The rat cytomegalovirus R32 gene encodes a virion-associated protein that elicits a strong humoral immune response in infected rats

Erik Beuken, Gert Grauls, Cathrien A. Bruggeman and Cornelis Vink

Department of Medical Microbiology, Cardiovascular Research Institute Maastricht, Maastricht University, PO Box 5800, 6202 AZ Maastricht, The Netherlands

A gene of rat cytomegalovirus (RCMV), designated R32, has been identified that encodes a homologue of the human cytomegalovirus (HCMV) pp150 (ppUL32) major tegument phosphoprotein. The R32 ORF has the capacity to encode a 667 amino acid polypeptide (pR32) with a calculated molecular mass of 73 kDa. The predicted amino acid sequence of pR32 shows similarity to that of polypeptides predicted to be encoded by the HCMV UL32, murine cytomegalovirus M32 and human herpesvirus types 6 and 7 U11 genes. The R32 gene is transcribed as a 2.5 kb mRNA during the late phase of RCMV infection in rat embryo fibroblasts in vitro. To study expression of the pR32 protein in vitro and in vivo, a rabbit polyclonal antiserum was raised against a recombinant protein that comprised amino acids 252–522 of pR32. By using this antiserum, pR32 could be detected predominantly in the cytoplasm of RCMV-infected fibroblasts at 24 and 48 h post-infection in vitro. The pR32 protein was also detected within virions isolated from the culture medium of RCMV-infected cells. Expression of pR32 in vivo was observed within the cytoplasm of salivary gland epithelial cells of RCMV-infected rats. In addition, recombinant pR32 was found to react with sera from rats that were previously infected with RCMV, whereas reactivity was not seen with sera from mock-infected rats. Together, these findings indicate that RCMV pR32 represents the homologue of HCMV ppUL32, both in primary structure and in function.

Introduction

The virion of human cytomegalovirus (HCMV) can be divided roughly into three major structures: the capsid, the envelope and a region between capsid and envelope termed the tegument (for reviews see Spaete et al., 1994; Mocarski, 1993). The HCMV tegument consists of at least seven proteins, the majority of which are phosphorylated (Spaete et al., 1994). The most prominent tegument proteins are encoded by the UL32 (pp150), UL69, UL82 (pp71), UL83 (pp65) and UL99 (pp28) ORFs. Although the proteins encoded by UL69, UL82 and UL83 were detected in the nucleus of HCMV-infected cells, the UL99-encoded polypeptide was observed in extra-nuclear compartments (Gallina et al., 1996; Landini et al., 1987; Schmolke et al., 1995). The UL32-encoded phosphoprotein, pp150, was reported to have a predominantly cytoplasmic distribution (Hensel et al., 1995; Sanchez et al., 1998; Scholl et al., 1988). This protein constitutes approximately 20% of the virion protein content (Kimrie & Gibson, 1983; Gibson, 1983; Jahn et al., 1987b). It is translated from a late, 6.2 kb mRNA and is predicted to consist of 1048 amino acids (Jahn et al., 1987a). An indication that pp150 plays an important role in HCMV replication has come from a study by Zipeto et al. (1993), who described a variant HCMV strain containing an in-frame, three-nucleotide deletion in the UL32 gene. Attempts to plaque-purify this strain were unsuccessful, suggesting that this mutant was growth-defective and, as a consequence, that pp150 is essential for virus replication (Zipeto et al., 1993). It should be noted, however, that rescue experiments, in which the wild-type UL32 gene was allowed to complement the mutated UL32 gene from the variant HCMV strain, were not performed. A vital function of pp150 in HCMV replication was also inferred from experiments in which HCMV infection was studied in astrocytoma cells that stably express UL32 antisense mRNA. This antisense mRNA strongly interfered with virus...
maturation during the late phase of infection (Meyer et al., 1997).

Of all HCMV virion proteins, pp150 is the strongest immunogen (Jahn et al., 1987 b). High antibody titres to this protein have been found, irrespective of the stage of infection, in nearly 100% of HCMV-seropositive individuals. Only during the early phase of naturally occurring primary HCMV infections was the antibody response to pp150 found to be either low or absent (Landini et al., 1988). Consequently, (recombinant) pp150 is widely used in HCMV serological assays within diagnostic laboratories.

Currently, few tegument proteins of animal CMVs have been studied in detail. Among these are the products of murine CMV (MCMV) genes M82, M83 and M84, encoding homologues of the HCMV UL82 and UL83 tegument phosphoproteins (Cramer et al., 1996), and the product of the MCMV M99 gene, encoding the homologue of HCMV pp28 (Cramer et al., 1994). However, putative homologues of the HCMV major tegument protein pp150 from animal CMVs have hitherto not been subjected to any detailed study. In this report, we describe the identification and characterization of the rat cytomegalovirus (RCMV) R32 gene, encoding the homologue of HCMV pp150.

**Methods**

- **Virus and cell culture.** Growth and infection of primary rat embryo fibroblasts (REF) with RCMV (Maastricht) was done as described previously (Bruggeman et al., 1985). The purification of RCMV genomic DNA was carried out as described by Vink et al. (1996).

- **Cloning and sequencing of RCMV DNA.** RCMV genomic DNA was digested with SphI and separated on a 1% agarose gel. After transfer to a nylon membrane (Hybond-N+, Amersham), the DNA was hybridized to a probe that was generated from the RCMV EcoRI fragment A, which has a length of approximately 50 kb (Meijer et al., 1986). Labelling, hybridization and detection experiments were performed with digoxigenin DNA-labelling and chemiluminescence detection kits (Boehringer Mannheim). Hybridizing SphI fragments were cloned as follows. RCMV DNA was digested with SphI and electrophoresed through agarose. RCMV EcoRI fragment A-specific SphI fragments were excised, purified and cloned into the SphI site of vector pUC119. The resulting constructs were sequenced and the presence of HCMV UL32-homologous regions was confirmed by alignment with sequences from the EMBL nucleic acid sequence database. Thus, a 3-6 kb RCMV SphI fragment was identified within plasmid pAS2B, which contains an ORF with significant identity to the HCMV UL32 gene. This novel RCMV ORF was termed R32. Both strands of the 3-6 kb SphI fragment were sequenced using the thermostequence cycle sequencing kit (Amersham) with Cy5-labelled primers and an ALFexpress automated DNA sequencer (Pharmacia Biotech). Sequence analysis was done using the program PC/GENe version 2.11 (Intelligenetics).

- **Poly(A)^+ RNA isolation and Northern blot analysis.** Poly(A)^+ RNA from RCMV- and mock-infected REF cells was isolated at immediate-early (in the presence of cycloheximide), early (in the presence of phosphonoacetic acid) and late times of infection and at 2, 8 and 48 h post-infection (p.i.) using a QuickPrep Micro mRNA purification kit (Pharmacia Biotech), as described previously (Beisser et al., 1998a). Poly(A)^+ RNA from mock-infected cells was isolated in a similar fashion as late mRNA, except that RCMV infection was omitted. Infections with RCMV were done with an m.o.i. of either 1 (for isolation of mRNA at immediate-early and early times and at 2 and 8 h p.i.) or 0.01 (for isolation of mRNA at late times and at 48 h p.i.). Electrophoresis of RNA and transfer to Hybond-N+ membranes has been described previously (Brown & Mackey, 1997). A 402 bp XhoI–BamHI fragment, derived from plasmid p026 (Beisser et al., 1998a), was used as an R32-specific probe. Labelling, hybridization and detection experiments were performed using digoxigenin DNA-labelling and chemiluminescence detection kits (Boehringer Mannheim) and Dig Easy Hyb hybridization solution (Boehringer Mannheim).

- **Generation of pR32 bacterial expression constructs.** To generate a construct that expresses part of the R32-derived protein fused to glutathione-S-transferase (GST), plasmid pAS2B was digested with SalI and the resulting 807 bp fragment was cloned into the SalI site of vector pRP269, generating plasmid p132. Vector pRP269 is derived from pGEX-1N (Pharmacia Biotech) and contains an extended polylinker-cloning site including more unique restriction endonuclease cleavage sites than pGEX-1N. Plasmid p132 encodes a protein, termed GST–pR32, consisting of GST fused at its C terminus to amino acids 252–522 of pR32.

To generate a construct that expresses a part of pR32 fused to thioredoxin and a tag consisting of six consecutive histidine residues (6 × His), an 842 bp BamHI–HindIII fragment from p132 was cloned into the BamHI and HindIII sites of vector pET-32b (+) (Novagen), resulting in plasmid p135. This plasmid encodes a protein, termed 6H–Trx–pR32, containing amino acids 252–522 of pR32 fused to the C terminus of the thioredoxin protein, which in turn is fused to the C terminus of a 6 × His tag.

- **Expression and purification of GST–pR32 and 6H–Trx–pR32.** Construct p132 was introduced into Escherichia coli strain AD202. The resulting strain was grown overnight at 37 °C in LB medium containing 50 µg/mL ampicillin. The culture was diluted 1:100 in 150 ml LB medium with ampicillin and grown at 37 °C to an OD600 of 0.8. Protein production was induced by addition of IPTG to a final concentration of 0.2 mM. After incubation for 3 h at 37 °C, the bacteria were harvested by centrifugation. The GST–pR32 protein was purified by single-step affinity chromatography using glutathione–Sepharose 4B (Pharmacia Biotech), as recommended by the manufacturer.

Construct p135 was introduced into E. coli strain BL21 (DE3) plysS. The resulting strain was grown overnight at 37 °C in LB medium containing 50 µg/mL ampicillin and 20 µg/mL chloramphenicol. The culture was diluted 1:100 in 150 ml LB medium containing ampicillin and chloramphenicol and grown at 37 °C to an OD600 of 0.8. Protein production was induced as described above. The 6H–Trx–pR32 protein was purified as follows. The bacterial pellet was resuspended in 10 ml buffer A (300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.8). After sonication of the suspension on ice, Triton X-100 was added to a final concentration of 1 mM. The bacterial lysate was then centrifuged for 20 min at 12000 g (4 °C). Ten ml of a 50% slurry of Ni2+–nitroacetate acid (NTA)–agarose (Qiagen), equilibrated previously in buffer A containing 5 mM imidazole, was added to the supernatant. The suspension was stirred for 1 h at 4 °C and subsequently poured into a column. Non-specifically bound proteins were washed with the Ni-NTA–agarose by three subsequent washes (of 4 ml each) with buffer A plus 5 mM imidazole, 10 mM imidazole and 20 mM imidazole, respectively. The 6H–Trx–pR32 protein was eluted from the column with 5 ml buffer A containing 300 mM imidazole. Fractions of 1 ml were collected, analysed by SDS–PAGE, pooled and dialysed against PBS at 16 h at 4 °C.
Characterization of the RCMV R32 gene

Fig. 1. The position of the R32 gene within the RCMV (Maastricht) genome and predicted amino acid sequence encoded by R32. (a) Localization of R32. The R32 gene was localized to a 3623 bp SphI fragment that was derived from EcoRI fragment A and cloned in plasmid pAS2B. The EcoRI restriction map of the RCMV genome has been described previously by Meijer et al. (1986). The black arrow shows the position and orientation of the R32 gene within the SphI fragment. This fragment also contains part of the R33 gene (Beisser et al., 1998a), which is transcribed in the opposite direction to R32. (b) Multiple alignment of amino acid sequences predicted to be encoded by the RCMV R32 (pR32), MCMV M32 (pM32) and HCMV UL32 (pUL32) genes. Numbering of amino acid residues is shown at the left of the aligned sequences. Residues that are identical between at least two of the aligned sequences are boxed. Gaps introduced into the alignment are depicted as dashes. The MCMV sequence was derived from Rawlinson et al. (1996) and the HCMV sequence was from Jahn et al. (1987a).

### Generation of a polyclonal antiserum directed against 6H–Trx–pR32.
A rabbit was immunized by intramuscular injection of 250 µg purified 6H–Trx–pR32 in Specol adjuvant (ID-DLO, Lelystad, The Netherlands). Two additional booster injections, each containing 250 µg purified 6H–Trx–pR32, were given at weeks 7 and 12. At week 16, blood was obtained and serum was prepared as described previously (Harlow & Lane, 1988). This serum was found to be strongly reactive to both 6H–Trx–pR32 and GST–pR32 by Western blot analysis.

### Generation of sera from RCMV- and mock-infected rats.
Male, specific-pathogen-free Lewis/N RT1 rats (Central Animal Facility, Maastricht University) were kept under standard conditions. The animals were immunocompromised by 5 Gy total-body Röntgen irradiation 1 day before infection, as described previously (Stals et al., 1990). Rats (10 weeks old, 250–300 g) were either mock-infected or infected with 5 × 10^5 p.f.u. RCMV. Serum was obtained from the rats at either day 28 or day 90 p.i.

### SDS–PAGE and Western blot analysis.
Proteins were separated on SDS–polyacrylamide gels, essentially as described by Laemmli (1970). After electrophoresis, gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose (PROTRAN, 0–2 µm; Schleicher & Schuell). Blots were developed by successive incubations with either rabbit anti-6H–Trx–pR32 antiserum (1:1000 diluted) followed by peroxidase-conjugated, rabbit anti-rat immunoglobulins (1:1000 diluted; P0450, Dako). The blots were stained by incubation in a solution containing diaminobenzidine.

### Indirect immunofluorescence.
REF were grown in 96-well tissue culture plates (Costar). At several times after infection with RCMV, cells were fixed, permeabilized and washed. Rabbit anti-6H–Trx–pR32 antiserum, diluted 1:100 in PBS, was added and incubated for 30 min at 37 °C. After washing, the cells were incubated with FITC-conjugated swine anti-rabbit immunoglobulins (P0205, Dako), washed and examined with an Axiovert 100 fluorescence microscope (Zeiss). Staining of cells with monoclonal antibody RCMV8 was done as described previously (Bruning et al., 1987).

### Immunohistochemistry.
Serial tissue sections (4 µm) of the (submaxillary) salivary glands of RCMV- and mock-infected rats were mounted on glass slides and deparaffinized. Sections were incubated successively with the anti-6H–Trx–pR32 antiserum, biotin-conjugated, swine anti-rabbit immunoglobulins (P0205, Dako), washed and examined with an Axiovert 100 fluorescence microscope (Zeiss). Staining of cells with monoclonal antibody RCMV8 was done as described previously (Bruning et al., 1987).
Table 1. Consensus sites for post-translational modification within the predicted amino acid sequence of RCMV pR32

The data were generated by screening the R32-encoded amino acid sequence against the PROSITE pattern library by using the computer program MOTIF (Hofmann et al., 1999).

<table>
<thead>
<tr>
<th>Consensus site</th>
<th>Number</th>
<th>Location</th>
</tr>
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<tbody>
<tr>
<td>Phosphorylation:</td>
<td>4</td>
<td>45–48, 70–73, 423–426, 563–566</td>
</tr>
<tr>
<td>Tyrosine kinase</td>
<td>3</td>
<td>18–25, 93–101, 568–576</td>
</tr>
<tr>
<td>N-Glycosylation</td>
<td>1</td>
<td>652–655</td>
</tr>
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Fig. 2. Transcription of the RCMV R32 gene. Transcripts from the R32 gene of RCMV were detected by Northern analysis as described in Methods. Poly(A)+ RNA was isolated from RCMV- and mock (M)-infected REF at various times (0, 2, 8 and 48 h p.i.) as shown above the lanes. The estimated length of the R32 transcripts is shown on the right-hand side in kb.

Results

Identification, cloning and sequence analysis of the RCMV R32 gene

In order to identify a putative homologue of the HCMV UL32 gene within the genome of RCMV, we assumed that this homologue would be located at a position within the genome similar to that of HCMV UL32 (Chee et al., 1990). This assumption was based on the observation that the RCMV genome is collinear with the genomes of HCMV and MCMV (Beuken et al., 1996; Vink et al., 1997; Beisser et al., 1998a,b). Previously, we identified and characterized the RCMV R33 gene, which is the homologue of the HCMV UL33 gene (Beisser et al., 1998a). Since HCMV UL33 is positioned directly 3’ of the UL32 gene (Chee et al., 1990), a potential RCMV homologue of UL32 was anticipated to be located at the 5’ side of, and adjacent to, the RCMV R33 gene, within the RCMV EcoRI fragment A (Beisser et al., 1998a; Meijer et al., 1986). In order to clone the potential RCMV UL32 homologue, we generated a panel of SphI plasmid subclones of the EcoRI fragment A, which has a length of approximately 50 kb (Fig. 1a; Meijer et al., 1986). The inserts of each subclone were sequenced and the resulting sequences were checked for the presence of ORFs that shared identity with UL32. Thus, a 3–6 kb SphI fragment was identified that contained sequences with significant identity to HCMV UL32. The homologous sequences are located within an ORF with a length of 2001 bp (Fig. 1a). The ATG initiation codon of this ORF is separated by only 88 bp from the initiation codon of the R33 ORF, which runs in the opposite direction within the RCMV genome. The 2001 bp RCMV ORF has the capacity to encode a 667 amino acid protein (Fig. 1b) with a calculated molecular mass of 73 kDa and a theoretical isoelectric point of pH 5.61. The amino acid sequence of this protein shows similarity to those of the polypeptides predicted to be encoded by MCMV M32 (37.4% identity; Rawlinson et al., 1996), HCMV UL32 (18.2% identity; Jahn et al., 1987b) and the U11 genes of human herpesvirus types 6 and 7 (17.9% and 15.9% identity, respectively) (Gompels et al., 1995; Nicholas, 1996). On the basis of these similarities, the 2001 bp RCMV ORF that we identified was termed R32, analogous to the nomenclature of the corresponding HCMV and MCMV genes (Chee et al., 1990; Rawlinson et al., 1996). Fig. 1(b) shows a multiple alignment of the predicted amino acid sequences derived from the R32, M32 and UL32 genes. The alignment was generated by a CLUSTAL W multiple sequence alignment (Thompson et al., 1994), with a PAM250 protein distance matrix (pairwise comparison).
Characterization of the RCMV R32 gene

Fig. 3. Purification of recombinant pR32 and the generation of rabbit antiserum against the purified protein. (a) Purification of 6H–Trx–pR32. The purification was carried out by affinity chromatography over Ni-NTA–agarose as described in Methods. During the purification, samples were analysed by SDS–PAGE and Coomassie blue staining. A photograph of the stained gel is shown. Samples include the supernatant of the crude bacterial lysate that was added to the Ni-NTA–agarose (lane 2), the unbound fraction (lane 3) and the eluted fractions after addition of buffer containing increasing concentrations of imidazole (lanes 4–12). In lanes 6 and 7, the fractions are shown that were eluted with the first and last 1 ml ‘wash’, respectively, with buffer containing 20 mM imidazole. The eluted protein shown in lanes 9–12 was pooled, dialysed and used for immunization. Sizes of protein markers (lane 1) (Pharmacia Biotech) are indicated to the left of the gel. (b) Western blots probed with either anti-6H–Trx–pR32 rabbit antiserum or preimmune serum. Approximately 100 ng of the following purified proteins was loaded in duplicate on a 10% SDS–polyacrylamide gel: 6H–Trx–IE-1 (lanes 2 and 7), 6H–Trx–pR32 (3, 8), GST (4, 9) and GST–pR32 (5, 10). After transfer of the proteins to a nitrocellulose filter, half of the filter was incubated with the anti-6H–Trx–pR32 rabbit antiserum (Immune; lanes 1–5), whereas the other half was incubated with the preimmune rabbit serum (Preimmune; lanes 6–10). Sizes of prestained protein markers (lanes 1 and 5) (New England Biolabs) are indicated to the left of the gel.

alignment gap penalty = 3; multiple alignment gap penalty = 10; gap extension penalty = 10). Relatively high identity can be seen among the N-terminal halves of these sequences, whereas rather low identity is found among the C-terminal halves. Although the R32- and M32-derived sequences can be aligned conveniently, large gaps were introduced in the alignment to accommodate the UL32-derived sequence, which probably relates to the UL32-encoded sequence being considerably larger than the corresponding RCMV and MCMV sequences.

The predicted sequence of the R32-encoded protein, which was designated pR32, was investigated for the presence of consensus sites for post-translational modification. A total of 35 consensus phosphorylation sites was found, scattered throughout the sequence (Table 1). Nineteen of these represent casein kinase II phosphorylation sites. The other sequences represent phosphorylation sites for protein kinase C (nine), cAMP- and cGMP-dependent protein kinase (four) and tyrosine kinase (three). In addition to these sites, the pR32 sequence was found to contain 13 consensus N-myristoylation sites and a single putative N-glycosylation site. Also, two putative bipartite nuclear localization signals were identified, at positions 423–440 (RRASVGAASADRPRART) and 548–565 (RKAAAQRNSGASYAKRRP).

Transcription of the RCMV R32 gene

In order to examine transcription of the RCMV R32 gene, Northern blot experiments were performed. As shown in Fig. 2, an R32-specific transcript with a length of approximately 2.5 kb was detected at 48 h p.i. (lane 5), but not at earlier times (lanes 2–4). Similar transcripts of the R32 gene have previously been identified in a study on the neighbouring R33 gene (Beisser et al., 1998a). As expected, R32 transcription was not observed in mock-infected REF (Fig. 2, lane 1). To investigate the kinetics of transcription of R32 further, poly(A)RNA isolated from RCMV-infected REF was analysed at either immediate-early (in the presence of cycloheximide), early (in the presence of phosphonoacetic acid) or late times after infection. R32 transcripts could be detected exclusively at late times of infection (data not shown). These data indicate that RCMV R32 is a late gene, similar to HCMV UL32 (Jahn et al., 1987a).

From the estimated length of the R32 transcript (2.5 kb), it can be hypothesized that this transcript comprises the
complete, 2001 bp R32 ORF. Within the RCMV genome, the R32 ORF is flanked by a potential TATA box at positions −51 to −45 (5’ TATTATAA 3’) relative to the predicted ATG initiation codon of R32. At its 3’ end, the R32 ORF lacks a consensus polyadenylation sequence (5’ AATAAA 3’). However, three adenine-rich sequences are located immediately downstream of the R32 stop codon: (A)\(_1\) at positions 32–42, 5’ (A)\(_3\)TA 3’ at positions 79–87 and 5’ ATAT(A)\(_4\) 3’ at positions 99–106 relative to the stop codon. Whether any or all of these sequences actually plays a role in transcription of the R32 gene was not investigated further.

**Generation of a polyclonal antiserum directed against recombinant pR32**

In order to study the expression of the R32-encoded protein, we set out to generate rabbit polyclonal antibodies against recombinant pR32. For this purpose, we expressed a protein containing amino acids 252–522 of pR32 fused to a combined 6 × His–thioredoxin tag (6H–Trx–pR32). The region between amino acids 252 and 522 of pR32 was selected for expression because it largely overlaps the most hydrophilic part of the protein, located roughly between amino acid residues 280 and 600 (data not shown). Protein 6H–Trx–pR32 was expressed in *E. coli* and purified by affinity chromatography (Fig. 3 a). Purification resulted in a major, ~50 kDa protein that was approximately 80% pure, as judged by SDS-PAGE and Coomassie blue staining (Fig. 3 a, lanes 9–12). The majority of the faster-migrating, minor species in lanes 9–12 was found to represent degradation products of the full-length ~50 kDa 6H–Trx–pR32 (see below). The fractions shown in lanes 9–12 were pooled, dialysed and used for immunization of a rabbit as described in Methods. The reactivity of the anti-6H–Trx–pR32 antiserum generated against 6H–Trx–pR32 was tested by Western blot analysis. As shown in Fig. 3 (b), the antiserum reacted with 6H–Trx–pR32 as well as with the GST fusion protein (GST–pR32), containing the same pR32 sequence as that in 6H–Trx–pR32 (Fig. 3 b, lanes 3 and 5). This shows that the anti-6H–Trx–pR32 antiserum contains antibodies that are directed specifically against the pR32 part of the fusion proteins. As might be expected, cross-reactivity of the antiserum was seen with another 6H–Trx–pR32 antiserum that contained part of the RCMV IE-1 protein (Beisser et al., 1998 b) (lane 4). Since the antiserum did not react with GST alone (lane 4), it is likely that the reactive, faster-migrating species from

![Fig. 4. The RCMV pR32 is expressed within the cytoplasm of infected cells. REF were grown in wells of microtitre plates and infected with RCMV at an m.o.i. of 0.01. At various times p.i., cells were fixed and stained by indirect immunofluorescence using the anti-pR32 rabbit antiserum. The time-points investigated are 0 (a), 8 (b), 24 (c) and 48 (d) h p.i. Magnification, × 400.](image-url)
Characterization of the RCMV R32 gene

Fig. 5. The RCMV pR32 protein is associated with virions. Virions were purified by high-speed centrifugation of culture supernatant of RCMV-infected REF and analysed by SDS–PAGE and Western blotting. The Western blot was probed with the anti-pR32 rabbit antiserum. As a control, supernatant from mock-infected cells was included in the analysis (lane 2). This supernatant was treated in a similar fashion as the supernatant from RCMV-infected cells (lane 3). Major reactive species of pR32, with estimated molecular masses of approximately 80 and 85 kDa, are indicated (lane 3). A minor, non-specific protein species of approximately 60 kDa can be seen in both lanes 2 and 3. Sizes of prestained protein markers (lane 1) (Biorad) are indicated to the left of the gel.

Localization of pR32 within RCMV-infected cells and in virions

In order to detect expression of pR32 in vitro, REF were infected with RCMV and investigated at various times after infection by indirect immunofluorescence, using the anti-6H–Trx–pR32 antiserum. A fluorescent signal was detected within RCMV-infected cells at 24 and 48 h p.i. (Fig. 4c, d) but not at 0 or 8 h p.i. (Fig. 4a, b). This indicates that the anti-6H–Trx–pR32 antiserum is able to recognize the authentic, virus-encoded pR32. The signal was predominantly cytoplasmic and diffuse at both 24 and 48 h p.i. However, granular staining could also be observed at 24 h p.i. (Fig. 4c), both in the cytoplasm and near nuclear membranes. The late kinetics of expression of pR32 are in agreement with the kinetics of transcription of R32.

On the basis of the similarity between RCMV R32 and HCMV UL32, it might be speculated that the R32-encoded protein is a virion constituent and, more specifically, a tegument protein. To investigate the putative presence of pR32 within RCMV virions, extracellular virus particles were purified from the culture supernatant of RCMV-infected REF and analysed by Western blotting. The anti-6H–Trx–pR32 antiserum reacted strongly with two proteins with molecular masses of approximately 80 and 85 kDa (Fig. 5, lane 3). Several other, minor species also reacted with the antiserum. A monoclonal antibody directed against an early RCMV protein (RCMV8), which is expressed in the nucleus of RCMV-infected cells but not in virions (Bruning et al., 1987), did not react with the RCMV virion proteins (data not shown). The estimated molecular masses of the major immunoreactive virion proteins do not correspond to the calculated molecular mass of pR32 (73 kDa). However, since the predicted amino acid sequence of pR32 contains numerous consensus post-translational modification sites, it is likely that the 80 and 85 kDa polypeptides represent differentially modified versions of pR32. We therefore conclude that pR32 is a virion-associated protein, with an estimated molecular mass of 80–85 kDa.

The expression of pR32 in salivary glands of RCMV-infected rats

The rat salivary gland is a preferred site of replication and persistence of RCMV (Bruggeman et al., 1985). To investigate the expression of the pR32 protein within the salivary gland of RCMV-infected rats, sections were prepared from salivary
glands of immunocompromised, RCMV-infected rats. Successive sections were then subjected to immunological staining with either anti-6H–Trx–pR32 antiserum or monoclonal antibody RCMV8 (Fig. 6 a, b). Strong cytoplasmic staining of RCMV-infected epithelial cells can be observed with the anti-6H–Trx–pR32 antiserum (Fig. 6 a). No signal was detected in the nuclei of these cells. Staining with the antiserum was dependent on RCMV infection, since it was not seen in salivary gland sections from mock-infected rats (data not shown). Similar to results described previously, the use of the RCMV8 monoclonal antibody resulted in nuclear staining of RCMV-infected cells (Fig. 6 b). These data show clearly that pR32 is expressed within the cytoplasm of RCMV-infected cells from rat salivary glands.

Humoral immunogenicity of pR32 during RCMV infection

As described above, the HCMV pp150 protein is a very strong immunogen, inducing high antibody titres in a very high percentage of HCMV-infected individuals. To study whether the RCMV homologue of pp150 elicits a similarly strong humoral immune response in rats, sera from both RCMV- and mock-infected rats were analysed for reactivity against pR32. To this end, GST and GST–pR32 proteins were separated by SDS–PAGE and blotted onto nitrocellulose. The blot was probed with sera from seven RCMV-infected rats and seven mock-infected rats. Most of the sera were taken at day 90 p.i., whereas two of the sera, both from RCMV-infected rats (sera 8 and 10), were taken at day 28 p.i. As shown in Fig. 7, sera from mock-infected rats were unable to recognize either GST–pR32 or GST efficiently (lanes 1–7). By contrast, the sera from RCMV-infected rats reacted strongly with GST–pR32, but not with GST (lanes 8–14). This indicated that these sera recognized the pR32 part of the fusion protein specifically, but not the GST part. The reactive protein species of higher mobility than full-length GST–pR32 in lanes 8–14 are likely to represent degradation products of the fusion protein. In conclusion, our results indicate that a strong humoral immune response against pR32 is induced in rats during infection with RCMV.

Discussion

In this report, we describe the cloning and characterization of the RCMV R32 gene, which encodes the homologue of the HCMV pp150 (or ppUL32) tegument protein. The R32 gene bears similarity to MCMV M32 (Rawlinson et al., 1996) as well as to HCMV UL32, which encodes pp150 (Jahn et al., 1987 a). These three genes are conserved not only in sequence, but also in orientation and position within their respective viral genomes. The 2001 bp R32 ORF runs towards the left-hand end of the genome and is spaced 88 bp from the R33 ORF, which runs in the opposite direction. Similarly, the MCMV M32 and HCMV UL32 ORFs are separated by only 50 and 70 bp from the M33 and UL33 ORFs, respectively (Rawlinson et al., 1996). The R32 and UL32 genes are also very similar in their kinetics of transcription: both genes are transcribed exclusively in the late phase of infection of fibroblasts in vitro. Similar to its putative HCMV counterpart, the RCMV R32-derived protein (pR32) might be phosphorylated. This notion is based on two observations. First, the predicted amino acid sequence of the protein was found to contain 35 consensus phosphorylation sites. Second, the estimated molecular mass of pR32 (80–85 kDa) was considerably larger than the theoretical molecular mass of the protein (73 kDa). It is, of course, possible that this difference in molecular mass is, at least in part, due to other post-translational modifications, like myristoylation or N- or O-linked glycosylation. Nevertheless, the pR32 virion protein was found to be resistant to digestion with N-glycosidase F (data not shown), which indicates that pR32 is probably not modified by N-linked glycosylation, similar to HCMV ppUL32 (Benko et al., 1988). It is also unlikely that the pR32 protein is modified by O-linked glycosylation. This was inferred from its insensitivity to alkaline β-elimination (data not shown). In contrast to pR32, however, ppUL32 was reported to be modified by O-linked N-acetylglucosamine (Benko et al., 1988).

In order to be able to study expression and localization of pR32 within RCMV-infected cells, a rabbit polyclonal antiserum was raised against an E. coli-produced part of pR32. In agreement with the transcription data, pR32 was found to be expressed at late times after infection of REF with RCMV in vitro, starting at 24 h p.i. The protein was localized predomi-
nantly within the cytoplasm of infected cells. In addition to diffuse cytoplasmic staining by the anti-pR32 antiserum, granular staining could also be observed, in particular at 24 h p.i. Although the granular staining was localized mainly in the cytoplasm, it was also apparent near nuclear membranes. A similar staining of ‘vesicle-like’ structures has been reported previously for the HCMV counterpart of pR32, pp150 (Hensel et al., 1995). Hensel and co-workers reported pp150 to have both a cytoplasmic and nuclear distribution within HCMV-infected fibroblasts in vitro (Hensel et al., 1995). Other groups, however, reported pp150 to be localized predominantly to the cytoplasm (Sanchez et al., 1998; Scholl et al., 1988), similar to our results for RCMV pR32 (Fig. 4). Also, staining of pR32 was seen exclusively within the cytoplasm of salivary glands cells from RCMV-infected rats (Fig. 6a). Since we only investigated the in vivo expression of pR32 at a single time after infection, it is possible that the protein is expressed within the nuclei of these cells at different times. The presence of two different consensus bipartite nuclear localization signals within pR32 indicates that the protein might indeed be transported to the nuclei of infected cells. It has been suggested previously that pp150 is acquired during nucleocapsid assembly in certain nuclear subcompartments within HCMV-infected cells (Hensel et al., 1995). This acquisition of pp150 may precede the packaging of DNA within the nucleocapsids. Since pp150 was found to be absent from nuclear dense body-like structures, the protein was hypothesized to play a role in maintaining a specific nucleocapsid–nuclear membrane interaction (Hensel et al., 1995). Whether a similar function in virion assembly can be attributed to the RCMV pR32 protein will have to be investigated in future studies, which would include immunoelectron microscopy and biochemical experiments.

As has been reported for HCMV ppUL32, the RCMV pR32 protein is the target of a strong humoral immune response. Sera from seven infected rats were each found to contain antibodies that reacted with recombinant pR32. These antibodies could already be detected by Western blotting at day 28 p.i. Although we showed only that pR32 is a virion-associated protein, it is likely that it is located in the tegument of the virions. This notion is based on the similarity between pR32 and the HCMV pp150 tegument protein. Since antibodies against HCMV tegument proteins are generally non-neutralizing and non-protective, it is likely that the anti-pR32 antibodies from rat sera are similarly non-protective. However, as with the detection of anti-HCMV antibodies, the detection of anti-RCMV antibodies is extremely useful as a serological marker of the course of infection.

In conclusion, we have identified the RCMV R32 gene, which is likely to represent the homologue of the HCMV UL32 gene. The R32-encoded protein, pR32, is expressed predominantly within the cytoplasm of infected cells, both in vitro and in vivo, and is associated with RCMV virions. A strong humoral immune response is elicited against pR32 upon infection of rats with RCMV, similar to the response seen with pp150 in HCMV-infected individuals. Once again, these data demonstrate the close similarity between RCMV and HCMV.

We thank Patrick Beisser and Joanne van Dam for their critical reading of the manuscript.

References


Received 29 March 1999; Accepted 1 July 1999