Structural and immunological characterization of human cytomegalovirus gp55-116 (gB) expressed in insect cells

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The gene encoding the major envelope glycoprotein complex, gp55-116 (gB), of human cytomegalovirus (HCMV) was expressed at high levels in insect cells utilizing a recombinant baculovirus. The mature intracellular form of the insect-derived gp55-116 was a protein of Mr 150K which contained approximately 50K of N-linked oligosaccharides. The oligosaccharide linkages were almost exclusively endoglycosidase H-sensitive. The 150K protein was processed, presumably by proteolytic cleavage, to yield at least one of the previously defined cleavage products of the gp55-116. This processing step was significantly less efficient in insect cells than the analogous step in mammalian cells. Finally, the insect-derived gp55-116 was highly immunogenic in experimental animals and readily recognized by antibodies contained within HCMV-immune human serum, suggesting that this recombinant protein warrants further study as a potential HCMV subunit vaccine candidate.

Introduction

Human cytomegalovirus (HCMV), the largest of the human herpesviruses, is a well recognized pathogen in humans. Immunocompromised allograft recipients suffer severe and sometimes fatal infections with HCMV in the post-transplant period (Ho, 1982). Recent reports have stressed the importance of HCMV in patients with human immunodeficiency virus infections (Drew & Mintz, 1984). In addition, HCMV is the most common cause of congenital viral infections with an annual incidence of 1% in the U.S.A. (Alford et al., 1980; Stagno et al., 1983). Although only 5 to 10% of congenitally infected infants suffer demonstrable sequelae following this intra-uterine infection, this represents an estimated 2000 damaged infants every year in the U.S.A. (Alford et al., 1980; Stagno et al., 1983). Protective immune responses against HCMV are as yet undefined; however, numerous immune effector functions have been described following HCMV infection (Bets & Schmidt, 1981; Ho, 1982; Meyers et al., 1980; Quinnan et al., 1982). Recently investigators have attempted to study the virus-encoded targets of these responses (Borysiewicz et al., 1988; Forman et al., 1985; Liu et al., 1988; Van Der Voort et al., 1989). Several reports have focused attention on the envelope glycoproteins because of their immunogenicity as well as their surface expression on infectious virions and virus-infected cells (Liu et al., 1988; Van Der Voort et al., 1989).
yotic recombinant expression systems have been utilized (Britt et al., 1988; Cranage et al., 1986; Spaete et al., 1988). At this time we are unaware of any report which details the efficient large scale production of this material from a recombinant system.

The use of baculovirus-based expression systems has been shown to be useful in the isolation of large quantities of recombinant proteins (Fraser, 1989). Many mammalian viral genes have been successfully expressed in baculovirus at surprisingly high levels (Estes et al., 1987; Hu et al., 1987; Inumaru & Roy, 1987; Matsuura et al., 1986; Murphy et al., 1988; Zhang et al., 1988). Unfortunately, insect cells do not always authentically glycosylate and process the heterologous protein, resulting in aberrant forms with altered immunogenicity. Because this result cannot be predicted in advance, we expressed the HCMV gp55-116 gene in a baculovirus and analysed its processing and immunogenicity. Although the insect cell-derived HCMV gp55-116 was not glycosylated and processed authentically, it retained its immunogenicity. This expression system should prove invaluable for production of therapeutic and diagnostic HCMV reagents.

**Methods**

Cells and virus. *Spodoptera frugiperda* cells, clone SF9 (SF), were maintained in Grace’s medium supplemented with 10% (v/v) foetal bovine serum, 100 units/ml penicillin and 50 μg/ml gentamicin. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) was obtained from Dr Max Summers, Texas A & M University, College Station, Tex., U.S.A. Wild-type viral DNA was purified by the method of Summers & Smith (1987). The construction of the recombinant vaccinia virus containing the intact HCMV gp55-116 (VVCMV) has been described previously (Britt & Auger, 1986). Polyhedron-negative plaques were identified and analysed by light microscopy. At this time the supernatant was picked using a Pasteur pipette. Initially, screening was carried out with 50 μl of medium containing suspected recombinant baculovirus. Following a 5 day incubation period, the plates were fixed in absolute ethanol and recombinant-positive plaques were identified by an immunofluorescence assay using murine monoclonal antibody 7-17 and fluorescein isothiocyanate-conjugated anti-mouse IgG (Britt, 1984). Plaques expressing gp55-116 were subjected to three cycles of plaque purification prior to use. One plaque-purified virus, Bae 3.1, was selected for further characterization.

Radiolabelling of Bac 3.1 or wild-type-infected SF cells. Cultures of SF9 cells were infected with either Bac 3.1 or wild-type AcMNPV at an m.o.i. of approximately 10 to ensure uniform infection of the monolayer. Approximately 36 h later, the infected cells were washed extensively with TN medium without methionine and pulse-labelled with 50 μCi/ml of [35S]methionine. Pulse–chase analyses were carried out essentially as described (Britt & Auger, 1986) with a pulse period of 5 or 10 min followed by removal of the radioactive medium and replacement with Grace’s medium. Radiolabelling with [3H]glucosamine or [3H]mannose was done 36 h following infection using approximately 50 μCi/ml of radioactive sugar in an 8 h pulse labelling.

Inhibition of N-linked glycosylation was accomplished by incubation of infected SF cells in medium containing 1 μg/ml of tunicamycin (TM; Boehringer Mannheim) for 90 min in TN medium without methionine. The medium was replaced with one containing 1-0 μg/ml TM and 50 μCi/ml of [35S]methionine and labelling was carried out for 4 to 6 h. Radioisotope incorporation was monitored by measurement of trichloroacetic acid-precipitable counts following solubilization of infected cell proteins.

Immunoprecipitation, SDS-PAGE and Western immunoblotting. Following radiolabelling, individual cultures were rapidly frozen at −70 °C and then thawed. Infected cell proteins were solubilized in RIPA buffer (1-0%, Nonidet P40, 1-0%, deoxycholate, 0-1% SDS, 0-05 M-Tris-HCl, 0-15 M-NaCl pH 7-4) at 4 °C for 20 min. Immunoprecipitation with murine monoclonal antibodies, collection of immune complexes with *Staphylococcus aureus* Cowan I strain and analysis by SDS–PAGE in 7.5% acrylamide gels have been previously described (Britt, 1984; Britt & Auger, 1986). SDS–PAGE and estimation of migration using Mr standards was carried out as described by Brett (1984).

Western immunoblotting was done as described by Britt & Auger (1986). Samples for Western immunoblotting were prepared by immediate solubilization of infected cell pellets in disruption buffer (2% SDS, 5% 2-mercaptoethanol, 0-15 M-Tris–HCl pH 8-0) followed by heating to 100 °C for 3 min.

Endoglycosidase treatment. Endoglycosidase H (Endo H, endo-β-N-acetylglucosaminidase H; Boehringer Mannheim) treatment of immune-precipitated protein was performed as described previously (Britt & Vugler, 1989).

Neutralization assays. Neutralizing activity was assayed by a rapid (16 h) immunofluorescence assay (Andreoni et al., 1989). Briefly, 0-2 ml of mouse serum diluted in medium was added to 0-2 ml of titrated virus containing 10% (v/v) guinea-pig complement. Following a 60 min incubation at 37 °C, 0-1 ml of the mixture was added to replicate wells of a 96-well microtitre tissue culture plate containing SF cells. After an approximate 2 h adsorption period, the inoculum was removed, the monolayers were washed once with medium, fresh medium was added and the plate was incubated overnight at 37 °C. The following day the medium was removed, the monolayer washed once with phosphate-buffered saline (PBS, pH 7-4) and fixed in absolute ethanol. Following fixation, the monolayers were stained with monoclonal antibody P63-27 (Andreoni et al., 1989), which is reactive with the major HCMV immediate protein, pp72, and the number of antigen-positive cells was quantified by immunofluorescence. Results are expressed as the mean percentage reduction of fluorescent nuclei (infectivity). The standard error of the mean of this assay is at most 10%, with usual values of 5%.
Results

Expression of HCMV gp56-116 in insect cells

Protein expression of recombinant baculovirus-infected cells was monitored by immunofluorescence using HCMV gp55-116 specific monoclonal antibodies. Cells infected with recombinant virus Bac 3.1, which contained the intact gene encoding HCMV gp55-116, exhibited bright immunofluorescence indicating expression of this HCMV protein in insect cells (data not shown). We next examined the expression of HCMV gp55-116 in insect cells by subjecting equivalent quantities of [35S]methionine-radiolabelled infected cell proteins from recombinant virus 3-1- or wild-type AcMNPV-infected cells to SDS-PAGE. Bac 3.1-infected cells contained a protein of estimated Mr 150K which was not seen in wild-type-infected cells (Fig. 1). The level of expression was significant as it accounted for an estimated 10% of the infected cells' [35S]methionine-radiolabelled proteins (Fig. 1). Immunological characterization of the insect cell-derived gp55-116 was carried out by immunoblotting of wild-type- and 3.1-infected cells using HCMV gp55-specific monoclonal antibody 7-17 (Britt & Auger, 1986). As controls, VVCMV (Britt et al., 1988) or recombinant vaccinia virus-infected cells expressing the human immunodeficiency virus gag-pol gene products (VVHIV) were included in the experiments. Monoclonal antibody 7-17 recognized a protein of approximate Mr, 150K (gp150) expressed by Bac 3.1 and a more broadly migrating species of Mr, 150K to 170K in VVCMV-infected cells (Fig. 2). Faster migrating species of Mr, 55K (gp55) and Mr, 52K (gp52) were seen in VVCMV-infected and Bac 3.1-infected cells, respectively (Fig. 2). As previously reported, monoclonal antibody 7-17 recognized a single species of Mr, 55K in gradient-purified HCMV virions (Fig. 2). These results were consistent with earlier studies and indicated that at least two of the four previously defined intracellular forms of the gp55-16 complex were present in Bac 3.1-infected insect cells (Britt & Auger, 1986). The increased rate of migration of the gp52 in baculovirus-infected cells, as compared to the migration of gp55 in VVCMV-infected cells, suggested that post-translational modifications of this protein may be different in insect cells compared to mammalian cells. Furthermore, the relative amount of gp52 compared to the higher Mr, form, gp150, in Bac 3.1-infected insect cells was much reduced as compared to near equivalent amounts of the two forms seen in VVCMV-infected cells, suggesting that cleavage of the precursor protein was less efficient within insect cells.

Processing of the HCMV gp55-116 (gB) in insect cells

The synthesis of the HCMV gp55-116 in insect cells was studied by pulse-chase analysis followed by immune
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Fig. 3. Pulse-chase analysis of Bac 3.1-infected cells (a) Bac 3.1- or (b) wild-type (wt)-infected cultures were pulse-labelled with [35S]methionine for 10 min and chased for the following time periods: lane 1, 0 min; 2, 10 min; 3, 20 min; 4, 40 min; 5, 80 min; 6, 160 min; 7, 320 min; 8, 18 h. Infected cell proteins were precipitated with antibody 7-17 and analysed by SDS–PAGE.

Precipitation and SDS–PAGE. Monoclonal antibody 7-17 precipitated a protein of estimated Mr 150K following a 10 min pulse labelling (Fig. 3). During the ensuing chase periods there was a slight but perceptible increase in the Mr of this form suggesting some type of post-translational modification(s) (Fig. 3). Approximately 80 min into the chase period, the gp52 was detected (Fig. 3, lane 5) and this species increased in quantity during the next two chase intervals. In addition, 160 min into the chase period a faint but detectable, broadly migrating form of estimated Mr 80K to 100K was also seen (Fig. 3, lane 6). This could possibly represent the insect cell form of the second component of the mature complex, gp116. A prolonged chase period of 18 h revealed the gp150 form and trace quantities of the gp52 form (Fig. 3, lane 8). These results suggested that the approximate Mr 150K protein initially seen was modified, possibly by oligosaccharide trimming, until a slightly faster migrating form, gp150, was generated. In addition, the previously recognized gp52 was apparently produced by a post-translational event, most likely by proteolytic cleavage of the gp150.

The carbohydrate modification of insect cell-derived gp55-116 was investigated next. The predominant

Fig. 4. SDS–PAGE analysis of HCMV gp55-116 precipitated from recombinant baculovirus-infected insect cells. Equivalent TCA-precipitable counts of (a) wild-type- or (b) recombinant virus (Bac 3.1)-infected cell proteins radiolabelled with either [3H]glucosamine (lanes 1 and 3) or [3H]mannose (lanes 2 and 4) were immune-precipitated with monoclonal antibody 7-17 and analysed by SDS–PAGE.

Fig. 5. Effect of TM on synthesis of HCMV gp55-116 in insect cells. (a) Equivalent TCA-precipitable counts of recombinant virus (lanes 1 and 3) or wild-type- (lanes 2 and 4) infected [35S]methionine-radiolabelled cell proteins from cultures treated with TM or left untreated (control) were subjected to SDS–PAGE. The arrow indicates the unique species present in the TM-treated cultures. (b) Infected cell proteins from Bac 3.1-infected cultures treated with TM or left untreated (control) were immune-precipitated with antibody 7-17 and analysed by SDS–PAGE.
Intracellular form, gp150, contained both glucosamine and mannose as evidenced by immunoprecipitation of this form following radiolabelling of infected cell proteins with either \(^{3}H\)glucosamine or \(^{3}H\)mannose (Fig. 4). We failed to detect incorporation of either radiolabelled sugar into the gp52, most likely owing to the low carbohydrate content of this cleavage product and inefficiency of cleavage of the gp150 in insect cells.

Table 1. Immunogenicity of insect-derived gp55-116

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<tr>
<th>Mouse†</th>
<th>1:100 Reduction in infectivity* (%) at serum dilutions of</th>
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<td>Control</td>
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* Reduction of input virus (neutralization) was determined using a 16 h fluorescent nuclei reduction assay as described in Methods. Results are expressed as percentage reduction at indicated serum dilution.
† Individual mice were given \(10^6\) Bac 3.1-infected SF cells emulsified in complete Freund's adjuvant followed 10 days later with a similar amount of cells emulsified in incomplete Freund's adjuvant. Mice were bled 7 days later and serum was assayed as above. Control mice were immunized with a prokaryotic cell-derived HCMV DNA-binding protein emulsified in complete Freund's adjuvant.

(Britt, 1984; Britt & Vugler, 1989). To determine the extent of glycosylation, Bac 3.1-infected cells were radiolabelled with \(^{35}S\)methionine in the presence of TM and analysed by SDS-PAGE. A protein of estimated \(M_r 100K\) could be seen in cultures incubated in TM and this species was specifically immunoprecipitated with monoclonal antibody 7-17 along with minor, more slowly migrating proteins which were probably products of incomplete inhibition of \(N\)-linked glycosylation by TM (Fig. 5). Thus, the insect cell-derived gp150 precursor contained some 50K of \(N\)-linked sugars, a finding similar to that reported in mammalian cells (Britt & Vugler, 1989). The structure of the carbohydrate linkages present in gp150 were next characterized using Endo H. Following a pulse–chase experiment with similar chase intervals as in Fig. 3, half of the immunoprecipitated sample was treated with Endo H and the other half left untreated. The samples were then analysed by SDS-PAGE. During all chase intervals the gp150 appeared to be sensitive to Endo H, with the size being reduced to approximately \(M_r 100K\) following Endo H treatment (Fig. 6). Interestingly, at 80 min into the chase period the migration of the Endo H-treated form was retarded slightly as compared to that seen in previous chase intervals (Fig. 6). This decreased migration was also seen in the subsequent chase intervals and in the migration of species detected following a 24 h chase (Fig. 6), suggesting that at these time points some oligosaccharide side-chains were Endo H-resistant. In this experiment, the Endo H sensitivity of the cleavage product gp52 could not be determined as only trace amounts of this protein were present.
Fig. 7. Reactivity of human anti-HCMV serum with recombinant baculovirus-derived HCMV gp55-116 (gB). [35S]Methionine-labelled (a) wild-type baculovirus or (b) recombinant Bac 3.1-infected cell proteins were immune-precipitated with serum from a seronegative individual (lane 1), monoclonal antibody 7-17 (lane 2) or serum specimens from individuals with seroimmunity to HCMV (lanes 3 to 6). Precipitated proteins were analysed by SDS-PAGE as described in Methods.

Immunogenicity of insect cell-derived HCMV p55-116

The immunogenicity of the insect cell-derived gp55-116 was investigated initially in vivo by injecting Bac 3.1-infected cells into mice. After two immunizations all animals developed significant quantities of neutralizing antibodies indicating the immunogenicity of this insect cell-derived protein (Table 1). The reactivity of human HCMV-immune serum against the insect cell-derived gp55-116 was next analysed by immune precipitation and SDS-PAGE. Serum from a seronegative individual failed to precipitate the insect cell-derived gp150, whereas serum specimens from four seroimmune individuals precipitated the gp150 (Fig. 7). Thus, insect cell-derived gp150 appeared to be immunogenic in experimental animals and, perhaps more importantly, was readily recognized by antibodies found in individuals with naturally acquired immunity to HCMV.

Discussion

The use of a recombinant baculovirus allowed significant levels of expression of the HCMV gp55-116 (gB) gene in insect cells. Although we have not carried out an analytical purification of the insect cell-derived gp55-116, our initial experiments have suggested that nearly 10% of the total [35S]methionine-radiolabelled infected cell protein was gp55-116. This level of expression was significantly higher than we have previously obtained using a recombinant vaccinia virus containing the HCMV gp55-116 gene (Britt et al., 1988). Furthermore, after additional modifications of the vaccinia virus transfer vector in which the HCMV gp55-116 gene was placed under control of the stronger p11 late promoter (W. J. Britt & E. B. Stephens, unpublished results), we could still demonstrate at least 10-fold more antigenically reactive intracellular gp150 in insect cells than in cells infected with the recombinant vaccinia virus. Thus, it appeared that expression of the gp55-116 in insect cells might offer a useful system for obtaining sufficient quantities of this protein for future biochemical and immunological studies. Preliminary experiments have shown that approximately 1 to 2 mg of antigenically reactive gp55-116 was obtained by passage of a detergent-solubilized lysate from 10⁸ infected SF cells over an immunoaffinity matrix (data not shown).

Maturation of the intracellular precursor of gp55-116 within insect cells involved a processing step, possibly trimming of carbohydrate side-chains, which yielded a fully modified form of estimated Mr, 150K. In contrast, processing of the gp55-116 intracellular precursor, gp150, within mammalian cells involved additional carbohydrate modification to yield a final, fully glycosylated form of estimated Mr, 170K (Britt & Vugler, 1989). In both insect cells and mammalian cells, these fully glycosylated forms were then cleaved into the mature smaller Mr polypeptides. In agreement with previous studies, we found that glycosylation of the HCMV gp55-116 within insect cells was not authentic (Hurwitz, 1988; Kuroda et al., 1986, 1987). Our results suggested that the vast majority of the oligosaccharide side-chains on the gp150 contained Endo H-sensitive linkages, whereas the majority of oligosaccharides present on the gp170 found in mammalian cells contained Endo H-resistant, complex sugars (Britt & Vugler, 1989). The demonstration of a small number of Endo H-resistant linkages on the insect cell-derived gp150 raised the possibility that previously described glycoprotein processing pathways unique to insect cells were operative (Hsieh & Robbins, 1984). We could not demonstrate endoglycosidase D-sensitive sugar linkages on either the gp150 or gp52 expressed in insect cells (data not shown), suggesting that the insect cell analogue of mammalian complex oligosaccharide linkages, mannose₂₉(N-acetylglucosamine)₂, were not present on the HCMV gp55-116 expressed in insect cells (Hsieh & Robbins, 1984). In addition, preliminary experiments also suggested that only Endo H-sensitive carbohydrate modifications were present on the gp52, providing additional evidence for the presence...
of only one type of oligosaccharide side-chains on the gp55-116 molecules (data not shown). Conversely, because we have not characterized the second component of the gp55-116 complex, gp116, following expression of the gp55-116 gene in insect cells, we cannot completely rule out the possibility that this form may contain significant quantities of Endo H-resistant sugars. This possibility must be considered in the light of previous studies in HCMV-infected mammalian cells which indicated that 20 to 30% of the mass of this protein consisted of complex oligosaccharides (Britt & Vugler, 1989). Our results, however, make this possibility unlikely as we could not detect significant quantities of Endo H-resistant oligosaccharides on fully processed gp150, the penultimate form within insect cells. Finally, resistance to either Endo H or Endo D might have occurred in a homologous site as defined previously in the gp55-116 of HCMV strain Towne (Spaete et al., 1988). Because anti-gp55 antibodies recognized only one lower Mr form, gp52, it seems likely that cleavage of the precursor occurred at a single site, and not randomly along the molecule. The availability of significant quantities of gp150 and gp52 should allow us to address this question directly in the future. Although we could demonstrate cleavage of the gp150 precursor, this processing step was much less efficient in insect cells than that observed in mammalian cells. We estimated that the cleavage product, gp52, represented at most 10% of the intracellular gp150 in insect cells, whereas the vaccinia recombinant virus-derived cleavage product, gp55, represented approximately 50% of its intracellular precursor (Fig. 2). The reason for the inefficiency in the cleavage of the precursor protein in insect cells is unknown. Previous studies utilizing recombinant baculovirus-expressed viral glycoproteins have yielded conflicting results. In some cases, endoproteolytic cleavage has been documented, such as in fowl influenza virus haemagglutinin (Kuroda et al., 1986, 1987), but not in the human immunodeficiency virus gp160 (Rusche et al., 1987) or the haemagglutinin of human influenza virus (Possee, 1986). As yet, there are no explanations for these discrepant findings, although at least one report suggested that many of the proteolytic enzymes responsible for protein maturation in mammalian cells were not present in insect cells (Lebaq-Verheyden et al., 1988). An alternative and more simplistic explanation of our results would be that the enzymic activity responsible for cleavage of the precursor was saturable and unable to process efficiently the large amount of protein present. It was also of interest that cleavage of the gp150 precursor into the smaller Mr form occurred within a similar post-labeling chase interval as that previously shown for cleavage of the gp55-116 precursor, gp170, in HCMV-infected human fibroblast cells (Britt & Auger, 1986). This finding suggested the kinetics of intracellular transport were similar in both cell systems. In addition, we have been able to demonstrate the gp55-116 on the surface of infected insect cells by two different techniques (data not shown), providing additional evidence that the intracellular transport of this glycoprotein was similar in both insect and mammalian cells. Thus, it was unlikely that inefficient cleavage of the intracellular precursor was a result of aberrant or delayed intracellular transport.

Authentic processing and glycosylation of the HCMV gp55-116 in insect cells was apparently not required for immunogenicity. Immunization of mice with Bac 3.1-infected SF cells led to the production of high levels of neutralizing antibodies. The level of neutralizing antibodies generated in these mice was comparable to the levels previously reported in mice immunized with a replicating vaccinia gp55-116 recombinant virus (Britt et al., 1988). In addition, the baculovirus-derived gp55-116 was readily recognized by human anti-gp55-116 antibodies produced following naturally acquired HCMV. Thus, it appeared the insect cell-derived gp55-116 was antigenically authentic. Because of the high level of expression of this HCMV protein in insect cells and its apparent antigenicity, this recombinant-derived product might be an important component of a future non-replicating vaccine to limit the morbidity of HCMV infections.

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References


