The gpl16 of the gp58/116 complex of human cytomegalovirus represents the amino-terminal part of the precursor molecule and contains a neutralizing epitope

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The glycoprotein complex gp58/116 of human cytomegalovirus (HCMV) represents a dominant antigen for the humoral immune response. We have used the human monoclonal antibody C23, which is capable of neutralizing HCMV in tissue culture without the addition of complement, to study the origin of gp116 as well as the amino acid sequence recognized by the antibody. Our results show that gpl16 is derived from the same open reading frame as gp58 and that it represents the amino-terminal portion of the precursor protein. Using prokaryote-expressed β-galactosidase–gpl16 fusion proteins, the binding site of C23 was located to between amino acids 27 to 84 of the amino-terminal portion of gp116. Analyses of HCMV-positive human sera revealed that this portion of the molecule is immunogenic during natural infection.

Introduction

Human herpesviruses contain a number of gene families in which the individual members are conserved in the arrangement of the respective open reading frames on the viral genomes as well as at the amino acid level of the encoded polypeptides. Among the envelope glycoproteins, gB of herpes simplex virus (HSV), gpII of varicella-zoster virus (VZV), gp110 of Epstein-Barr virus and gp58 of human cytomegalovirus (HCMV) represent members of one of these families (for review see Mach et al., 1989). When amino acid sequences of the primary translation products are compared, the proteins show approximately 20 to 30% similarity. A major difference, however, is found in the post-translational modification of the respective gene products. Glycoprotein B and gp110 represent polypeptides of approximately 125K and 110K respectively (Spear, 1985; Gong et al., 1987). The gpII complex of VZV consists of two polypeptides of 66K and 68K, respectively. The disulphide-linked components are derived from a glycosylated precursor molecule of 149K via proteolytic cleavage and represent the two halves of the precursor (Montalvo & Grose, 1987).

The genomic location of the sequence encoding gp58 was identified within the HindIII F fragment of HCMV strain AD169 (Mach et al., 1986). The open reading frame has the capacity to encode a polypeptide of 906 amino acids (aa) with a theoretical M, of 102K (Cranage et al., 1986). However, the gene product(s) have not been fully characterized. Using inhibitors of glycosylation, a primary translation product of approximately 105K has been identified which, after glycosylation to a 160K to 170K species, is proteolytically processed into a 58K protein (Britt & Vugler, 1989; Rasmussen et al., 1988). The carboxy-terminal part of the precursor molecule is represented by gp58 (Mach et al., 1986). In infected cells as well as in purified virus particles, gp58 is linked via disulphide bonds to a heterogeneous protein of approximately 116K in size (Britt, 1984). Although it seems generally accepted that the proteins are not antigenically related, the origin of gpl16 remains controversial. Data from several laboratories have indicated that the gp58/116 complex is derived from a single precursor protein and, therefore, probably represents the amino-terminal portion (Britt & Vugler, 1989; Gretch et al., 1988; Spaele et al., 1988). Others have suggested that the amino-terminal half of the precursor is degraded after cleavage and the two proteins are derived from different genes (Cranage et al., 1986; Stannard et al., 1989).

Of the HCMV glycoproteins, gp58 induces a dominant humoral immune response and is capable of eliciting neutralizing antibodies (Britt et al., 1988). A number of neutralizing monoclonal antibodies reacting with the gp58/116 complex have been isolated from murine (Britt, 1984; Kari et al., 1986; Rasmussen et al., 1985) and more recently from human (Masuho et al., 1987) sources. Using murine monoclonal antibodies, a highly conserved
linear neutralizing epitope has been identified on gp58 (Utz et al., 1989). This epitope has also been shown to be immunogenic in humans (Britt et al., 1990a). In the light of this dominant immune response and the fact that gp58 represents a likely candidate for a subunit vaccine, it is necessary to define the origin and the immunogenicity of gp116.

In this study we have used the human monoclonal antibody C23 isolated by Masuho et al. (1987) to investigate the nature of the gp58/116 complex. The results demonstrate that gp116 is derived from the amino-terminal sequence of the gp58/116 open reading frame. In addition, the binding site of C23 was identified within aa 27 to 84 of HCMV strain AD169.

**Methods**

**Virus and cell culture.** HCMV strains AD169 and Towne were propagated in human foreskin fibroblasts (HFF) by standard procedures. Extracellular virus particles were purified from tissue culture supernatant by high speed centrifugation (1 h, 10 °C, 27000 r.p.m., Beckman SW27 rotor).

**Radiolabelling of HCMV proteins.** Subconfluent monolayers of HFF cells were infected with HCMV strain AD169, at 2 p.f.u./cell. The cultures were washed twice with RPMI 1640 medium without methionine 3 days post-infection and further propagated in the same medium containing 100 μCi of [35S]methionine per ml. After 48 h, virus particles were harvested from tissue culture supernatant by centrifugation at 24000 r.p.m. for 1 h at 10 °C in a Beckman SW27 rotor. The isolated virus particles were suspended in phosphate-buffered saline containing 0.1 mM-PMSF and stored at −20 °C.

**Immunoprecipitation of HCMV proteins.** The radiolabelled HCMV particles were detergent-solubilized by addition of extraction buffer, composed of 0.1% SDS, 10% Nonidet P-40, 1% deoxycholate and 0.1 mM-PMSF in Tris-buffered saline (0.05 M-Tris-HCl, 0.15 M-NaCl pH 7.3). After incubation on ice for 15 min, the lysate was precleared with preimmune rabbit serum and Protein A-Sepharose (Pharmacia), followed by centrifugation at 3000 g for 5 min at 4 °C. For precipitation, purified anti-HCMV monoclonal antibodies or rabbit sera were mixed with the labelled precleared lysate and incubated overnight at 4 °C. The immune complexes were collected by addition of Protein A-Sepharose (final concentration 10 mg/ml, 2 h on ice) and subsequent centrifugation (3000 g, 5 min, 4 °C). After four washes with extraction buffer and four washes with high salt buffer (0.05 M-Tris–HCl, 0.5 M-NaCl, 5 mM-EDTA pH 7.3), the bound material was eluted in SDS sample buffer or an identical buffer without 5% 2-mercaptoethanol. The samples were boiled for 3 min and applied to 10% polyacrylamide gels as described (Laemmli, 1970). The gels were impregnated with 20% PPO in DMSO and autoradiographs were prepared.

**Recombinant plasmids.** Standard DNA cloning techniques were used in the construction of gp16 expression clones (Maniatis et al., 1982). All DNA fragments were derived from plasmid pBSXC4, which encodes the entire gp58/116 gene product, and were inserted into the expression vector pSEM2 (kindly provided by Dr Stefan Knapp, Behringwerke, Marburg, F.R.G.). This plasmid contains multiple cloning sites following a truncated gene encoding the first 375 aa of Escherichia coli β-galactosidase. Plasmid pMBG58 expresses aa 484 to 650 of gp58 as a β-galactosidase fusion protein.

**Synthesis of bacterial fusion proteins, SDS–PAGE and Western blot analyses.** The fusion proteins were produced in E. coli strain W3110.

**Results**

**Antibody C23 recognizes the diffuse migrating protein in the gp58/116 complex**

Antibody C23 is capable of efficiently neutralizing a variety of HCMV strains in the absence of complement. In immunoprecipitation analyses, the antibody precipitated two proteins from HCMV-infected cells of 130K and 55K respectively, indicating that the proteins recognized may constitute the gp58/116 glycoprotein complex. In the initial report, however, this was not demonstrated unequivocally. In order to define the target antigen of C23 precisely, we carried out immunoprecipitation analyses with C23 and the murine monoclonal antibody 27-287 in parallel. The epitope recognized by 27-287 has been identified in the region around aa 616 of gp58 (Utz et al., 1989). HCMV-infected HFF were labelled metabolically with [35S]methionine, extracellular HCMV particles with antibodies C23 (lane 1 and 6), 27-287 (lanes 2 and 7), a rabbit serum raised against non-infectious enveloped particles (lane 4) and a rabbit preimmune serum (lane 5). Proteins were analysed on 10% SDS–polyacrylamide gels with 2-mercaptoethanol (lanes 1 to 5) or without 2-mercaptoethanol (lanes 6 and 7). Lane 3 contains M, markers. (b) Western blot analysis of proteins from extracellular HCMV particles with antibodies C23 (lane 1) and 27-287 (lane 2). Proteins were separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Protein A coupled to horseradish peroxidase and dianisobenzidine staining was used as a detection system.
gel electrophoresis under reducing conditions revealed a well-resolved polypeptide of about 58K and a diffusely migrating protein in the range between 90K and 130K (Fig. 1a). Under non-reducing conditions, several high \( M_r \) complexes of between 150 and 300K were observed (Fig. 1a). An identical precipitation pattern was obtained with both C23 and 27-287, indicating that C23 was recognizing the gp58/116 complex. The differences in the \( M_r \)s compared to the original report most probably result from the different gel systems used for the analysis of the proteins. Alternatively, the 130K polypeptide observed by Masuho et al. (1987) could represent the uncleaved precursor molecule present in infected cells. Control reactions, with a rabbit hyperimmune serum raised against non-infectious enveloped particles, precipitated between 15 and 20 polypeptides, whereas a non-immune serum did not recognize any viral protein. In Western blots in the presence of reducing agents, the two antibodies gave different results. As has been shown before (Utz et al., 1989), 27-287 recognized a single protein species with an apparent \( M_r \) of 58K, whereas C23 reacted with the diffusely migrating protein in the \( M_r \) range between 90K and 130K (Fig. 1b). Under non-reducing conditions, both antibodies again detected several high \( M_r \) complexes which migrated similarly to those detected in the immunoprecipitations (data not shown). These results suggested that the epitope recognized by C23 is located in the gp116 component of the gp58/116 complex. In addition, the positive reaction of C23 with gp116 under denaturing conditions indicated that the antibody was recognizing a linear epitope.

Antibody C23 reacts with sequences from the amino-terminal part of the gp58/116 gene product

In order to investigate the possibility that gp116 represents the amino-terminal portion of the gp160 precursor molecule, sequences from the corresponding part of the gene were expressed in \( E. coli \). Neither the amino nor the carboxy terminus of gp116 of HCMV strain AD169 has been determined experimentally so two assumptions were made. Firstly, it has been shown that the protein of the Towne strain of HCMV is cleaved at the sequence RTKR/S 461 (Spaete et al., 1988). Strain AD169 carries a very similar sequence, RTRR/S 460, at the same position in the molecule so it was assumed that this sequence represents the cleavage site. Secondly, using the algorithm of Perlman & Halvorson (1983), the amino-terminal signal sequence was placed between aa 1 and 29. Signal sequences are normally removed from glycosylated envelope proteins and do not represent part of the mature polypeptide. We therefore decided to express sequences between nucleo-
tides 81 (aa 27) and 1451 (aa 484) in the pSEM system. In this system, fusion proteins between a truncated \( \beta \)-galactosidase (aa 1 to 375) and HCMV sequence can be generated. Although these plasmids have been successfully used in our laboratory to express a variety of HCMV genes, including sequences from the 3' end of the same gene, it proved to be difficult to construct plasmids expressing amino-terminal sequences. Four plasmids (pHM 90-1, pHM 90-4, pHM 90-5, pHM 90-7) could be generated which were stable at the DNA level (Fig. 2). Of these, three (pHM 90-4, pHM 90-5, pHM 90-7) also synthesized stable fusion proteins of 72K, 56K and 54K respectively. pHM 90-1 did not synthesize a detectable fusion protein of the expected size (102K; Fig. 3a). However, in Western blots using HCMV-positive human sera, proteins between 50K and 60K could be detected, indicating that the fusion protein was degraded after synthesis (data not shown). Sequences between aa 100 and 256 could not be expressed. \( E. coli \) extracts containing the respective fusion proteins were analysed in Western blots for recognition by antibody C23. Proteins pHM 90-5 and pHM 90-7 gave strong positive signals. pHM 90-1 was also recognized but, owing to degradation of the fusion protein, the corresponding signal was not confined to a single band (Fig. 3b). This indicated that the HCMV sequence recognized by antibody C23 is located between aa 27 and 84 of the gp58/116 gene product. It also provided proof that gp116 of the gp58/116 complex was derived from the amino-terminal part of the gp160 protein.
Further characterization of the antigenic site recognized by C23

DNA sequence information for the gp58/116 gene is available for HCMV strains AD169 and Towne (Cranage et al., 1986; Spaete et al., 1988). At the amino acid level, the two proteins show 95% similarity (Spaete et al., 1988). The differences, however, are not distributed randomly across the entire molecule. Instead, the amino-terminal part of gp116 carries a cluster of divergent amino acids, including deletions (Fig. 4a). Between residues 30 and 70 the homology is 55%. Antibody C23 was binding to a sequence derived from this part of the molecule and, therefore, the analysis of polypeptides of the Towne strain could give additional information about the epitope recognized by C23. As can be seen from the Western blot analysis shown in Fig. 4(b), C23 recognized gpl16 in both strains. The sequence expressed by pHM 90-7 was composed of the non-homologous region between amino acids 30 and 70, as well as an additional 17 aa which are identical in AD169 and Towne. Given the specificity of antigen-antibody interactions, the recognition of both HCMV strains by C23 suggested that the binding site is localised between Asn 70 and Val 84.

Sequences encoded by pHM 90-7 are immunogenic in humans

Antibody C23 was generated from lymphocytes of an HCMV-seropositive patient, indicating that, during natural infection, the amino-terminal part of gp116 is capable of inducing a humoral immune response in at least some individuals. Whether the production of antibodies against this part of the molecule was a more general phenomenon was tested in Western blots using 19 HCMV-positive and one negative human sera. HCMV particles as well as the plasmids pHM 90-4, pHM 90-5 and pHM 90-7 were used as antigens. The construct pMBG 58 was included in the analysis. This plasmid expresses 166 aa (484 to 650) of gp58, including the sequence around aa 614, which have been shown to contain a highly conserved linear epitope recognized by all gp58-positive sera tested so far (Uitz et al., 1989; Britt et al., 1990b).

Three different patterns of recognition were observed (Fig. 5). Fig. 5(b) shows a serum that reacted with both pHM 90-7 and pMBG 58, whereas Fig. 5(a) and (c) were representative of sera containing antibodies against only pHM 90-7 or pMBG 58. There was good correlation between recognition of pMBG 58 and gp58 in viral particles (Fig. 5a, c). Owing to the diffuse migration of gpl16 in polyacrylamide gels, a correlation with the recognition of pHM 90-7 could not be established. Fig. 5(d) shows the reaction pattern of an HCMV-positive,
gp58/116 K-negative serum. Of the 19 HCMV-positive sera tested, two recognized pHM 90-7 exclusively, eight reacted with pMBG 58 alone and six gave positive results with both proteins (Table 1). pHM 90-4 was recognized by one serum. The signal strength of pHM 90-5 and pHM 90-7 did not differ to a significant extent, indicating that aa 84 to 100 do not contain an additional epitope (data not shown). However, the signals were not analysed quantitatively. Therefore we cannot exclude the possibility that some sera contain low amounts of antibodies reactive with aa 84 to 100.

**Discussion**

Numerous studies have analysed HCMV glycoproteins and glycoprotein complexes present in the viral envelope. Our study has focused on the nature of gp116 in the gp58/116 complex. This complex has also been termed gA (Pereira et al., 1984), gB (Cranage et al., 1986), gp55/116 (Britt, 1984), gp55-130 (Rasmussen et al., 1985) and gC1 (Gretch et al., 1988) by other investigators.

So far this protein has not been analysed directly because of its low abundance in virions compared to other viral polypeptides, its diffuse migration in SDSPAGE and the lack of immunological reagents specific for this polypeptide. However, the formation of a stable, disulphide-linked complex of gp116 with gp58 has allowed the generation of data about synthesis, modification and processing of gp116 through the aid of gp58-specific immunological reagents. From these studies it was apparent that the formation of gp116 was synchronized with the cleavage of a glycosylated precursor molecule of approximately 160K (Britt & Auger, 1986; Rasmussen et al., 1985). Data from this laboratory have shown that gp58 represented the carboxy-terminal part of the precursor protein which, by analogy to the corresponding gene in HCMV Towne, most probably starts at aa 460 of the primary translation product (Mach et al., 1986; Spaete et al., 1988). However, the relationship of gp116 to gp58 remains controversial. Some investigators suggested that gp116 resembles the amino-terminal part of the precursor molecule (Britt & Vugler, 1989; Gretch et al., 1988; Spaete et al., 1988). Others suggested that the amino-terminal half is rapidly degraded after cleavage (Cranage et al., 1986) and/or that the components of the complex are derived from different genes (Stannard et al., 1989). Our data with monoclonal antibody C23 clearly demonstrate that gp116 is derived from sequences upstream of the cleavage signal at aa 460. The formation seems, therefore, to resemble that of the homologous gpl1 complex of VZV (Montalvo & Grose, 1987). The theoretical Mr of the amino acid backbone of gp116 is 52.6K, which is in good agreement with the proposed size of the deglycosylated gp116 protein (Britt & Vugler, 1989).

Of the 19 potential sites for N-linked glycosylation (Kornfeld & Kornfeld, 1985) located on the extracellular portion of gp58/116, 15 are located in the gp116 part. It therefore seems likely that the heterogeneous electrophoretic behaviour was a consequence of differential glycosylation and/or some other post-translational modification such as phosphorylation or sulphation or both. Both types of modification have been described on
enveloped glycoproteins of human herpesviruses (Edson et al., 1987; Gabel et al., 1989; Hope et al., 1982; Montalvo & Grose, 1987).

The data indicating that gp116 and gp58 are products of different genes were based on results obtained with an antiserum raised against a recombinant vaccinia virus containing the entire gp58/116 open reading frame (Stannard et al., 1989). Although this serum was capable of immunoprecipitating the gp58/116 complex, it did not recognize gp116 in Western blots. These results can be explained by a lack of gp116-specific antibodies. We have been unsuccessful in our attempts to raise gp116-specific polyclonal antisera of sufficient titre using various synthetic peptides or prokaryote-expressed fusion proteins (M. Mach, unpublished results). Moreover, an antiserum raised against HCMV envelope glycoproteins failed to recognize gp116 in Western blots (Lehner et al., 1989). Whether this was a result of low immunogenicity of the polypeptide or the high and probably variable amount of sugars on the molecule was not clear. Alternatively, the glycosylation and/or processing steps in recombinant vaccinia virus-infected cells might be different from that in HCMV-infected fibroblasts, leading to a gene product preventing the production of antibodies specific for gp116 in HCMV virions.

Studies have indicated that a significant proportion of neutralizing antibodies in human convalescent sera are directed against the gp58/116 complex (Britt et al., 1990a). Because we have used fragments of gp116 expressed as bacterial fusion proteins, our analysis of antibody recognition sites was limited to sequences without higher order structure or modification. Consequently, our results revealed only the minimum number of binding sites because we have ignored antibodies directed against sequences with tertiary structure or modification. A linear neutralizing epitope has recently been identified on gp58 (Utz et al., 1989). This epitope is conserved between different HCMV strains and is recognized by all gp58-positive human sera tested so far (Britt et al., 1990b). gp116 represents the extracellular domain of the complex and, therefore, a detailed analysis of this molecule is important.

The use of prokaryotic systems for the determination of linear antibody binding sites is especially advantageous because it allows the determination of a variety of antibody recognition sites once the expression plasmids have been constructed. Following this strategy, the binding site of antibody C23 was mapped between aa 27 and 84 of HCMV strain AD169. This region most probably represents the amino terminus of gp116 because aa 1 to 29 contain the signal sequence which is removed from the molecule (Perlman & Halvorson, 1983). However, based on several assumptions, the neutralizing site is predicted to be localized on a stretch of 17 aa between residues 68 and 84. The assumptions are that the minimum length required for antibody binding is between five and seven aa (Houghten, 1987); any change in the amino acid composition of this minimal recognition sequence results in loss of binding (Schoofs et al., 1988); and, between positions 27 and 70, the longest stretch of homologous aa between strains AD169 and Towne is four residues. The strong positive reaction of C23 with both strains therefore indicates that the recognition site is located between aa 68 and aa 84.

The location of a linear neutralizing epitope on the amino terminus of gp116 is similar to the situation found in HSV. In a recent study, Pereira et al. (1989) have mapped a linear neutralizing domain on the homologous glycoprotein B of HSV-1 between aa 1 and 47. HSV gB has been implicated in the attachment of virus to the cell surface (WuDunn & Spear, 1989). Whether gp58/116 serves a similar function for HCMV has yet to be shown.

The amino-terminal sequence between aa 27 and 84 seemed to be immunogenic during natural infection. In addition, it also represented an immunodominant domain on the amino-terminal part of gp116. Antibody recognition by human sera, however, was less consistent than that observed with the immunoreactive sequences on gp58. Of 19 HCMV-positive sera tested, we found eight that were reactive with pMBG 58 protein but non-reactive with pHM 90-7. On the other hand, two sera that were negative for pMBG 58 recognized pHM 90-7. Antibodies against the sequence between aa 256 and 484 were detected in only a single serum sample, indicating that this part of the molecule did not induce conformation-independent antibodies to a significant extent. This again is similar to HSV gB, where only discontinuous epitopes have been found in the corresponding part of the protein (Pereira et al., 1989). The analysis of sequences between aa 100 and 256 will depend on the use of a different expression system or the use of synthetic peptides. Currently we do not have an explanation for the problems involved in expressing this part of the molecule. There are several possible interpretations for the differential recognition of the pHM 90-7-encoded sequence. Firstly, the sequence divergence observed in strains AD169 and Towne might not be limited to aa 30 to 70 but may extend further into the molecule in some strains. Consequently, antibodies specific for the respective strain would not recognize AD169 sequences. Secondly, the region covered by pHM 90-7 contains three potential sites for N-linked glycosylation plus an additional site (NTT 89) directly adjacent to pHM 90-7. Differential usage of these sites by different strains might inhibit formation of antibodies against linear epitopes in this region. Thirdly, the synthesis of antibodies might be dependent on the time of infection. Correlation between
time of infection and appearance of antibodies to specific viral proteins has been shown (Mirolo et al., 1987). It has to be mentioned, however, that all 26 clinical isolates tested so far were reactive with C23, indicating that this epitope is conserved at the amino acid level.

Neutralizing human monoclonal antibodies could be used to limit the consequences of HCMV infection in immunocompromised transplant patients. So far, hyperimmune sera have been used with conflicting results (Bowden et al., 1986; Snydman et al., 1987). Antigenic strain variability could pose a problem for the applicability of monoclonal antibodies for immune prophylaxis. In fact, it has been reported that various neutralizing antibodies show a marked difference in the recognition of different HCMV isolates (Baboonian et al., 1989; Masuho et al., 1987; Rasmussen et al., 1985). The differences were observed only with antibodies recognizing conformational epitopes and/or depending on complement for neutralization. The currently available antibodies against linear epitopes (Utz et al., 1989; Masuho et al., 1987) seem to be superior in this respect because they appear to interact with highly conserved regions of gp58/116. The production of antibodies against linear sequences might therefore be advantageous. Prokaryote-expressed fusion proteins might constitute a useful source of antigen for the development of such antibodies.

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