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A Major Neutralizing Domain Maps within the Carboxyl-terminal Half of the Cleaved Cytomegalovirus B Glycoprotein

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SUMMARY

Cytomegalovirus (CMV) encodes several glycoproteins reported to be structural homologues of glycoproteins encoded by herpes simplex virus type 1 (HSV-1). To map the antigenic and functional domains on the 907 amino acid CMV glycoprotein B (gB), we cloned and expressed a subfragment of *Bam*HI fragment R of the CMV (Towne) genome into an expression vector and reacted the resulting gene product with a panel of monoclonal antibodies. Our results showed that the DNA fragment encodes related glycoproteins which we previously designated gA and which others have reported to be homologous to HSV-1 gB in CMV (AD169). Analyses of the processing of CMV gB transiently expressed in eukaryotic cells showed that glycosylation occurred independently of viral infection. Ten antibodies with complement-dependent and independent neutralizing activity reacted with a truncated derivative of gB that contained 619 amino-terminal residues but lacked the transmembrane and intracellular regions of the molecule. Twelve additional antibodies reacted with a CHO cell line expressing a 680 amino-terminal derivative of gB. All of the reactive antibodies precipitated the 447 residue carboxy-terminal cleavage product of gB from extracts of CMV-infected cells. These results showed that the neutralizing epitopes map in at least two domains of gB which are located in a discontinuous segment of 219 amino acids between residues 461 and 680 from the amino terminus of the molecule.

Cytomegalovirus (CMV) is a human herpesvirus which causes life-threatening disease in immunocompromised patients and is a leading cause of birth defects (for review see Meyers, 1985; Stagno *et al.*, 1982, 1983). Several CMV glycoproteins have been identified in immune precipitates obtained with convalescent-phase sera (Cremer *et al.*, 1985; Middeldorp *et al.*, 1985; Pereira *et al.*, 1982a, 1983) and with monoclonal antibodies (Britt & Auger, 1985; Gretch *et al.*, 1988; Kari *et al.*, 1986; Pereira & Hoffman, 1986; Pereira *et al.*, 1982b, 1984; Rasmussen *et al.*, 1985b, 1984). Of particular interest is a family of polymorphic, antigenically related glycoproteins that elicit neutralizing antibodies (Britt, 1984; Pereira *et al.*, 1984; Rasmussen *et al.*, 1985a). A gene reported to encode these glycoproteins has been mapped on the genome of CMV (strain AD169) and has been shown to be homologous to the herpes simplex virus type 1 glycoprotein B gene (Cranage *et al.*, 1986; Mach *et al.*, 1986). Analysis of the nucleotide sequence predicted that the 907 amino acid gene product is cleaved to generate a 447 amino acid fragment from the carboxy-terminal half of the glycoprotein (Spaete *et al.*, 1989). In the present study, we cloned the *Bam*HI fragment R from CMV (strain Towne) corresponding to the region

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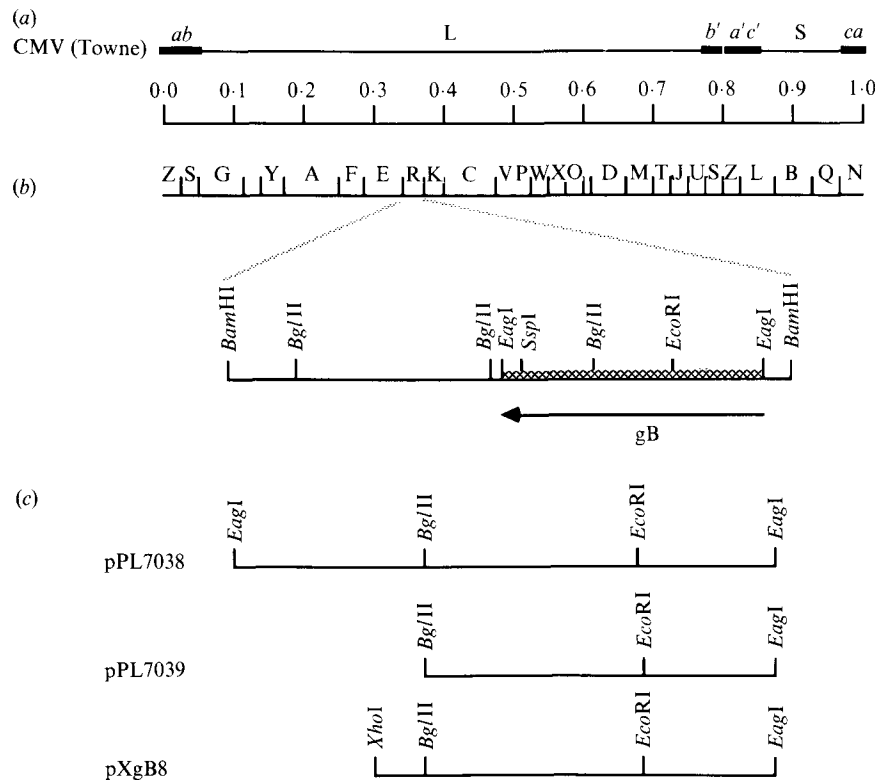


Fig. 1. (a) Schematic diagram of the CMV (Towne) genome and location of the DNA fragment used in this study. The thin lines represent the unique sequence of the long (L) and short (S) components whereas the filled rectangles represent the terminal sequences *ca* and *ab* internally repeated as the inverted sequences *b'a'c'* (a) (Mocarski *et al.*, 1985). (b) Map of *Bam*HI restriction fragments and the expanded scale of *Bam*HI fragment R, which contains the coding sequence for the CMV gB gene. The arrow indicates the direction of transcription (Cranage *et al.*, 1986; Mach *et al.*, 1986). (c) Construction of plasmid pRB7030 by subcloning the *Eag*I fragment, which contains the entire gB coding region, from *Bam*HI fragment R into the *Eag*I site of pMT11(*Eag*I). Plasmid pPL7038 was constructed by inserting the gB gene from pRB7030 into the *Bgl*II site in the proper transcriptional orientation of the expression vector p91023B. Plasmid pPL7039 was constructed by cloning the *Bgl*II subfragment from pRB7030 into the *Bgl*II site of p91023B. The construction of pXgB8 has been described (Spaete *et al.*, 1989).

of the genome that encodes the HSV-1 gB homologue and expressed the gene product transiently in eukaryotic cells to map the epitopes of monoclonal antibodies to a family of CMV glycoproteins.

Fig. 1 illustrates the *Bam*HI restriction map of the CMV (Towne) genome and the plasmids constructed to express gB (a, b and c). The fragment that contains the coding sequence corresponding to the region containing gB in the CMV (AD169) genome was excised with *Eag*I from the *Bam*HI fragment R of strain Towne and ligated to the vector pMT11(*Eag*I) to create pRB7030 (b) [pMT11(*Eag*I) had been made by digesting pMT11 with *Ava*I, filling in ends with Klenow polymerase and ligating to form an *Eag*I site.] The CMV gB gene was transferred into pPL7038 by cloning the *Eag*I subfragment from pRB7030 in the appropriate orientation into the *Bgl*II site of the expression vector p91023B which contains the adenovirus late promoter, VA genes, and simian virus 40 origin (Wong *et al.*, 1985) (c). A truncated derivative, pPL7039, was constructed by cloning the *Eag*I–*Bgl*II subfragment which encodes the amino-terminal 619 residues of gB into p91023B (c). The derivative (gB₆₁₉) encoded approximately two-thirds of the amino-terminal region of gB but lacked the hydrophobic transmembrane sequence and the

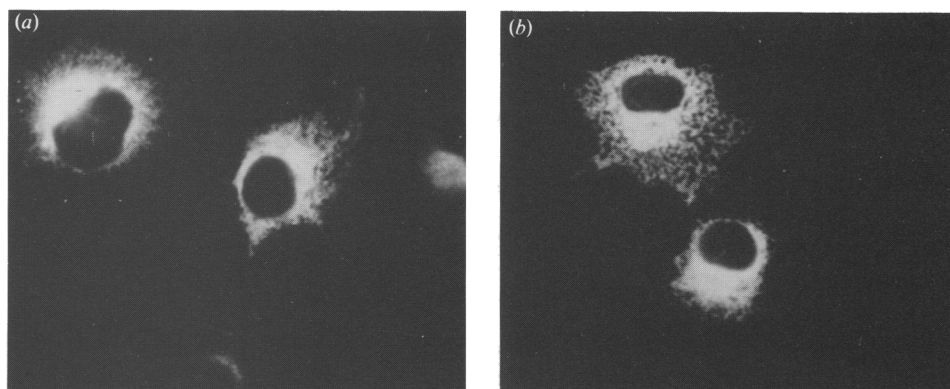


Fig. 2. Immunofluorescence staining of CMV (Towne) gB transiently expressed in COS-1 cells transfected with plasmid DNA containing the intact gB gene (a) and the truncated derivative of gB (b).

carboxy terminus of the intact molecule. The construction of plasmid pXgB8 and the properties of CHO cell line 67, a methotrexate-amplified derivative of line 9-14 that stably expresses the amino-terminal 680 amino acids of CMV (Towne) truncated gB, have been reported (Spaete *et al.*, 1989).

Our next step was to express CMV (Towne) gB and to map the antigenic domains on the glycoprotein with a panel of monoclonal antibodies. For these experiments, COS-1 cells were transfected with plasmid DNA containing the intact gB and gB_t genes using published procedures (Graham & van der Eb, 1973). After 48 h, the transfected cells were assayed for expression of the gene products by immunofluorescence after acetone fixation using a mixture of antibodies previously shown to react with a family of CMV glycoproteins (CH45, CH87, CH28, CH407 and CH51) (Pereira *et al.*, 1982*b*, 1984) and with fluorescein-conjugated anti-mouse immunoglobulin. Fig. 2(a) shows the pattern of cytoplasmic immunofluorescence and perinuclear Golgi localization obtained by staining cells expressing the intact glycoprotein with the antibodies. These results showed that the CMV (Towne) genome indeed encodes this family of related glycoproteins and that the CMV gB homologue in strain Towne maps in the same region as in the AD169 genome. Analysis of cells expressing gB_t (Fig. 2b) also showed cytoplasmic fluorescence as did reactions with line 67 (data not shown).

We and others have reported that the major CMV glycoprotein is processed from a precursor polypeptide (95K) into heterogeneous, more slowly migrating forms (107K to 160K) (Pereira *et al.*, 1984; Rasmussen *et al.*, 1988). We noted also that an antigenically related, faster migrating form (58.5K) was generated, apparently by post-translational cleavage of the larger forms. To characterize the processing of CMV gB in the absence of other viral gene products, we did a series of experiments using transfected cells. Fig. 3 shows the polypeptides precipitated by reacting antibodies with extracts of cells transfected with the intact CMV gB gene and labelled with [³⁵S]methionine in medium with monensin or tunicamycin or medium alone (a). Cells were extracted in buffer containing 1% NP40 and sodium deoxycholate and 10⁻³ M each of *N*-*p*-tosyl-L-lysine chloromethyl ketone and L-1-tosylamide-2-phenylmethyl chloromethyl ketone. Cell extracts were incubated with a pool of monoclonal antibodies to CMV gB and adsorbed to Protein A-Sepharose beads. Samples were denatured in 2% 2-mercaptoethanol, 1% SDS boiled and electrophoresed in 9% polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide. The protein (pgB) made in tunicamycin-treated cells was approximately 95K and a more slowly migrating (140K to 160K) form was precipitated from untreated transfected cells (Fig. 3a, lane 2). The present results agree with studies on CMV-infected cells, show that glycosylation of gB is carried out by cellular enzymes, and indicate that many of the *N*-linked glycosylation sites are utilized. Analysis of the precipitates obtained with monensin-treated cells showed that the processing of gB was not significantly altered by the drug (Fig. 3a, lane 3). Since monensin is

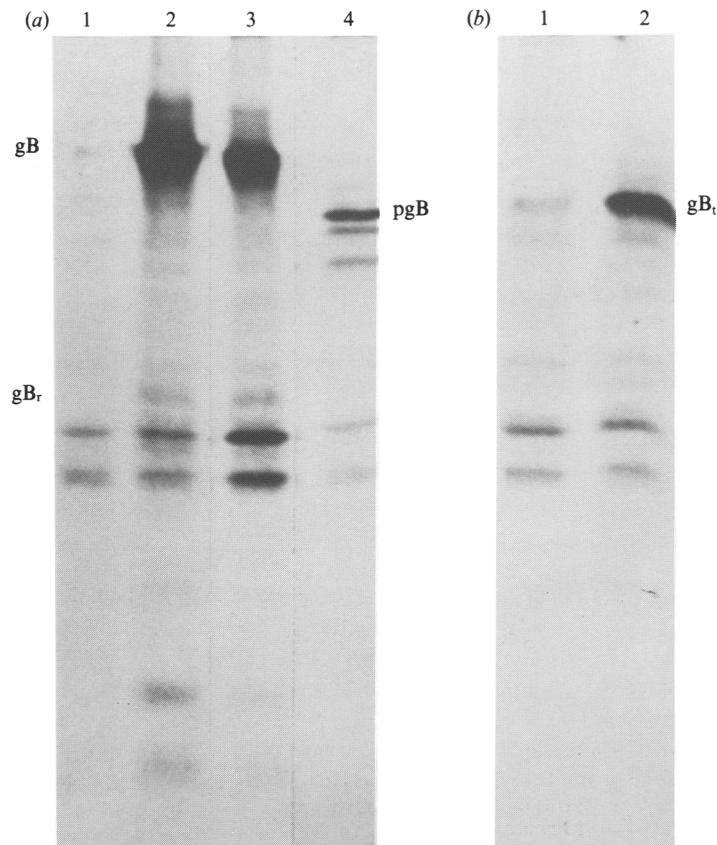


Fig. 3. Autoradiograms of electrophoretically separated polypeptides in immune precipitates obtained with monoclonal antibodies to CMV gB (a) and the truncated derivative of gB (b) transiently expressed in COS-1 cells. (a) After transfection with the intact gene, cells were pretreated with 10 μ g/ml tunicamycin (lane 4) or 1 μ M-monensin (lane 3) for 2 h, or untreated (lane 2), then labelled from 44 to 48 h after transfection with 100 μ Ci/ml of [35 S]methionine (sp. act. 1106 Ci/mmol) in methionine-free medium. (b) Lane 2 shows immune precipitates obtained with the truncated derivative of CMV gB (gB_t) reacted with the antibody pool. gB_r is a gB-related peptide (447 amino acid carboxy terminal cleavage fragment). pgB is the partially glycosylated precursor of gB. Lanes 1 show the control reaction with negative ascites.

known to inhibit the addition of *O*-linked oligosaccharides causing membrane proteins to accumulate in Golgi-derived vacuoles (Johnson & Spear, 1983; Uchida *et al.*, 1979), our findings indicate that CMV gB may not contain appreciable amounts of *O*-linked carbohydrate.

We next studied the properties of the truncated derivative gB_t that contained the amino-terminal 619 amino acids of gB but lacked the carboxy terminus and transmembrane domains. In contrast to the results obtained with the intact gB gene, a single band of 105K was precipitated with a pool of antibodies from COS-1 cells transfected with the gB_t gene (Fig. 3b). A protein of this size would be predicted by partial glycosylation of the polypeptide encoded by this derivative. Although gB_t lacks the membrane anchor sequence, we failed to detect an appreciable amount of the truncated protein in the medium in this experiment.

We previously described the properties of a panel of 40 monoclonal antibodies to CMV gB (then designated gA) (Pereira & Hoffman, 1986; Pereira *et al.*, 1984). The antibodies were divided into five groups based on neutralization assays, reactivity with the denatured glycoprotein, and with a set of cleavage fragments generated by *Staphylococcus aureus* protease V-8. To map the antigenic domains of gB, we tested each of these antibodies by immunofluorescence for reactivity with cells transfected with plasmid DNA containing CMV

Table 1. Reactions of monoclonal antibodies with CMV (Towne) gB and truncated derivatives containing amino-terminal residues of gB*

Group	Monoclonal antibody	Neutralizing activity		Immunofluorescence			Immune precipitate†
		-C'	+C'	gB 907 aa	gB _{t(1)} 619 aa	gB _{t(2)} † 680 aa	C-terminal 461-907 aa
3	87-1	-	+	+	+	+	+
	92-1	-	+	+	+	+	+
	143-13	-	+	+	+	+	+
	177-3	+	+	+	+	+	+
	253-1	-	+	+	+	+	+
	358-5	-	-	+	+	+	+
	388-2	-	+	+	+	+	+
	424-1	-	+	+	+	+	+
	432-1	-	+	+	+	+	+
	442-1	-	+	+	+	+	+
2	105-7	-	+	+	-	+	+
	112-1	-	+	+	-	+	+
	130-9	-	+	+	-	+	+
	244-4	-	+	+	-	+	+
	382-2	+	+	+	-	+	+
	395-1	-	+	+	-	+	+
	409-2	-	-	+	-	+	+
	434-1	-	+	+	-	+	+
	436-1	-	+	+	-	+	+
	446-1	-	+	+	-	+	+
1	51-4	-	+	+	-	+	+
	114-5	-	+	+	-	+	+

* Abbreviations: C', complement; aa, amino acids.

† pXgB8 (Spaete *et al.*, 1989).‡ Pereira *et al.* (1984).

(Towne) gB, the truncated 619 amino acid derivative gB_{t(1)}, and CHO line 67 producing the 680 amino acid derivative gB_{t(2)}. We found that 33 of 40 antibodies reacted with cells transfected with the intact gB gene (data not shown). Immunofluorescence analyses revealed that 10 of 40 antibodies recognize gB_{t(1)} and that 22 antibodies react with line 67 producing gB_{t(2)} (Table 1). All of the antibodies precipitate the 447 amino acid carboxy-terminal cleavage fragment of CMV gB previously designated gA6 and recognize discontinuous epitopes (Pereira & Hoffman, 1986; Pereira *et al.*, 1984). Eighteen antibodies had complement-dependent neutralizing activity and two neutralized without complement.

The results of the present study are summarized in Fig. 4 which shows a topographic map of the epitopes on CMV gB. The glycoprotein contains at least two neutralizing domains mapping within a segment of 219 amino acids between residue 461, the first amino acid of the carboxy-terminal cleavage fragment of gB, and residue 680, the last amino acid in gB_{t(2)}. Domain 1 which is located between residues 461 and 619 contains 158 amino acids. Domain 2 maps within the proximal 61 carboxy-terminal amino acids and has been subdivided into two groups based on the reactivity of antibodies with *S. aureus* protease V-8 cleavage fragments (Pereira & Hoffman, 1986). The proximity of domains 1 and 2 suggests that they are assembled into a major discontinuous domain and that both are required to form the neutralizing epitopes mapping in this portion of the carboxy-terminal half of the glycoprotein. Fine mapping the epitopes by site specific mutagenesis should resolve this question. Our results indicate that this region of the molecule is highly immunogenic and plays an important role in the function of gB in CMV infection.

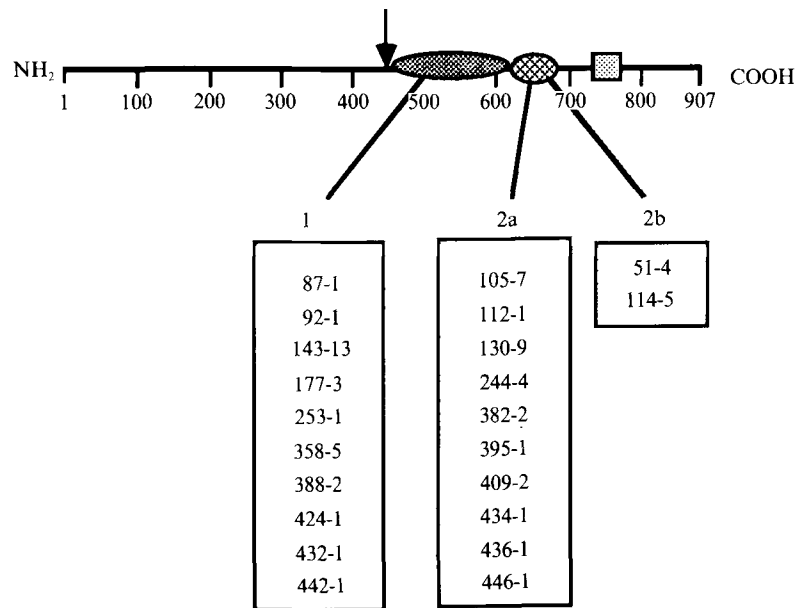


Fig. 4. Schematic representation of a topographical map of epitopes on CMV gB. Discontinuous neutralizing domains are indicated by ellipses. Amino acids are designated by numbers inclusive of the signal sequence. The map location of the site of cleavage of gB is marked by an arrow at amino acid 460 (Spaete *et al.*, 1989). Transmembrane anchor domain (box) and amino and carboxy termini are shown (Cranage *et al.*, 1986; Spaete *et al.*, 1989). Epitopes of antibodies mapping in the discontinuous neutralizing domains 1 (amino acids 461 to 619), 2a and 2b (amino acids 620 to 680) are listed. Properties of the antibodies mapped have been published (Pereira & Hoffman, 1986) and are shown in Table 1.

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