Expression of the murine cytomegalovirus glycoprotein H by recombinant vaccinia virus

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The sequence of the gene encoding glycoprotein H (gH) of murine cytomegalovirus (MCMV) strain Smith was determined and compared with the sequence of the gH of MCMV strain K181. Transcriptional analysis showed that gH is encoded by a large mRNA of 5.0 kb, which is synthesized late in infection. A recombinant vaccinia virus expressing the MCMV gH open reading frame was constructed (Vac-gH). Anti-MCMV serum precipitated a protein of 87K from Vac-gH-infected cells. Reactivity with a monoclonal antibody showed the identity of the MCMV gH with a 87K envelope glycoprotein described previously by Loh and Qualtiere. Immunization of mice with the Vac-gH recombinant gave rise to an anti-gH serum, which neutralized MCMV without complement in vitro.

Murine cytomegalovirus (MCMV) is a close relative of human cytomegalovirus (HCMV), which is a ubiquitous human pathogen. Most primary infections cause only mild if any symptoms of sickness. However, in an immunologically immature host, such as the fetus, as well as in immunocompromised individuals HCMV is a cause of serious illness and often death (Ho, 1991; Pass, 1991). Because of the species specificity of HCMV, the availability of an appropriate animal model is important. MCMV is widely used as such a model because of the many similarities between human and murine infection with respect to biology and pathogenesis.

To prevent the risks of intra-uterine HCMV infection attenuated virus strains are candidates for vaccines. Treatment of HCMV disease in immunodeficient patients is even more difficult and presently the combined application of drugs and anti-HCMV antibodies is regarded as being most successful. To avoid the risks associated with live vaccines, the application of subunit vaccines based upon the surface glycoproteins of the virus has been proposed. Envelope glycoproteins of herpesviruses are known to be antigens for both cellular and humoral immune response (Spear, 1985). Their property of serving as targets for neutralizing antibodies is of particular interest.

Two glycoproteins are known to be conserved among all herpesviruses, namely the glycoprotein B (gB) and the glycoprotein H (gH). The two glycoproteins are essential and probably play a role in virus entry and cell-to-cell spread. They are conserved in several aspects including genomic location of the genes, amino acid sequence, tertiary structure and probably function. The gB appears to be the major target for the host immune response against HCMV, and a high proportion of the neutralizing antibodies in human antisera are directed against gB (Britt et al., 1990). The recombinant protein induces a neutralizing antibody response (Cranage et al., 1986) and gB is also a target for human cytotoxic T lymphocytes (Borysiewicz et al., 1988).

So far, most investigations concerning glycoproteins have concentrated on gB, whereas much less is known about gH, although it is an essential protein as well and capable of inducing neutralizing antibodies. Recently, the gH gene of the MCMV strain K181 has been identified and sequenced (Xu et al., 1992). After having characterized and expressed the gB gene (Rapp et al., 1992) we examined the sequence and expression of the gH gene of the MCMV strain Smith with the intention of investigating the MCMV glycoproteins further in view of their usefulness as subunit vaccines.

The nucleic acid sequence of the gH gene of the MCMV strain Smith was determined by a modified version of the dideoxynucleotide sequencing method (Tabor & Richardson, 1987). The GCG software
package version 7.0 (Devereux et al., 1984) was used for the analysis of the nucleic acid sequence, for the deduction of the amino acid sequence, and for the comparison with the sequence of strain K181. Assuming no differences between the two MCMV strains regarding the genomic location of the gene, a 2.5 kb HindIII–EcoRI fragment of the HindIII C fragment of MCMV strain Smith was subcloned (pH1, Fig. 1) and sequenced at the EcoRI end. The nucleotide sequence of this region showed 95% identity to the published K181 strain gH sequence. Then, a further subclone was constructed from the HindIII C fragment (pH2, Fig. 1) which should contain the complete open reading frame (ORF). The DNA sequence was determined on both strands by either sequencing of pill and pH2 with synthetic oligo-nucleotide primers. The analysis revealed a single, long DNA fragment of 2175 nucleotides capable of encoding a protein of 725 amino acids. Thus, the gH of MCMV strain Smith is one amino acid longer than that of MCMV strain K181. The identity of the genes is 98% on the nucleotide level and 95% on the amino acid level. The differences occurring in the nucleotide sequences are clustered in three short regions (Fig. 2). The amino acid exchanges in region 1 result from several differences in the nucleotide sequences. The differences occurring in regions 2 and 3 are due to short frameshifts. Besides these regions there are only two other amino acid differences. One occurs at position 322, the other at position 664.

Genes of herpesviruses often show rather complicated transriptional patterns as exemplified by the mRNAs of the HCMV and MCMV polymerase and gB genes (Kouzarides et al., 1987; Elliott et al., 1991; Rapp et al., 1992). To elucidate the situation for the gH gene, whole-cell RNA was prepared from MCMV-infected cells at different times after infection following published procedures (Chirgwin et al., 1979), and Northern blot hybridization was performed. A double-stranded probe specific for the gH region revealed two fragments of 5.0 kb and 5.8 kb (data not shown). To discriminate between the gH encoding transcript and other transcripts of that region, strand-specific probes were prepared from a 1.1 kb fragment from the 3'-terminal half of the gH genes.
ORF (Fig. 3, probes a and b) and ‘run-off’ synthesis was carried out (Stürzl & Roth, 1990). Probe a, which is specific for the coding strand, reacted with the 5.0 kb band. The probe specific for the non-coding strand (b) hybridized with a transcript of 5.8 kb. Probe c, which is located in the HindIII D fragment about 2.0 kb downstream of the gH ORF (Fig. 3c) also reacted with the 5.0 kb RNA plus an additional RNA of 3.1 kb. All RNAs are expressed late in infection, i.e. after 16 h post-infection (p.i.) (Keil et al., 1984). Since they were not synthesized after the DNA polymerase inhibitor phosphonoacetic acid had been added to infected cells we conclude that the transcription of the gH gene is restricted to the late phase of infection.

A vaccinia virus recombinant (Vac-gH) expressing the complete gH ORF was generated as follows. For the construction of the recombinant plasmid pCS43-gH a 2.1 kb fragment was amplified by PCR from the HindIII C fragment of MCMV (Ebeling et al., 1983) using the primers HP1 and HP2 which included a BamHI restriction site for cloning purposes (Fig. 1). The generation of a fragment of the correct size was confirmed by gel electrophoresis. The fragment was isolated, cut with BamHI and inserted into the vaccinia virus recombination vector pCS43 (Arnold et al., 1990) downstream of the vaccinia virus early-late promoter p7.5. The correct position of the start codon was confirmed by sequencing of the resulting plasmid pCS43-gH. The vaccinia virus recombinants were produced following established procedures (Mackett et al., 1984) using the DNA of vaccinia virus strain Copenhagen and the temperature-sensitive mutant ts7 (Drillien & Spehner, 1983).

To detect gH expression in Vac-gH-infected cells two peptides representing amino acids 476 to 495 (PepgHI) and 504 to 523 (PepgHII) were selected from the deduced amino acid sequence of the ORF and anti-peptide sera were generated in rabbits. Western blotting with these sera confirmed the expression of the gH in Vac-gH-infected cells (data not shown). To characterize the gH gene products further, immunoprecipitations were carried out as described previously (Keil et al., 1985). Proteins of MCMV-infected cells were labelled with [35S]methionine from 20 to 28 h p.i. Similarly, proteins of Vac-gH-infected cells were labelled from 4 to 8 h p.i. Samples of cell lysates were incubated with anti-MCMV serum or ascitic fluid of the monoclonal antibody 8D1.22A (Loh & Qualtiere, 1988). The precipitated proteins were separated on 7.5% SDS-polyacrylamide gels and visualized by fluorography. In MCMV- and Vac-gH-infected cells, a protein with the Mr of 87K was recognized by the monoclonal antibody 8D1.22A (Fig. 4). Anti-MCMV serum also precipitated a 87K protein from Vac-gH-infected cells. In MCMV-infected cells a number of proteins are recognized by this serum, among them a protein with an Mr similar to the 87K protein precipitated from Vac-gH-infected cells, which probably
represents the MCMV-expressed gH. The protein found in Vac-gH-infected cells appeared to have a slightly lower Mr than the protein in MCMV-infected cells (Fig. 4). The positive reaction with the neutralizing monoclonal antibody 8D1.22A showed that the 87K envelope glycoprotein described previously (Loh & Qualtiere, 1988) is indeed the MCMV gH.

To determine the neutralizing capacity of an anti-gH serum raised in mice by infection with the Vac-gH recombinant a plaque reduction assay was performed as described previously (Rapp et al., 1992). An anti-Vac-gB serum and an anti-MCMV serum raised in mice by infection with MCMV were included in the plaque reduction assay for comparison. As a negative control a recombinant vaccinia virus expressing the immediate early protein pp89 was used, which does not induce a neutralizing antibody response (Jonjid et al., 1988). Sera against the vaccinia virus recombinants were generated by immunizing the mice intravenously with two doses of 1 × 10⁶ p.f.u. MCMV given 3 weeks apart. Animals were bled 3 weeks after the boost. Whereas the anti-Vac-pp89 serum was not able to neutralize the virus, the anti-gH, anti-gB and anti-MCMV antisera showed considerable neutralizing activity (Table 1). The anti-gH serum was capable of neutralizing MCMV in the presence or absence of complement. The complement-independent neutralizing activity of the anti-gH serum was about fivefold higher than that of the anti-MCMV serum.

Our data show that the sequences of the gH of the MCMV strains Smith and K181 are almost identical. The differences are clustered in three regions. The lower percentage of identity at the amino acid level (95%) compared to the nucleotide level (98%) is caused by two small frameshifts that result in completely different amino sequences (Fig. 2a and b, regions 2 and 3). However, we cannot rule out the possibility that these differences are due to sequencing mistakes, since they are located in GC-rich regions with palindromic structures. We believe that our sequence is correct since it was obtained by sequencing both strands of this region several times and using deaza-dGTP to avoid compressions. The identity found between the gH of different MCMV strains is comparable to that found with the HCMV gH (Pachl et al., 1989). In the case of the MCMV gB the comparison of the nucleotide sequence of the Smith strain gB to the 3'-terminal part of the K181 strain gB sequence also showed a high level of identity (97%) (Rapp et al., 1992; Elliott et al., 1991). However, as only part of the K181 strain gB sequence has been published, it is difficult to assess the full extent of the differences. For HCMV the gH sequence is more conserved between different strains than is the gB sequence (Chou, 1992), which is apparently the case for MCMV also.

One interesting finding was that gH is encoded by a 50 kb mRNA, which is about 3 kb longer than the ORF. The existence of additional, longer transcripts has also been reported for the HCMV and MCMV gB (Spaete et al., 1988; Rapp et al., 1992) and polymerase genes (Kouzarides et al., 1987; Elliott et al., 1991). It has been proposed that these HCMV transcripts represent read-through products of the gB transcript which would normally terminate at the polyadenylation signal within the DNA polymerase coding region (Kouzarides et al., 1987). In the case of the gH no smaller transcript was found, and we conclude that the protein is encoded by the 50 kb transcript. That means that the gH transcript does not terminate at the potential polyadenylation signal sequence immediately downstream of the ORF as proposed by Xu et al. (1992) but continues through to the next gene and probably stops after the dUTPase gene.

To conduct in vivo protection experiments the gH ORF was inserted into the vaccinia virus genome. The protein expressed by the recombinant vaccinia virus Vac-gH had approximately the same size (87K) as the gH protein in MCMV-infected cells (Fig. 4). The reaction with the monoclonal antibody 8D1.22A revealed the identity of the gH with the 87K protein described by Loh and Qualtiere. They have shown that this 87K protein is a glycoprotein and is present on the surface of the virion as determined by surface iodination reaction (Loh & Qualtiere, 1988). The small difference in size between the vaccinia virus-expressed gH and the authentic protein in MCMV-infected cells may be due to different glycosylation of the protein in Vac-gH-infected CV-1 cells in contrast to MCMV-infected mouse embryo fibroblasts. Alternatively, the difference may reflect improper processing of gH in Vac-gH-infected cells. Several authors have found that the gH homologues of other herpesviruses need an accessory small protein for proper folding, subsequent processing and transport to the cell surface (Hutchinson et al., 1992; Spaete et al., 1993). This small glycoprotein was named gL in herpes simplex virus (HSV) (Hutchinson et al., 1992). The vaccinia virus-expressed HCMV gH for example also shows a slightly reduced electrophoretic mobility compared to the authentic protein and is not transported to the cell surface (Cranage et al., 1988). In immunoprecipitation

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<th>Antiserum</th>
<th>Reciprocal of serum dilution for 50% plaque reduction</th>
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<td>Anti-Vac-pp89</td>
<td>&lt; 10</td>
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<tr>
<td>Anti-Vac-gB</td>
<td>200–700</td>
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<tr>
<td>Anti-Vac-gH</td>
<td>150–280</td>
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<td>Anti-MCMV</td>
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experiments with iodinated surface proteins we also found no evidence for the surface expression of the MCMV gH in Vac-virus-infected cells (data not shown). In cells that co-express gH and gL, transportation of the HCMV gH to the cell surface can be observed (Spaete et al., 1993). To what extent the improper processing of the gH has an effect on its antigenic properties remains to be established.

Typically for herpesviral gH homologues the MCMV gH is recognized by a monoclonal antibody (8D1.22A) that shows complement-independent neutralization (Loh & Qualtiere, 1988). In addition, immunization with the Vac-gH recombinant induced considerable complement-independent neutralization activity in mice. In fact, the neutralizing capacity of the Vac-gH-induced antisera was about fivefold higher than that of the anti-MCMV serum without complement. These results suggest that there are fewer anti-gH antibodies in the anti-MCMV serum than in the monospecific anti-gH serum. This is in accordance with the fact that gH is apparently less abundant in MCMV-infected cells than in Vac-gH-infected cells (data not shown). Furthermore, these data show that, even in the absence of the MCMV gL, the Vac-gH recombinant expresses the protein in a form that is strongly immunogenic and antigenic. This is apparently not the case for the HSV gH, because no or only a little neutralization activity was induced by the HSV gH expressed by vaccinia virus or baculovirus (Blacklaws et al., 1990; Forrester et al., 1991; Ghiasi et al., 1992).

Thus, the gL is not a stringent prerequisite for the formation of immunogenic domains of gH giving rise to neutralizing antibodies.

Generation of the vaccinia virus recombinants Vac-gB and Vac-gH provides a tool for studying the immune response against individual glycoproteins. Since the experimental vaccine induces a stronger protein-specific in vitro neutralization than the authentic MCMV-expressed protein, in vivo protection experiments will determine the active and passive protection potential of the two proteins. As outlined above, the two glycoproteins, especially gB, are extremely conserved in several aspects. Thus, the results of these studies will also be of general interest in view of the evaluation of the two glycoproteins as candidates for subunit vaccines.

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