column. Frozen serum free conditioned medium pooled from confluent cell cultures was thawed and the protease inhibitors traysol and *N*-ethylmaleimide added (final concentration of 1 U/ml and 1 mM, respectively). The medium was centrifuged at $3000 \times g$ for 15 min to remove cell debris and 5 M NaCl added to give a final concentration of 0.5 M. Optimal flow rate through the affinity column was 0.05 ml/min via a 50 ml superloop attached to a Pharmacia FPLC system. The column was washed with 10 column volumes of 0.5 M NaCl/PBS at 0.25 ml/min and with 0.15 M NaCl/PBS at 1 ml/min before elution with 0.1 M glycine-HCl, pH 2.5, at 0.1 ml/min. The eluted 1 ml fractions were immediately neutralized with 2 M Tris, pH 8.0, and pooled before adding sodium chloride and sodium acetate to give a final concentration of 0.05 M each for passage through a Pharmacia Mono S HR 5/5 cation exchange column as described previously [12].

A modification of the standard IgG cardiolipin ELISA with no serum-derived diluents or blocking agents was performed as previously described [12].

Peptide:*N*-glycanase F (New England Biolabs) digestions were performed with 1000 U (NEB units) of enzyme on 1.5 μg of purified recombinant β_2-glycoprotein I and 6.5 μg of native β_2-glycoprotein I. All samples were first denatured at 100°C for 10 min in 0.5% SDS and 1% mercaptoethanol before incubating at 37°C in 50 mM $NaPO_4$ buffer, pH 7.5, and 1% NP-40 for 18 h. SDS-PAGE was performed using slab gels with 10% polyacrylamide separating gel and 4% stacking gel and then silver stained. Western blotting was performed using Towbin transfer buffer overnight at 4°C and probed with 1/1000 dilution of affinity purified rabbit anti-β_2-glycoprotein I and 1/1000 dilution of sheep anti-rabbit alkaline phosphatase conjugated IgG.

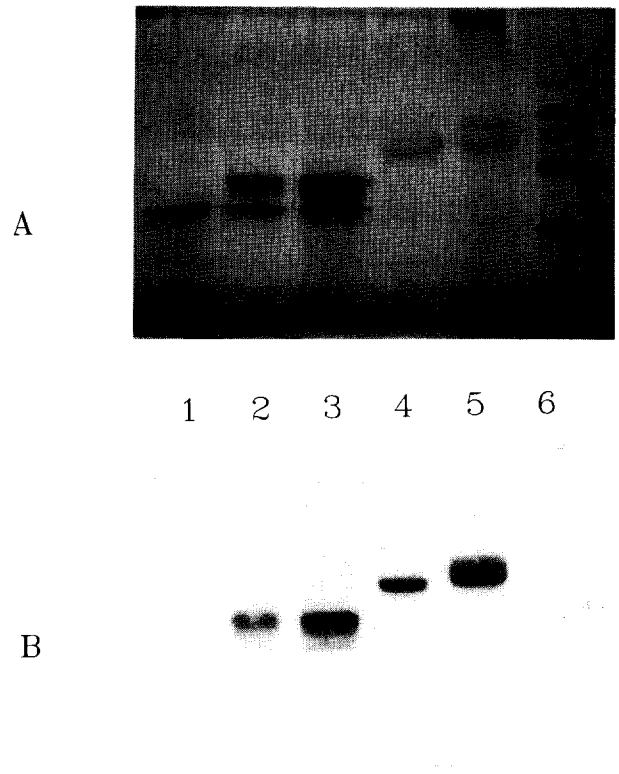

Fig. 1. SDS-PAGE (A) and Western blotting (B) analysis of β_2-GPI. Purified recombinant (lanes 3,5) and native β_2-GPI (lanes 2,4) before (lanes 4,5) and after deglycosylation (lanes 2,3). PNGase F alone (lane 1) and molecular weight markers (lane 6; 97,66,43,31,21 kDa).

3. RESULTS

Five pB2C6 transfected DG44 clones secreted β_2-glycoprotein I into serum free culture medium at a rate between 0.25 and 2.9 $\mu g/10^6$ cells/day in the absence of methotrexate. Recombinant β_2-glycoprotein I was purified from clone Y1B by immunoaffinity and elutes in the same fraction as native β_2-glycoprotein I during carion exchange chromatography. The yield of eluted β_2-glycoprotein I from the affinity column determined by radioimmunoassay was 60% but decreased to 44% after cation exchange.

Amino-terminus sequencing performed at Biotech Australia confirmed that the first 18 amino acids of the purified protein from the affinity column corresponded to β_2-glycoprotein I. Following cation exchange chromatography, two protein bands are revealed by SDS-PAGE and Western blot analyses (Fig. 1). Recombinant β_2-glycoprotein I gave a 61 kDa and a diffuse band of 50–56 kDa, both bands being larger than native β_2-glycoprotein I (49 kDa). They appear to be glycoforms as digestion with peptide:*N*-glycosidase F which cleaves all *N*-linked oligosaccharides from asparagine residues resulted in a single diffuse band of 36–42 kDa, the main portion of which co-migrates with similarly digested native β_2-glycoprotein I (40 kDa). This size coincides with the deglycosylation study of Walsh et al. [18].

In the modified IgG cardiolipin-ELISA, anti-cardiolipin antibodies from five autoimmune patients purified using affinity and cation exchange chromatography [14] had an absolute requirement for recombinant β_2-glycoprotein I in order to bind cardiolipin (Fig. 2). Binding was dose dependent up to 8 μg/ml, similar to native β_2-glycoprotein I (Fig. 3). In contrast, anti-cardiolipin fractions purified by cation exchange from one patient with infection bound cardiolipin without the need for recombinant β_2-glycoprotein I (Fig. 2) and was not dose responsive (Fig. 3).

4. DISCUSSION

Anti-phospholipid antibodies found in SLE and related disorders are associated with thrombotic disease. The finding that β_2-glycoprotein I constitutes part of the antigen targetted by these antibodies has raised interest in the nature of the epitope to which these auto-antibodies bind. Recently, there is evidence to suggest a lipid binding site at the carboxy-terminus of β_2-glycoprotein I [19,22]. The analysis and expression of mutants would delineate the sequences involved and we show here that the transfected Chinese hamster ovary cell line can syn-

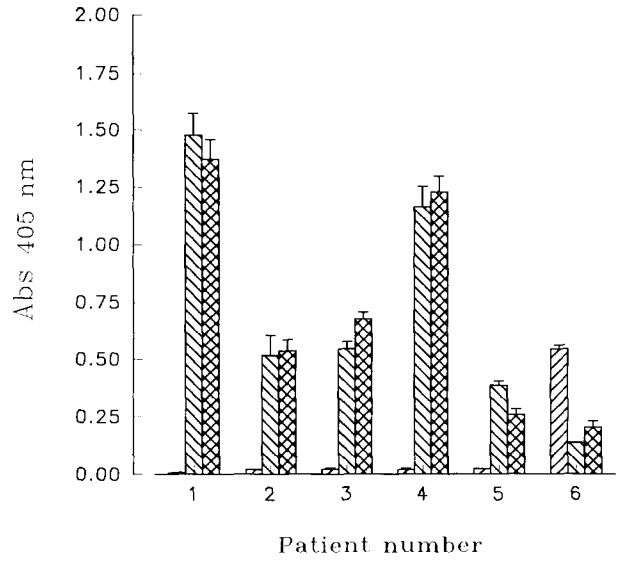

Fig. 2. Modified IgG CL-ELISA (4 μg/ml aCl) for autoimmune (patients 1–5) and infection group (patient 6) with and without β_2-GPI (8 μg/ml). ▨, no β_2-GPI; ▧, native β_2-GPI; ▩, recombinant β_2-GPI. Results are the mean ± S.E.M. of 3 samples.

thesize β_2-glycoprotein I with cofactor activity and thus provide the means to accurately express β_2-glycoprotein I mutants.

The difference in *N*-linked glycosylation between recombinant and native β_2-glycoprotein I appears not be significant with respect to cofactor activity. Anti-cardiolipin antibodies from autoimmune patients had an identical requirement for recombinant β_2-glycoprotein I to bind cardiolipin in ELISA whilst infective type aCL antibodies did not, both findings previously described for native β_2-glycoprotein I [14,15]. In addition, there is evidence to suggest that carbohydrate residues may not be important for anti-cardiolipin cofactor activity as all isoelectric subspecies of β_2-glycoprotein I with wide ranging p*I* have significant cofactor activity [19]. Most anticardiolipin antibodies, however, have a preference for human compared to bovine β_2-glycoprotein I [20] despite high homology (84%). A number of human β_2-glycoprotein isoforms have been described and there may be differences in the cofactor requirement of anti-cardiolipin antibodies for particular isoforms.

Oligosaccharide processing amongst mammalian cells is species and cell type dependent and is also influenced by the cell culture environment (reviewed in [21]). We are investigating whether the oligosaccharide structure of β_2-glycoprotein I differs when grown in serum containing medium and whether there are glycosylation differences between autoimmune and normal type β_2-glycoprotein I.

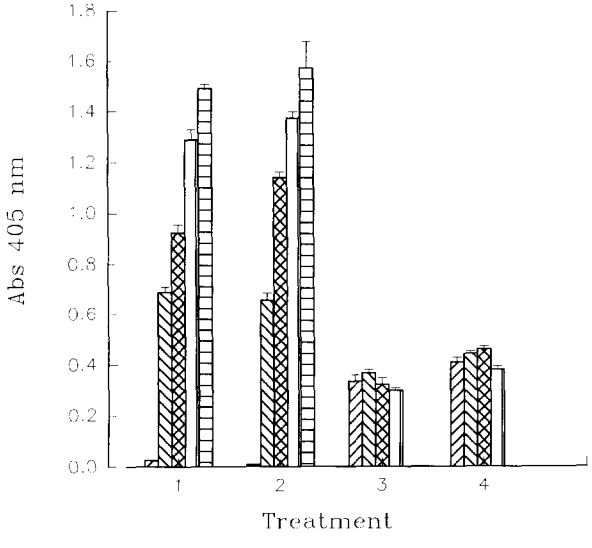

Fig. 3. Dose-response of recombinant (1,3) and native (2,4) β_2-GPI at (▨) 0 μg/ml, (▧) 1 μg/ml, (▩) 2 μg/ml, (▥) 4 μg/ml, (▤) 8 μg/ml against aCl (4 μg/ml) from one autoimmune patient (1,2) and one infection patient (3,4). Results are the mean ± S.E.M. of 3 samples.

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