The R27080 glycoprotein is abundantly secreted from human cytomegalovirus-infected fibroblasts

Jürgen Müllberg, Mei-Ling Hsu, Charles T. Rauch, Mary J. Gerhart, Ajamete Kaykas and David Cosman

Immunex Corporation, 51 University Street, Seattle, WA 98101, USA

A 45 kDa glycoprotein was purified from the culture media of human cytomegalovirus (HCMV)-infected fibroblasts. N-terminal sequencing revealed that the protein, R27080, is the translation product of the R27080 open reading frame of HCMV. R27080 is highly glycosylated and contains no cysteine or methionine residues. Proteolytic cleavage of R27080 by a furin-like enzyme was analysed in transfected COS-7 cells. R27080 is the first identified viral protein secreted from HCMV-infected cells.

The extremely large genomes of herpesviruses contain many genes that are non-essential for in vitro replication. These genes presumably encode proteins that function as modulators of the immune response, facilitate the spread of the virus in the infected host, mediate virus replication in specific cell types, or function in latency. The availability of the complete nucleic acid sequences of an increasing number of viral genomes allows the identification of open reading frames (ORFs) that are candidates for such functions. Many interesting examples of such genes have already been found, mostly on the basis of their homologies to cellular proteins. Viral forms of the genes presumably encode proteins that function as modulators of the immune response, facilitate the spread of the virus in the infected host, mediate virus replication in specific cell types, or function in latency. The availability of the complete nucleic acid sequences of an increasing number of viral genomes allows the identification of open reading frames (ORFs) that are candidates for such functions. Many interesting examples of such genes have already been found, mostly on the basis of their homologies to cellular proteins. Viral forms of the such genes have already been found, mostly on the basis of their homologies to cellular proteins. Viral forms of the functions of the cytokines IL-10, IL-17 and IL-6 were found in Epstein–Barr virus (Ryon et al., 1993; Swaminathan et al., 1993), herpesvirus saimiri (Yao et al., 1995) and human herpesvirus-8 (Neipel et al., 1993; Swaminathan et al., 1993), respectively. Genes encoding several G-protein-coupled transmembrane receptors are present in the coding region of HCMV (Chee et al., 1990; Gao & Murphy, 1994) and other herpesviruses (Ahuja & Murphy, 1993), and the HCMV UL18 ORF encodes a homologue of human MHC class I (Beck & Barrell, 1988). As an approach to look for novel, non-essential viral genes we analysed proteins secreted from HCMV-infected human foreskin fibroblasts (HFF). We identified a 45 kDa glycoprotein, which corresponds to a hypothetical protein of 103 amino acids encoded by the R27080 ORF, the product of a previously identified spliced transcript (Rawlinson & Barrell, 1993). This protein, R27080, shows no homology to any viral or cellular protein identified to date. R27080 appears to be the most abundant protein secreted from HCMV-infected fibroblast cells.

HFF were infected with the AD169 strain of HCMV at a multiplicity of 5 p.f.u. per cell. Proteins secreted by 5 × 10^7 cells in a period of 72 h post-infection (p.i.) to 96 h p.i. were purified from 250 ml culture media. The supernatant from [3H]glucosamine-labelled infected HFF was added as a tracer (90 c.p.m./ml). Glycoproteins were bound to 10 ml lentil lectin resin, the column was washed with 150 mM NaCl in 10 mM Tris (pH 7.5), and 0.2 M α-methyl glucopyranoside in washing buffer was used for elution. After trifluoroacetic acid was added to a final concentration of 0.1% the eluate was loaded onto a Vydac C4 reversed-phase column. Proteins detected in radioactive fractions of an acetoneitrile gradient (2% per min) were transferred onto PVDF membrane and subjected to N-terminal protein sequencing. The coding region of the R27080 ORF was amplified by PCR from genomic DNA of the AD169 strain using R27080-specific primers. Nucleotide sequences of the constant region of a human IgG1 heavy chain used in R27080 constructs were previously described (Fanslow et al., 1992; Spriggs et al., 1996). All R27080 constructs were inserted into the mammalian expression vector pDC409 (Giri et al., 1994). COS-7 cells were transiently transfected using DEAE-dextran (McMahan et al., 1991). Forty-eight hours after transfection cells were metabolically labelled with 50 μCi/ml [35S]cysteine/[35S]methionine (1:1 mixture), or a mixture of tritiated amino acids (Phe, Lys, Leu, Pro, Tyr), or [3H]glucosamine. For labelling of HFF, 200 μCi/ml [35S]cysteine/[35S]methionine or [3H]glucosamine was used. Radiochemicals were obtained from Amersham. Fc constructs of R27080 were precipitated with protein A–Sepharose (Pharmacia); an R27080 protein that contains the ‘Flag’ epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was precipitated in the absence of ammonium sulphate.
Fig. 1. Two-dimensional gel analysis of proteins from culture media of HCMV-infected HFF. Eighty hours p.i. cells were metabolically labelled with $[^{35}S]$cysteine/$[^{35}S]$methionine (A) or $[^{3}H]$glucosamine (B) for 16 h. Uninfected cells were used as controls.

Same way after preincubation with a Flag-specific antibody (Immunex Corporation) at a 1/100 dilution. R27080 Flag bound to protein A-Sepharose beads was treated with 20 U/ml protein N-glycosidase F (PNGaseF; Boehringer Mannheim) or 180 mU/ml neuraminidase (Boehringer Mannheim) for 20 h at 37°C under conditions recommended by the supplier. Radiolabelled proteins in cell lysates and culture media were analysed by one-dimensional or two-dimensional SDS–PAGE using precasted polyacrylamide gels (Novex). Gels were treated with the fluorographic intensifier solution Amplify (Amersham) and exposed to X-ray film.

In order to analyse proteins secreted from HCMV-infected cells, HFF infected with the AD169 strain of HCMV were metabolically labelled with $[^{35}S]$cysteine/$[^{35}S]$methionine or $[^{3}H]$glucosamine. Culture media of uninfected and infected cells were analysed by two-dimensional gel electrophoresis (Fig. 1). The analysis of supernatants of $[^{35}S]$cysteine/$[^{35}S]$methionine-labelled cells (Fig. 1A) showed a very similar pattern of protein species between uninfected and infected cells and a somewhat reduced incorporation of label into infected cell proteins. No newly arising $^{35}S$-labelled protein species were obvious in culture media of infected cells, even upon examination of longer autoradiographic exposures. In contrast to this result, the supernatants of infected HFF labelled with $[^{3}H]$glucosamine contained two glycoproteins which were not secreted from uninfected cells (Fig. 1B). We found a protein of high apparent molecular mass running slightly below the 200 kDa marker and a 45 kDa protein which was detected over a wide pH range, most likely due to heterogeneity in glycosylation (see below). The secretion of these proteins could result from viral gene expression or induction of cellular gene expression as a consequence of HCMV infection. To clarify the identity of these molecules, glycoproteins were purified from culture media of HCMV-infected HFF using affinity chromatography on lentil lectin followed by RP-HPLC. Supernatants of $[^{3}H]$glucosamine-labelled cells were added as a tracer. Proteins with apparent molecular masses of approximately 200 kDa, 45 kDa and 25 kDa detected in radioactive fractions were subjected to N-terminal protein sequencing. No amino acid sequence was obtained for the 200 kDa protein. The sequencing results of the 45 kDa protein and the 25 kDa protein were identical; both protein species were identified as R27080, a hypothetical HCMV protein coded by a previously identified spliced transcript (Rawlinson & Barrell, 1993). The amino acid sequence of R27080 predicted from translation of the R27080 ORF is shown in Fig. 2(A). A signal peptide of 20 amino acids followed by an additional eight amino acids is encoded by the first exon; the second exon codes the remaining 75 residues. Besides the initiating methionine, the sequence contains no additional cysteine or methionine residues, explaining the lack of detection of this protein by labelling with $[^{35}S]$cysteine/$[^{35}S]$methionine (Fig. 1A). Interestingly, two different N-terminal amino acid sequences were obtained in
ORFs within the genome of HCMV, thereby facilitating expression of additional viral proteins. However, in Northern blot analysis using the coding region of R27080 as a probe (Fig. 2B), we were unable to detect any mRNA species in HCMV-infected cells other than that shown to code for R27080 itself. As described by Rawlinson & Barrell (1993), the R27080 mRNA was most abundantly expressed at the late stage of HCMV infection (Fig. 2B).

In order to examine the post-translational modification of R27080, several constructs were made in which the coding region of R27080 was fused to different tags. A Flag epitope was added to the C terminus of R27080 (R27080–Flag). The constant region of a human IgG1 heavy chain (IgG1 Fc) was added to the C terminus of R27080 (R27080–Fc). A human IgG1 Fc was also added to the N terminus of R27080, either replacing the signal sequence (amino acids 1–20) but retaining the rest of the first exon including the potential furin-like cleavage site (Fc–fur–R27080), or replacing all of the first exon (amino acids 1–28) and fusing the IgG1 Fc directly to the second exon (Fc–R27080). These proteins were transiently expressed in COS-7 cells, metabolically labelled, and analysed. Transfection of untagged R27080 gave rise to aglycoprotein with an apparent molecular mass of about 25 kDa in culture media of cells labelled with [35S]cysteine/ [35S]methionine (lanes 1–3). Transfection of R27080–Flag and labelling with a mixture of tritiated amino acids also resulted in the detection of a secreted protein of similar size (Fig. 3A, lanes 1–3). Treatment with PNGaseF or neuraminidase plus O-glycosidase or a combination of the enzymes was used to assess the degree of glycosylation of R27080. As shown in Fig. 3A, PNGaseF treatment (lane 4) resulted in the detection of two smaller R27080 proteins with apparent molecular masses of about 20 kDa and 15 kDa. This observation fits well with the removal of one or two N-linked carbohydrates and is in agreement with the usage of the two potential N-glycosylation sites present in R27080 (Fig. 2A). O-Glycosidase treatment alone (lane 5) or in combination with PNGaseF (lane 6) showed no influence on the apparent molecular mass of R27080. In a similar experiment in which [3H]glucosamine-labelled R27080 was treated with PNGaseF and O-glycosidase we were still able to detect labelled R27080 protein (data not shown). In addition to two N-linked side chains, R27080 also appears to carry O-linked carbohydrates resistant to O-glycosidase treatment. The difference between the calculated mass of the R27080 protein of about 9.5 kDa and the apparent molecular mass detected in Fig. 3A is most likely due to the presence of glycosidase-resistant carbohydrate side chains.

To examine the proteolytic processing of R27080, R27080–Fc was expressed in COS-7 cells. The molecule was purified from culture media using protein A–Sepharose and subjected to N-terminal protein sequencing. The sequence that was predominantly detected was Ser-Val-Thr-Val-Glu-Gln-Pro-Ser-Thr-Ser-Ala-Asp-Gly-Ser-Asn, which is made up of the first 15 amino acids encoded by the second exon (Fig. 2A),
consistent with cleavage at the furin-like site, Lys-Ser-Lys-Arg. To visualize this cleavage more directly, COS-7 cells were transfected with the Fc–R27080 and Fc–fur–R27080 constructs and metabolically labelled with $[^{35}S]$cysteine/$[^{35}S]$methionine. Fusion proteins were analysed in cell lysates and culture media (Fig. 3B). These two IgG1Fc constructs of R27080 (lanes 3, 4, 7, 8) were only weakly expressed and secreted compared with the R27080–Fc version (lanes 2, 6). With all constructs, fusion proteins of similar apparent molecular mass were detected in cell lysates (lanes 2–4). In culture media both Fc–R27080 and Fc–fur–R27080 proteins appeared as 68 kDa proteins (lanes 7, 8) were only weakly expressed and secreted compared with (Fig. 3B). These two IgG1 Fc constructs of R27080 (lanes 3, 4, 7, 8) were only weakly expressed and secreted compared with the R27080–Fc version (lanes 2, 6). With all constructs, fusion proteins of similar apparent molecular mass were detected in cell lysates (lanes 2–4). In culture media both Fc–R27080 and Fc–fur–R27080 proteins appeared as 68 kDa proteins (lanes 7, 8), but the major band detected with the ‘cleavable’ Fc–fur–R27080 construct corresponded to a protein of about 35 kDa (lane 8), the size of the IgG1 Fc portion, set free upon limited proteolysis of the fusion protein. The use of $[^{35}S]$ for labelling fusion proteins did not allow the detection of the second cleavage product, corresponding to the R27080 moiety. Our data suggest that cleavage of R27080 by a furin-like protease occurs in the culture media and is not detected inside the cell. R27080 undergoes the same cleavage, regardless of whether it is secreted from infected HFF or transfected COS-7 cells.

Although several cytokine-like herpesvirus proteins have been identified as secreted products of virus-infected cells, it is perhaps surprising that no HCMV-encoded secreted proteins had been previously identified. The R27080 ORF was not noted in the initial sequencing of HCMV (Chee et al., 1990) due to its small size. Subsequently, a spliced transcript was identified that hypothetically encoded a small, secreted protein (Rawlinson & Barrell, 1993). We can speculate that the presence of R27080 in supernatants from HCMV-infected HFF was not previously detected because of the absence of methionine and cysteine, the amino acids most commonly used for metabolic labelling of proteins. However, $[^{3}H]$glucosamine labelling revealed the abundant secretion of R27080. This protein is a viable candidate to function as a ‘virokine’ by modulation of the immune response of the host. Future studies will be needed to identify its biological activities and host proteins with which it interacts.

The authors thank Dr Stefan Rose-John for discussion of the manuscript, Mari Hall and S. Sayah for expert help with the artwork and Anne Bannister for editorial assistance.

References


Received 13 July 1998; Accepted 15 October 1998