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# A Continuous Sequence of More Than 70 Amino Acids Is Essential for Antibody Binding to the Dominant Antigenic Site of Glycoprotein gp58 of Human Cytomegalovirus

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**Antigenic domain 1 (AD-1) on glycoprotein gp58 of human cytomegalovirus was characterized in detail, using mouse and human monoclonal antibodies as well as human convalescent sera. Series of procaryotically expressed fusion proteins and synthetic peptides of various lengths were used as sources of antigen. Binding of antibodies was found to depend on a continuous sequence of more than 70 amino acids between residues 552 and 635 of gp58. The fine specificities for sequences involved in antibody binding were (i) amino acids 557 to 635 for neutralizing as well as nonneutralizing mouse monoclonal antibodies, (ii) amino acids 552 to 630 for a neutralizing human monoclonal antibody, and (iii) amino acids 557 to 630 for antibodies present in human sera. Experiments involving fragments of AD-1, presented either as procaryotically expressed fusion protein or as synthetic peptides, indicated that the intact structure was required for recognition of AD-1 by antibodies.**

The humoral immune response against human cytomegalovirus (HCMV) is complex and involves reactivity against a number of polypeptides. Using different methods for detection of HCMV-specific antibodies such as immunoprecipitation or immunoblotting, between 15 and 20 distinct targets of human antibodies have been detected (1, 23, 31, 32, 40). Antibodies directed against the viral envelope are of particular importance for the host defense since they are potentially capable of neutralizing virus or eliminating infected cells. The genome of HCMV contains approximately 30 reading frames that have characteristics of glycoproteins and a number of additional genes encoding membrane proteins with multiple hydrophobic domains (8, 25). However, only three glycoprotein complexes, designated gCI, gCII, and gCIII, have been characterized in more detail (4, 5, 14, 16, 17, 19, 20, 26). Antibody reactivity against gCI, which seems to be the immunodominant glycoprotein of HCMV, is consistently detectable in conventional immunoblot assays or immunoprecipitations (22, 23).

In its nonreduced form, gCI consists of protein complexes of 160, 190, and 250 to 300 kDa (4, 16). These complexes can be dissociated into two proteins of 58 kDa (gp58) and 90 to 130 kDa (gp116) by the addition of reducing agents such as β-mercaptoethanol (β-ME), suggesting that they are linked by disulfide bonds. The gene encoding gCI has been identified, and the nucleotide sequences for the laboratory strains AD169 and Towne have been reported (10, 37). According to the nomenclature of Chee et al. (8), the open reading frame has been designated HCMVUL 55 in strain AD169. The primary translation product consists of a polypeptide of more than 900 amino acids (aa) which is cotranslationally modified to form a 150- to 160-kDa glycosylated precursor molecule (5, 16). Following transport into the Golgi apparatus, the protein undergoes endoproteolytic cleavage into the heavily glycosylated gp116 and the less heavily glycosylated gp58, with gp116 originating from the amino-terminal half and gp58 originating from the carboxy-terminal half of the precursor polypeptide (20, 27, 29).

Using immunoblot analysis with extracellular virus as an antigen, Landini et al. (23) reported that a majority of HCMV-positive human sera react with gp58. By using HeLa cell extracts infected with a gp58/116 recombinant vaccinia virus as a specific immunoadsorbent, it was shown that between 40 and 70% of the total neutralizing capacity of human sera was directed against this glycoprotein (6). Additional studies using procaryotically expressed fusion proteins constructed from overlapping fragments of the gp58/116 reading frame of strain AD169 identified antigenic regions at the amino terminus between aa 28 and 84 (29), at the carboxy terminus between aa 783 and 906 (22, 35), and around aa 616 (39). The latter site, termed antigenic domain 1 (AD-1), constitutes the dominant domain for the induction of antibodies. All gp58-positive human sera so far analyzed react with this domain. Mouse monoclonal antibodies directed against AD-1 can inhibit gp58-specific binding of antibodies in human convalescent sera by more than 50% (22). Neutralizing human as well as mouse monoclonal antibodies reactive with AD-1 have been isolated. However, by using mouse monoclonal antibodies, AD-1 has been shown to also bind nonneutralizing antibodies, which can compete with the neutralizing antibodies (39).

gp58/116 has been proposed as a vaccine candidate for HCMV, and in fact purified viral protein has been used to immunize a limited number of humans (15, 33). In light of this development, it is imperative to fully characterize the immune response elicited by this glycoprotein. The precise structure of AD-1 has not been determined; thus, antibody reactivity against AD-1 remains inadequately described. In

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this paper, we report on the characterization of this domain, using mouse as well as human monoclonal antibodies.

## MATERIALS AND METHODS

**Expression of gp58 fragments in *Escherichia coli*.** The DNA fragments used to generate the gp58 expression plasmids 58-Dde and 58-Hin were isolated from plasmid p5813, which contains a 900-bp *Pst*I-*Pst*I fragment (encompassing aa 484 to 783) of the *Hin*dIII-F region of HCMV strain AD169 (39). 58-Nci was derived from plasmid gig 58-1, which contains a 490-bp *Pst*I-*Sal*I fragment (aa 484 to 545) (22).

The DNA fragments were inserted into the expression vector pSEM, which contains the truncated gene coding for the first 375 aa of *E. coli* β-galactosidase 5′ to a multiple cloning site (21). All cloning procedures were performed by standard methods. Correct insertion of DNA fragments in expression plasmids was monitored by defining the nucleotide sequence at the fusion point between the vector and the viral gene by using the chain termination method with a T7 polymerase sequencing kit (Pharmacia, Freiburg, Germany).

Fusion proteins were produced in *E. coli* W3110. The cells were grown at 37°C to a density of approximately 1.0 $A_{660}$ unit, and production of fusion proteins was initiated by adding isopropylthiogalactopyranoside (IPTG) to a final concentration of 1 mM. The synthesis of fusion proteins was allowed to proceed for 4 to 5 h at 37°C. Cells were harvested by centrifugation, lysed in sodium dodecyl sulfate (SDS)-gel sample buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE).

To purify fusion proteins, cells of a 100-ml culture were resuspended in 4 ml of phosphate-buffered saline (PBS) containing DNase I (final concentration, 1 μg/ml) and lysozyme (final concentration, 2.5 μg/ml). After incubation on ice, the lysate was sonicated for 30 s and centrifuged. The sediment was washed twice in PBS, suspended in 2 ml of PBS, and added slowly to 4 ml of 6 M urea with continuous stirring on ice. After centrifugation, the soluble fraction, containing the enriched fusion proteins, was analysed by PAGE.

Gig 58 plasmids were constructed in the pATH expression vector and transfected into *E. coli* C600. Induction of fusion protein expression was done as described previously (38). Briefly, the cells were grown to a density of approximately 0.2 $A_{660}$ unit at 37°C, and synthesis of fusion protein was initiated by addition of indoleacrylic acid (final concentration, 10 μg/ml). Synthesis of fusion protein was allowed to continue for 4 h. Cells were harvested by centrifugation and analyzed by PAGE. The DNA sequences of gp58 inserts were characterized by determining the nucleotide sequence at the fusion point between the vector and the viral insert.

Purification of fusion proteins via fast protein liquid chromatography (FPLC) was performed exactly as described previously (13). Briefly, the solubilized fusion proteins were dialyzed against 10 mM Tris-Cl (pH 7.5)–1 mM EDTA, and chromatography on a Superose 12 HR 10/30 column (Pharmacia) was performed in the same buffer. The eluate was collected in fractions, and the fractions were analyzed individually by PAGE. The elution time of fusion proteins was generally in the range expected for proteins of 40- to 70-kDa molecular mass, excluding the possibility of formation of large aggregates.

**SDS-PAGE and immunoblotting.** SDS-PAGE and immunoblotting were done by standard procedures. Monoclonal antibodies and human sera were diluted in PBS containing 0.1% Tween 20. Antibody binding was detected after incubation with horseradish peroxidase-coupled anti-human or anti-mouse immunoglobulin G (IgG) and staining with 4-chloro-1-naphthol or diaminobenzidine. To reduce background staining of bacterial proteins, human sera used in immunoblot analysis were preincubated. The diluted sera were incubated for 1 h on ice with a mixture of β-galactosidase (final concentration, 2 μg/ml) and a lysate of an *E. coli* culture containing the pSEM-derived protein. After centrifugation, the supernatant was saved and the preincubation step was repeated twice.

**Exonuclease III-mung bean nuclease digestion.** The exonuclease III-mung bean nuclease deletion system was used to shorten the gp58 gene at the 5′ and 3′ ends. The expression plasmids gig 58-2 and Exo 58-315 were digested with restriction enzymes, creating a 5′ overhang at the end of the gp58 fragment and a 3′ overhang at the vector, which was resistant to exonuclease III digestion. Thirty micrograms of DNA was incubated with 600 U of exonuclease III at room temperature in EXOIII buffer (50 mM Tris [pH 8.0], 5 mM $MgCl_2$, 10 mM $ZnSO_4$, 10 mM β-ME). After 15, 30, 45, and 60 s, aliquots containing 5 μg of DNA were transferred to 175 μl of 8/7 mung bean nuclease buffer (57 mM sodium acetate [pH 5.0], 34 mM NaCl, 1.14 mM $ZnSO_4$) to terminate the reaction. After incubation at 68°C for 15 min, the single-strand extensions were removed by digestion with mung bean nuclease (3 U/μg of DNA) for 30 min at 28°C and then subjected to phenol-chloroform extraction and ethanol precipitation. The shortened fragments were religated and transfected in *E. coli* C600.

**Peptide synthesis.** Peptides were synthesized according to a method using fluoroenylmethoxycarbonyl-protected amino acids (34) applied on resin in individual solvent-permeable polypropylene bags as described for simultaneous multiple peptide synthesis by Houghten (18).

**Enzyme-linked immunosorbent assays (ELISAs).** FPLC-purified fusion protein BaMa-58 (300 μg/ml) was diluted to 1 μg/ml in 6 M urea and used at 50 μl per well to coat microtiter plates for 16 h at 4°C in a humid chamber. All subsequent steps were carried out at room temperature. Reaction wells were rinsed three times (5 min each) with buffer A (PBS, 0.05% Tween 20) and blocked for 2 h with PBS containing 2% fetal calf serum. Plates were again rinsed with buffer A and incubated with antibodies (50 μl per well) for 2 h. After three additional washes with buffer A, peroxidase-conjugated anti-human or anti-mouse IgG was added in appropriate dilution for 1 h. Plates were washed two times, and substrate (*o*-phenylenediamine; 2 mg/ml) was added for 10 min. The reaction was stopped by the addition of 100 μl of 2 N $H_2SO_4$, and the optical density was determined at 492 nm. Dilution of all antibodies was done in PBS–2% fetal calf serum.

To allow binding of antibodies to fusion proteins or synthetic peptides in solution, mixtures were incubated for 1 h at 4°C prior to addition to the reaction wells.

**Monoclonal antibody production and purification.** Hybridomas producing the mouse monoclonal antibodies were grown in RPMI 1640 medium containing 5 to 10% fetal calf serum. Antibodies were purified from tissue culture supernatant on protein A-Sepharose. PAGE analysis was performed to test the purity of the antibody preparation.

## RESULTS

**Antibody recognition of bacterial fusion proteins expressing AD-1.** We have previously shown that *E. coli* containing plasmid gig 58-2 expresses aa 549 to 645 of gp58 as a

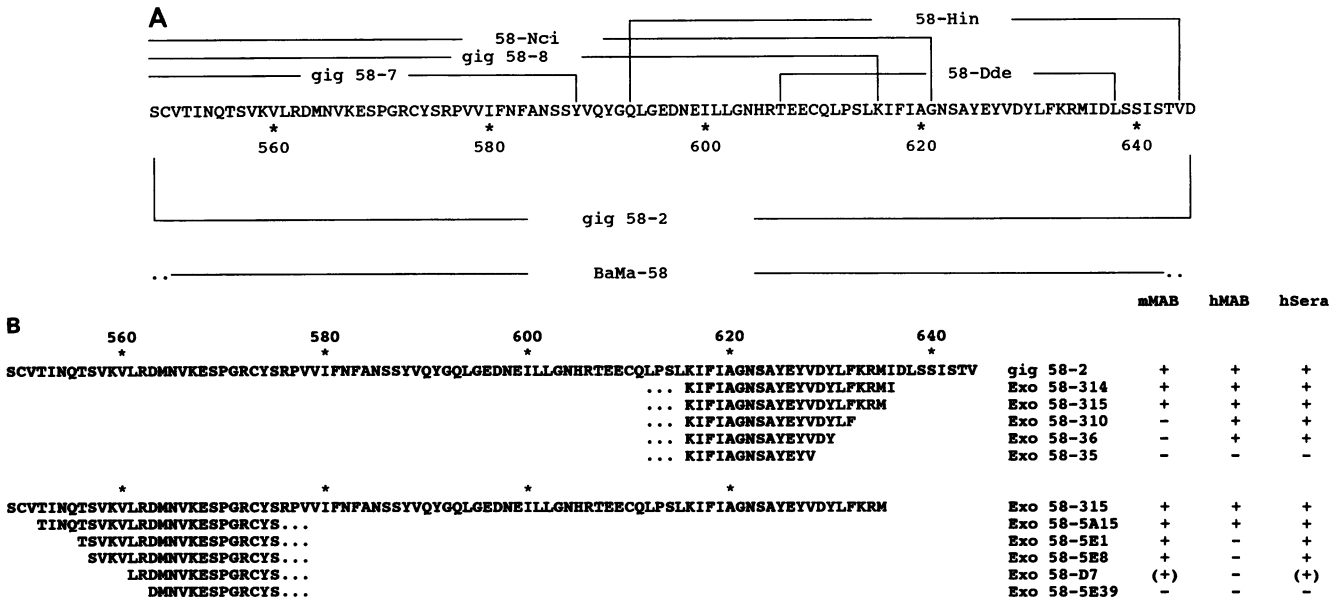


FIG. 1. (A) Expression plasmids used for the characterization of AD-1. The amino acid sequence of strain AD169 gp58 between residues 549 and 645 is given in single-letter code. The plasmids are indicated by brackets. BaMa-58 contains aa 484 to 650. (B) Encoded sequence of the Exo constructs and recognition by mouse and human monoclonal antibodies (mMAb and hMAb) and human sera (hSera).

procaryotic fusion protein. This gp58 fragment contained a structure sufficient for binding of AD-1-specific antibodies. Reactivity was maintained under denaturing conditions in immunoblots, suggesting that the antibody binding site was a continuous amino acid sequence. Insertion of 4 aa at position 616 resulted in the loss of binding of all tested monoclonal antibodies or human sera, further demonstrating the necessity of the continuous amino acid sequence surrounding position 616 for antibody recognition (22). However, using synthetic peptides of various lengths spanning aa 607 to 626, we could not determine the binding domain precisely, since little or no binding was observed in a variety of assays. The peptides that were tested included 12 nonamers, starting at position 607, each overlapping its neighbor by eight amino acids. In addition, a peptide covering residues 607 to 626 was used, and again no reactivity was observed (data not shown).

Several plasmids were then constructed for a detailed characterization of AD-1 (Fig. 1). The gig series of plasmids has been described elsewhere (22). Plasmids 58-Nci, 58-Dde, and 58-Hin contain aa 496 to 621, 607 to 638, and 593 to 644, respectively. Together, these constructs cover, in overlapping fragments, the region between aa 549 to 645, encoded by gig 58-2. Gig 58-2N contains a 4-aa (Ile-Ala-Met-Ala) insertion at position 616. In addition, plasmid BaMa-58, which encodes aa 484 to 650, was used in some experiments. The characteristics of the monoclonal antibodies are shown in Table 1. The neutralizing ($NT^+$) mouse antibody 7-17 as well as the $NT^+$ human antibody 89-104 are capable of neutralizing HCMV in tissue culture independent of complement. The other antibodies do not show neutralizing activity ($NT^-$). In addition, human sera were used in some experiments.

In the first set of experiments, bacterial lysates containing the fusion proteins were tested for recognition by the monoclonal antibodies in immunoblots. All antibodies gave identical results. The reactivities of antibodies 89-104 (human, $NT^+$) and 27-287 (mouse, $NT^-$) are shown as examples (Fig. 2). All fusion proteins except gig 58-2 were negative. This result indicated that the monoclonal antibodies, regardless of

TABLE 1. Specifications of monoclonal antibodies

| Antibody | Origin | Isotype | NT | Reference |
|---|---|---|---|---|
| 7-17 | Mouse | IgG3κ | + | 3 |
| 27-287 | Mouse | IgG2bκ | − | 39 |
| 27-156 | Mouse | IgG2bκ | − | 37 |
| 89-104 | Human | IgG1κ | + | 12 |

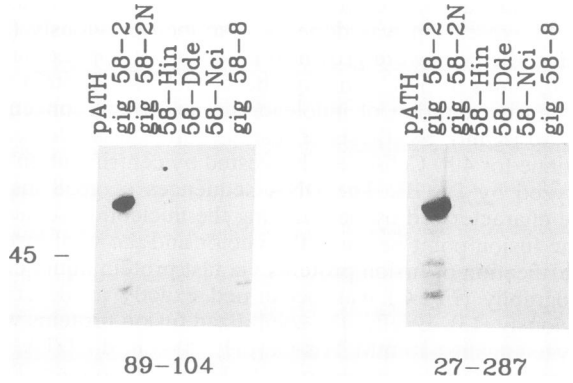


FIG. 2. Reactivity of monoclonal antibodies for bacterial fusion proteins. *E. coli* containing the indicated expression plasmids was induced and solubilized as described in Materials and Methods. Total cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, individual blots were incubated with monoclonal antibodies at 4 μg/ml (89-104) or 8 μg/ml (27-287). After the membranes were washed, they were incubated with peroxidase-conjugated anti-human (89-104) or anti-mouse (27-287) IgG and developed with 1,4-chloronaphthol and $H_2O_2$. The position of molecular mass standards (in kilodaltons) is indicated at the left.

origin and biological activity, have similar if not identical binding specificities. The results further suggested that the binding site is unusually complex. The difference in reaction between gig 58-2 and gig 58-2N indicated that amino acids close to residue 616 are essential for antibody binding. Fusion protein 58-Hin, covering 52 aa between aa 593 and 644, was negative. This construct extends 23 aa to the amino terminus and 28 aa to the carboxy terminus of aa 616. Moreover, 58-Nci ending at aa 621 was also not recognized by the antibodies. In summary, these data suggested that the sequence required for binding of antibodies was larger than 29 aa. To exclude structural influences from the procaryotic part of the fusion proteins, some of the experiments were repeated with proteins having different fusion partners. Identical results were obtained (data not shown).

To define the minimal sequence required for antibody binding, we constructed a series of gig 58-2 derivatives that had deletions at the $NH_2$ as well as the COOH end of the viral polypeptide. For this purpose, gig 58-2 DNA was first digested unidirectionally with exonuclease III at the 3′ end of the viral insert and religated. The fusion proteins were tested in immunoblots for reactivity with the monoclonal antibodies. The shortest construct that was positive for all antibodies was then used to create 5′ deletions of the viral sequence. Representative immunoblots of relevant constructs with antibodies 27-287 (mouse, $NT^-$) and 89-104 (human, $NT^+$) and a human serum are shown in Fig. 3. A summary of the results is presented in Fig. 1B. The smallest fusion protein showing a positive reaction with mouse monoclonal antibodies extended to the methionine at position 635 (Exo 58-315). Exo 58-310 ending at aa 632 was negative. All mouse monoclonal antibodies had the same pattern of reactivity. The human monoclonal antibody 89-104 ($NT^+$) had a slightly different reaction pattern (Fig. 1 and 3). Exo 58-310 was reactive, as was Exo 58-36 containing tyrosine 630 at the COOH terminus. Exo 58-35, ending at valine 628, was nonreactive with human antibody 89-104 ($NT^+$). Human serum had a recognition pattern similar to that of human antibody 89-104 ($NT^+$). Although the reaction was clearly positive, human sera in general showed a difference in signal strength between gig 58-2 and shorter fusion proteins (Fig. 3A). The difference could be confirmed with ELISAs using FPLC-purified fusion protein as the antigen (data not shown). These data indicate that the carboxy-terminal boundary for mouse monoclonal antibodies is located between aa 632 and 635, whereas for human antibody recognition, aa 629 to 630 are required.

The sequence requirements at the amino terminus of AD-1 for antibody recognition were investigated in the next group of experiments. Mouse monoclonal antibodies required a sequence beginning with serine at position 557 (Exo 58-5E8). Exo 58-5E39, beginning with aspartic acid at position 563, was not reactive with antibodies. The human monoclonal antibody required a slightly larger sequence for binding. The shortest fusion protein positive for recognition started at threonine 552 (Exo 58-5A15). At the amino terminus, human sera exhibited a recognition pattern different from that of the human monoclonal antibody in that they more closely resembled mouse antibodies. Fusion protein Exo 58-5E8 was positive with human sera; however, this fusion protein was nonreactive with human antibody 89-104 ($NT^+$) (Fig. 3B). Exo 58-D7, starting at leucine 561, gave inconsistent results with mouse monoclonal antibodies and human sera. Therefore, it can be concluded that for recognition of antigen in immunoblots, mouse antibodies required 79 aa (557 to 635), whereas for human monoclonal antibody 89-104 ($NT^+$), aa

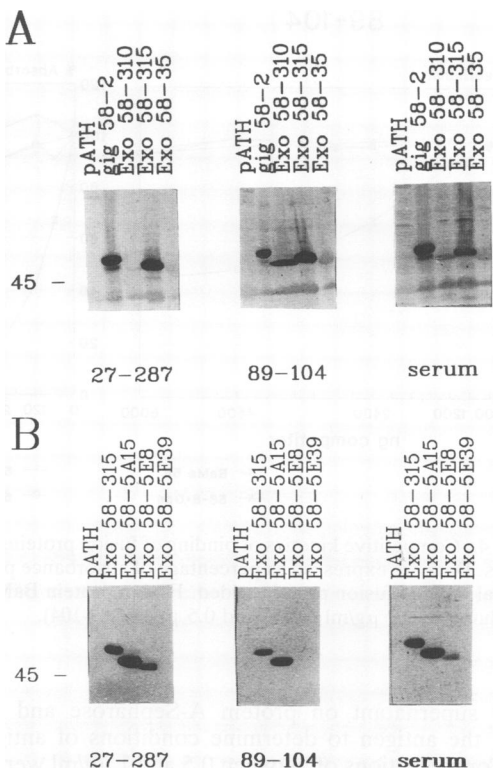


FIG. 3. Reactivity of monoclonal antibodies 27-287 and 89-104 and human serum for the Exo fusion proteins. *E. coli* lysates containing the indicated fusion proteins were separated by SDS-PAGE and immunologically detected as described in the legend to Fig. 2. The human serum was used in a 1:150 dilution. Panels A and B represent constructs with 3′ and 5′ deletions, respectively. For amino acid sequences encoded by the plasmids, see Fig. 1B. Positions of molecular mass standards (in kilodaltons) are indicated at the left.

552 to 630 were essential. Human sera gave positive signals with antigens containing aa 557 to 630 (74 aa).

Considering the length of this domain, we have postulated that structural features other than primary sequence play an essential role in antibody recognition. The reactivity of the domain in immunoblot assays also suggested this structure was resistant to denaturing agents to a certain extent.

**Reactivity of antibodies with antigen in solution.** The experiments thus far described were done with antigen bound to a solid support, likely fixing antigen in a stable conformation. Recognition of soluble antigen could be different. We therefore developed an ELISA in which antibody binding to the fusion proteins in solution could be determined. In this assay, antibody binding to the fixed antigen was measured in the presence of fusion protein in solution. Fusion proteins gig 58-7, gig 58-8, 58-Nci, 58-Dde, and 58-Hin were purified by low-pressure liquid chromatography to remove *E. coli* proteins which could give rise to nonspecific binding of the antibodies. As estimated from PAGE, the fusion proteins were 80 to 95% pure (data not shown). For unknown reasons, gig 58-2 fusion protein could not be purified to levels comparable to those of the other polypeptides and was therefore replaced by fusion protein BaMa-58. BaMa-58 was positive with all monoclonal antibodies in immunoblots (data not shown). Microtiter plates were coated with 50 ng of BaMa-58 per well. Antibodies were purified from tissue

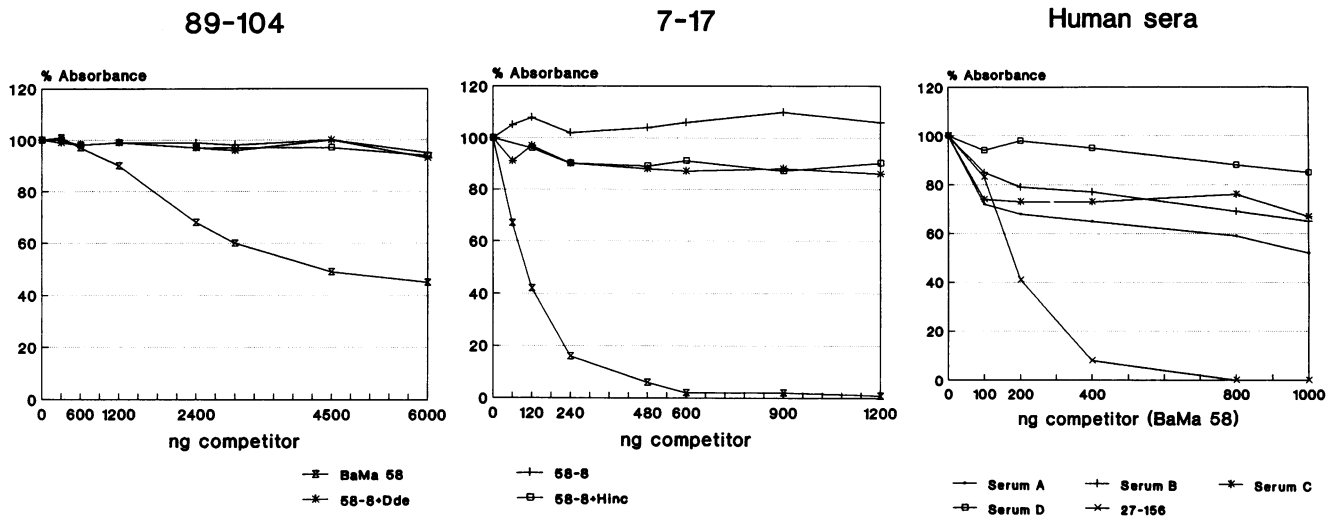


FIG. 4. Competitive kinetics of binding of fusion proteins to monoclonal antibodies. ELISAs were performed as described in Materials and Methods. Data are expressed as percentage of absorbance plotted as a function of the amount of competitor, with 100% absorbance indicating the signal with no fusion protein added. Fusion protein BaMa-58 was used at 50 ng per well to coat microtiter plates. Monoclonal antibodies were diluted to 0.7 μg/ml (7-17) and 0.5 μg/ml (89-104).

culture supernatant on protein A-Sepharose and titrated against the antigen to determine conditions of antigen excess. Concentrations of between 0.5 and 1 μg/ml were found to be optimal. Antibodies were incubated with increasing amounts of fusion proteins for 1 h, and the ELISA was performed as described in Materials and Methods. Results of representative experiments using antibodies 7-17 (mouse, $NT^+$) and 89-104 (human, $NT^+$) are shown in Fig. 4. Binding of mouse antibody 7-17 ($NT^+$) was inhibited by fusion protein BaMa-58. A 50% reduction in signal was obtained with 100 ng of protein, increasing to 94% with 500 ng (Fig. 4). The other polypeptides when tested alone or in combinations did not compete for binding of antibodies in amounts of up to 1,200 ng. The combinations tested included gig 58-7 plus 58-Dde, gig 58-7 plus 58-Hin, gig 58-8 plus 58-Dde, gig 58-8 plus 58-Hin, 58-Nci plus 58-Dde, and 58-Nci plus 58-Hin. Similar results were obtained for the other mouse monoclonal antibodies (data not shown). Binding of human antibody 89-104 ($NT^+$) was less susceptible to competition by BaMa-58. With 50 to 500 ng of competitor, no decrease in binding was observed; however, 2,400 ng inhibited binding by 50% (Fig. 4). As with the mouse antibodies, no influence was seen with the other fusion proteins individually or in combinations. Human sera gave intermediate results. Between 15 and 57% reduction in signal was observed when 1 μg of fusion protein BaMa-58 was used as a competitor (Fig. 4).

Finally, antibodies were tested with a set of overlapping peptides of various lengths which spanned the entire region containing AD-1. Peptides gB10 through gB18 represent 20-mers covering aa 550 to 645, with a 10-amino-acid overlap between peptides. In addition, two larger peptides of 40 aa (M51) and 26 aa (M47) were used. The sequences covered by the individual peptides are shown in Fig. 5A. When the peptides bound to the plates were used as antigens, indeterminant results were obtained. With some of the antibodies, a weak reaction with a number of different peptides was noted (data not shown). When the peptides were used in solution in the competition ELISA at amounts of between 12.5 and 100 ng, no inhibition was observed for individual peptides or combinations (Fig. 5B). In this assay, human monoclonal antibody C23, which is reactive with the amino-terminal part of gp116 of HCMV, was included as a control (28). Binding of C23 to purified fusion protein HM90-5, encompassing aa 27 to 84 of gp116, was inhibited 100% by the addition of 37.5 ng of a synthetic peptide containing the sequence N-70ETIYNTTLKYGDVVGV-86. It should be noted that on the molar level, the concentration of peptides in solution was approximately 30-fold higher than in the assay using fusion proteins.

Together with the results obtained for procaryotic fusion proteins, the data indicated the antibody binding domain represented by AD-1 required the entire structure of this polypeptide. It should be mentioned, however, that parts of AD-1 were immunogenic in experimental animals. Rabbit sera, raised against peptides gB10, gB11, gB13, gB15, gB16 and M51, respectively, were reactive in immunoblots and ELISAs with BaMa-58 (data not shown).

## DISCUSSION

We have investigated the specificity of mouse and human antibodies reactive with the dominant antibody binding site AD-1 on gp58 of HCMV strain AD169. Previous results with bacterially derived fusion proteins and immunoblots under denaturing conditions indicated that AD-1 represents a continuous or linear domain (22, 39). However, data from this investigation strongly argue against this previous interpretation. Continuous antibody binding sites have been characterized for numerous antibodies and generally have been found to be 5 to 18 aa in length, irrespective of whether the antibodies originate during natural infection or after immunization with synthetic peptides or entire proteins. These short sequences most probably do not represent a complete epitope since it has been shown that the interaction of antigen and antibody is dependent on conformation of both partners (for a review on the nature of epitopes, see reference 24). The minimal sequence required for binding of AD-1-specific antibodies consists of more than 70 aa, and hence it has to be postulated that the recognition event is dependent on a conformational domain contained in AD-1.

A

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          560                 580                 600                 620                 640
           *                   *                   *                   *                   *
SCVTINQTSVKVLRDMNVKESPGRCYSRPVVIFNFANSSYVQYGQLGEDNEILLGNHRTEECQLPSLKIFIAGNSAYEYVDYLFKRMIDLSSISTVD
 CVTINQTSVKVLRDMNVKES (gB10)
           VLRDMNVKESPGRCYSRPVV (gB11)
                     PGRCYSRPVVIFNFANSSYV (gB12)
                               IFNFANSSYVQYGQLGEDNE (gB13)
                                         QYGQLGEDNEILLGNHRTEE (gB14)
                                                   ILLGNHRTEECQLPSLKIFI (gB15)
                                                         RTEECQLPSLKIFIAGNSAY (gB16)
                                                               KIFIAGNSAYEYVDYLFKRM (gB17)
                                                                     EYVDYLFKRMIDLSSISTVD (gB18)
                    RCYSRPVVIFNFANSSYVQYGQLGEDNEILLGNHRTEECQ (M51)
                                                   ILLGNHRTEECQLPSLKIFIAGNSAY (M47)
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B

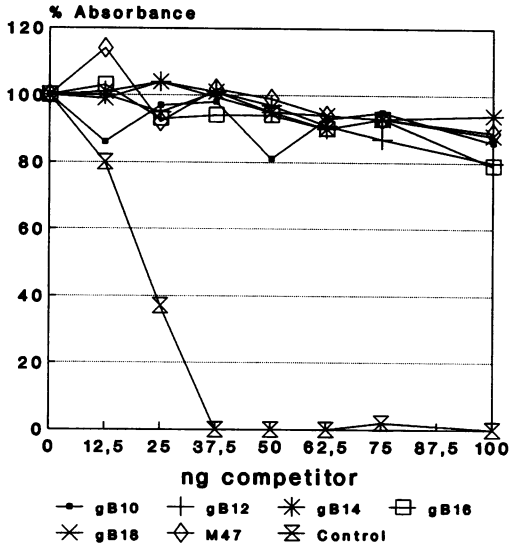


FIG. 5. (A) Synthetic peptides encompassing AD-1. (B) Competitive kinetics of binding of synthetic peptides to monoclonal antibody 27-287. Experimental conditions were as described in the legend to Fig. 4. Control indicates the control reaction involving fusion protein HM90-5 as the immobilized antigen, human monoclonal antibody C23, and the peptide N-70ETIYNTTLKYGDV VGV-86 (28).

This structure has to be highly stable and largely independent of native modifications such as glycosylation. Two potential sites for N-linked glycosylation are located within AD-1; NSS-587 is located within the region recognized by murine and human antibodies, and NQT-556 is located outside the binding domain for mouse antibodies but at the border of the domain for human monoclonal antibody 89-104 ($NT^+$).

Upon examination of AD-1, we found several features worth noting. First, AD-1 contains two cysteine residues at positions 573 and 610, making the formation of a disulfide bond possible. Formation of -S-S-linked loops has been postulated for other highly immunogenic structures such as the principal neutralization domain of human immunodeficiency virus type 1, and binding of antibodies to residues from the putative loop has been demonstrated (28). Although we cannot rule out entirely the formation of such a structure for AD-1, several results argue against it. Addition of reducing agents such as β-ME did not influence the capacity of antibodies to bind to AD-1. The structure was also maintained in immunoblots involving denaturation of antigen with SDS and β-ME. In addition, peptide M51 containing both cysteine residues was nonreactive with any of the antibodies tested. The complete loss of reactivity with fusion proteins mutated at position 616, which is outside the hypothetical loop structure, argued against a recognition of the loop itself. Instead, it indicated that residues outside the loop were critical for reactivity. The formation of a disulfide bond between the cysteine residues of AD-1 and the procaryotic fusion partner also does not contribute to the structure, since AD-1 alone was sufficient for antibody binding (22a). The dual occurrence of the motif Ser-Pro-X-X-Cys-573 and the reverse orientation at positions 614 to 610 was striking, and computer predictions indicate turns of the polypeptide in these areas, probably contributing to the overall structure of AD-1. On the basis of experiments using mouse monoclonal antibodies and chymotrypsin-generated fragments of gCI, antibody binding sites dependent on disulfide bonds on the gp58 molecule between residues 514 and 635 have been postulated. However, these sites were completely destroyed by denaturing the protein with SDS and β-ME (19).

The complexity of AD-1 was mirrored by the primary structure of one of the antibodies reacting with it. Sequence data for human antibody 89-104 ($NT^+$) indicated an unusual structure, especially in the CDR3 region of the heavy chain (30). This segment of the antibody does not match any published D segment and is one of the largest D regions described, possibly reflecting the necessity of interaction with an unusual antigenic structure.

AD-1 is a structure that is conserved between HCMV isolates, as indicated by two lines of evidence. (i) Comparison of nucleotide sequences from different isolates has revealed a high degree of homology in this area of the gene (9, 25). (ii) We have been unable so far to identify HCMV isolates which were not recognized by AD-1-specific monoclonal antibodies. Recently Darlington et al. (11) have reported on the sequence variations of clinical HCMV isolates within a part of AD-1, specifically aa 603 to 635. Single and double amino acid changes were detected in few (9%) isolates, and in the absence of immunological data, it was speculated that these variations could represent escape mutants. Our own analysis of the entire region (25) has revealed similar mutations; however, as stated above, all clinical isolates were recognized by AD-1-specific antibodies. The high degree of conservation suggests an important function of this part of the molecule for the replication cycle of HCMV.

Highly antigenic structures are capable of inducing antibodies against a number of different, in some cases overlapping, core sequences within a single region. This has been shown, for example, for the principal neutralizing domain of human immunodeficiency virus or hemagglutinin of influenza virus (7, 36). For AD-1, a similar phenomenon might occur during natural infection. Whereas no difference was observed between the recognition of 89-104 (human, $NT^+$) and human sera at the carboxy-terminal end of AD-1, a

difference of at least 5 aa was found at the amino terminus, indicating that antibodies with different specificities were induced during natural infection. Antibodies of the 89-104 (human, $NT^+$) type seem to represent a minor fraction, since they were not detected in immunoblots with the Exo plasmid-encoded proteins. This assay, however, does not allow the detection of low-level antibodies because of the background reaction with the procaryotic fusion partner. The fact that fusion protein gig 58-2, comprising the entire antigenic region, consistently showed the strongest reactivity with human sera also pointed to the possibility that antibodies with different specificities were induced during natural infection. Recent results obtained with a number of human monoclonal antibodies suggest that in fact antibodies with different specificities were induced during natural infection (31a). What fraction of human antibodies induced during natural infection and differing from 89-104 (human, $NT^+$) in their specificity is neutralizing remains to be determined. The more homogeneous response of the murine antibodies probably reflected the genetic restriction exerted by the murine major histocompatibility complex, since these antibodies were derived from a single inbred mouse strain.

In summary, our results have shown that the immunodominant domain of gp58 of HCMV consists of a continuous sequence of more than 70 aa which formed a highly stable structure. The domain had the capacity to bind antibodies with different biological activities. Since gp58/116 has been proposed as a candidate for a subunit vaccine, it will be important to determine which types of antibodies, i.e., neutralizing or nonneutralizing, are induced by this domain during natural infection. Such studies are in progress.

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