Cloning and epitope mapping of a functional partial fusion receptor for human cytomegalovirus gH

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A cDNA clone encoding a partial putative human cytomegalovirus (HCMV) gH fusion receptor (CMVFR) was previously identified. In this report, the cDNA sequence of CMVFR was determined and the role of this CMVFR in HCMV/cell fusion was confirmed by rendering fusion-incompetent MOLT-4 cells susceptible to fusion following transfection with receptor cDNA. Blocking experiments using recombinant gH or either of two MAb (against recombinant gH or purified viral gH:gL) provided additional evidence for the role of gH binding to this protein in virus fusion. An HCMV-binding domain of 12 aa in the middle hydrophilic region of CMVFR was identified by fusion blocking studies using synthetic receptor peptides. The 1368 bp cDNA of CMVFR contained a predicted ORF of 345 aa with two potential membrane-spanning domains and several possible nuclear localization signals. A search of sequence databases indicated that CMVFR is a novel protein. Further characterization of this cell membrane protein that confers susceptibility to fusion with the viral envelope should provide important information about the mechanism by which HCMV infects cells.

Introduction

More than 60% of the general adult population is infected with the betaherpesvirus human cytomegalovirus (HCMV) (Ho, 1991). HCMV rarely causes disease in immunocompetent individuals; however, in immunocompromised individuals (including allograft recipients and patients with AIDS), HCMV infection can cause sight-threatening and/or life-threatening illness (Ackermann et al., 1988; Betts et al., 1975; Ho, 1977; Ho et al., 1975; Pass et al., 1978; Rubin et al., 1977; Whelchel et al., 1979); HCMV may also be a cause of atherosclerosis (Melnick et al., 1995) or coronary restenosis following angioplasty (Speir et al., 1992; Michelson et al., 1977; Reynolds, 1978; Smith & de Harven, 1974). Three HCMV envelope glycoprotein complexes have been identified that may be involved in HCMV infection: gcI (55 and 93–116 kDa) consisting primarily of gB, the major envelope glycoprotein and essential for virus infectivity; gcII (47–52 and > 200 kDa), which is unique to CMV; and gcIII (86:32 and 145 kDa) consisting primarily of gH:gL, which is essential for virus infectivity (Britt & Auger, 1986; Forrester et al., 1992; Gretch et al., 1988; Kari & Gehrz, 1988; Lehner et al., 1989; Nowak et al., 1984; Pereira et al., 1982; Rasmussen et al., 1985). Cell proteins that are possibly involved in HCMV attachment and entry include heparan sulfate proteoglycan (Compton et al., 1993) and two cell membrane proteins. One of these proteins, a 30–36 kDa protein (Adlish et al., 1990; Taylor & Cooper, 1990), which may be annexin II (Wright et al., 1994, 1995), has been shown to bind to gB (Pietropaolo & Compton, 1997). The other is a 92–5 kDa protein that binds to gH:gL (Keay & Baldwin, 1991).

Our laboratory previously reported that antibodies that mimic gH:gL and bind to the 92-5 kDa cell membrane protein

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inhibit HCMV/cell fusion specifically (Keay & Baldwin, 1991). In addition, others have found that gH:gL is involved in cellular egress of infectious virions (Desai et al., 1988), as well as cell-to-cell spread of virus (Rasmussen et al., 1991; Milne et al., 1998), a function shared by other herpesvirus gH:gL homologues (Forrester et al., 1992).

Preliminary characterization of the 92-5 kDa cell membrane protein indicates that it is a constitutively phosphorylated glycoprotein with primarily N-linked sugar residues (mannose and complex oligosaccharides) (Keay & Baldwin, 1992). Experiments using specific kinases or phosphatase inhibitors also indicated that phosphorylation of this receptor may be important in mediating HCMV infection (Keay & Baldwin, 1996). Furthermore, this 92-5 kDa receptor protein appears to mediate transmembrane signalling events, resulting in increased inositol triphosphate and intracellular calcium ion concentrations, as well as Sp1 and NF-κB activation following exposure to HCMV or cross-linked MAbs that bind to the receptor (Keay et al., 1995; Yurochko et al., 1997).

We previously isolated cDNA clones encoding part of the 92-5 kDa receptor and demonstrated that purified recombinant proteins expressed by HB101 Escherichia coli could block HCMV/cell fusion and plaque formation (Baldwin et al., 1996). We therefore sought to determine whether this cloned receptor protein could also mediate HCMV/cell fusion by transfecting fusion-incompotent cells (MOLT-4, unpublished data) with the receptor cDNA and by correlating expression of cell membrane receptor protein with the ability to fuse with octadecyl rhodamine B chloride (R123)-labelled HCMV. The sequence of the functional receptor cDNA was determined and synthetic peptides were generated to map the region involved in HCMV/cell fusion.

### Methods

#### Antibodies. MAbs 4-3-5 and 6-5-1 (MAb) that mimic gH:gL of HCMV were generated and purified as previously described (Keay et al., 1988). BALB/c mouse immunoglobulin M (IgM)-kappa chain control antibody and IgG<sub>κ</sub> control antibody (both from Southern Bio-technology) were purified by the same method. MAbs 1G6 (kindly provided by Lucy Rasmussen, Division of Infectious Diseases, Stanford Medical School, Stanford, CA, USA) and 11-1-1 (kindly provided by William Britt, Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL, USA) that are directed against HCMV gH:gL or recombinant gH, were generated as previously described (Rasmussen et al., 1984; Simpson et al., 1993) and purified using protein A Sepharose (Zymed) according to the manufacturer’s instructions.

#### Recombinant gH. Recombinant HCMV gH (rgH) was produced using a vaccinia virus vector (VV-gH; kindly provided by William Britt) and transfection of African green monkey kidney (AGMK) cells. Briefly, AGMK cells were inoculated with VV-gH at an m.o.i. of 30 in Eagle’s minimum essential medium (MEM) containing 2.5% foetal bovine serum (FBS), 1% antibiotic/antimycotic and 1% t-glutamine; the inoculated medium was incubated at 37 °C, 5% CO<sub>2</sub> for 24–48 h and the rgH was then harvested and purified as previously described for HCMV gB (Simpson et al., 1993; Britt et al., 1995). Mock gH was prepared by the above procedure from cells that had not been inoculated with VV-gH.

#### DNA sequencing and sequence analysis. Plasmid DNA was isolated by the alkaline lysis method (Birnboim & Doly, 1979). Nucleotide sequences of the plasmid inserts were determined using the dyeoxo chain termination method (Sequenase Version 2.0; Amersham) or cycle sequencing (ThermoSequenase; Amersham). Oligonucleotides were synthesized at the Biopolymer Core Facility at the University of Maryland. Nucleotide and amino acid sequence analyses were performed using the University of Wisconsin Genetics Computer Group version 8.0 program.

#### Bioinformatics. Human embryonic lung (HEL) fibroblasts (ATCC CCL-137) and AGMK cells (BioWhittaker (70-104B)) were grown in MEM containing 10% FBS, 1% antibiotic/antimycotic and 1% t-glutamine (Keay et al., 1988). MOLT-4 cells (ATCC CRL-1582) were grown in RPMI-1640 medium containing 10% FBS, 1% antibiotic/antimycotic and 1% t-glutamine. HCMV strain AD169 was passaged and harvested as previously described (Keay et al., 1995). Virus titre was determined by plaque formation in HEL cells (Keay et al., 1988).

#### Isolation of HEL cDNA clones. Clones were isolated from two HEL cDNA jgt11 expression libraries (Clontech) using 4-3-5 and 6-5-1 as previously described (Baldwin et al., 1996).

#### Isolation of insert and subcloning into vectors. The purified cDNA inserts (purification described by Baldwin et al., 1996) were cleaved from the phage by digestion with EcoRI (Gibco BRL), run on a 1× NuSieve agarose gel (FMC Bioproducts) and purified by Wizard PCR prep (Promega). These inserts were then subcloned into pSecTagA, B or C (encodes an Ig kappa chain leader sequence for transport to the cell membrane) (Invitrogen) and/or pGEX-4T-1 (Pharmacia) which was previously digested with EcoRI and treated with calf intestinal alkaline phosphatase (Pharmacia). Competent One Shot TOP10F<sup>+</sup> E. coli (Invitrogen) was transformed with 611-pSecTagA, B or C, and competent BL21 E. coli was transformed with 611-pGEX-4T-1, 131-pGEX-4T-1 or 31-pGEX-4T-1 as previously described (Baldwin et al., 1996). Subclones containing inserts were identified by digestion of the plasmid DNA with EcoRI and analysis on a 1% agarose gel. The protein encoded by 611-pSecTagA was designated CMVFR.
**Transfection of MOLT-4 cells.** Vector pSecTagA and pSecTagC, B- or C-containing 611 cDNA in E. coli were purified using a Qiagen plasmid maxi kit. MOLT-4 cells were then transfected by electroporation. Briefly, 6 × 10⁶ MOLT-4 cells in complete medium were mixed with 20 µg cDNA, incubated for 10 min at 37 °C and then put on ice for 5 min. The culture was then electroporated in 0.4 cm Gene Pulser cuvettes (Bio-Rad) at 260 V and 960 μF and put on ice for 2 min. Cells were transferred into T25 flasks with 10 ml complete medium and incubated for 48–72 h at 37 °C. Further incubation for 2–4 weeks at 37 °C with complete medium containing Zeocin (50 µg/ml; Invitrogen) was used to select transfected cells.

**Immunofluorescence assay.** MOLT-4 cells transfected with 611-pSecTagA or controls (pSecTagA alone, 611-pSecTagB or 611-pSecTagC, or non-transfected MOLT-4 cells) were plated onto 8-well LabTek slides (Nunc) and incubated for 2 days at 37 °C. The cells were then examined for fluorescence using a Nikon diaphot inverted phase microscope.

**Fusion assay.** HCMV (m.o.i. of 20) was labelled with a fluorescent amphiphile (R14) as previously described (Keay & Baldwin, 1991), then incubated with transfected MOLT-4 cells [611-pSecTagA or controls (pSecTagA alone, 611-pSecTagB or C, or non-transfected MOLT-4 cells)] for 30 min at 37 °C. Cells were rinsed with PBS and quantification of the fluorescent signal was obtained using a Zeiss axioplan microscope connected to a Perceps image analyser.

**Western blot and dot blot analyses.** Segments A–D and control proteins were run on 10% Tricine–SDS–polyacrylamide gels (Schagger & von Jagow, 1987) and transferred in Towbin buffer (25 mM Tris, 192 mM glycine, pH 8.3) containing 10% methanol to either nitrocellulose or PVDF membranes (both from Bio-Rad). Nitrocellulose blots were blocked in 2% polyvinylpyrrolidone-40 in PBS for 1 h at room temperature, incubated with 50 µg 4-3-5 or control BALB/c IgM in PBS for 2 h at 37 °C, followed by 1:2000 alkaline phosphatase-labelled goat anti-mouse Ig in PBS for 1 h at room temperature, and developed with BCIP/NBT (Kirkegaard and Perry). PVDF blots were stained (50% methanol, 0.1% Coomassie R250) and destained (30% ethanol, 5% glacial acetic acid) and the degree of staining was used to determine protein concentration.

For dot blots, varying amounts (1, 2 or 4 µg) of peptides 1, 2, 1–11A or 1–10B were blotted onto nitrocellulose membranes. Membranes were then blocked in 15% Blotto (4-3-5 and IgM blocker; Keay et al., 1988) or 20% Blotto/1% Tween-20 (6-5-1 blocker) for 1 h at room temperature and incubated with 25 µg 4-3-5, 6-5-1 or BALB/c IgM control in PBS for 2 h at 37 °C followed by 1:250 horseradish peroxidase-labelled goat anti-mouse IgM (Zymed) in PBS for 1 h at room temperature prior to development with a-chloronaphthol.

**Statistical analysis.** Significant differences between independent means were determined at the 95% confidence level (P < 0.05) using a statistical program from Decision Analyst.

**Accession number.** The accession number for the nucleotide sequence of CMVFR is AF169251.

### Results

**Sequencing of clones that express partial HCMV fusion receptor**

Three clones were previously identified from two different HEL 1g11 expression libraries and shown to bind specifically to both MAb_2 (Baldwin et al., 1996). The nucleotide sequences of these cDNA were determined to be identical; the 1368 bp cDNA and derived amino acid sequences are shown in Fig. 1.

As determined by a ‘21-residue window’ with a mean hydrophobicity value greater than 0.42 (Eisenberg et al., 1984), two possible transmembrane regions exist within the predicted 345 aa ORF of the receptor peptide: aa 73–93 (mean hydrophobicity value = 0.53) and aa 161–181 (mean hydrophobicity value = 0.48) (Fig. 1). Three putative nuclear localization signals were also found throughout the cDNA. N-linked glycosylation sites were not found, however, indicating that the N-linked glycosylation sites implied by previous receptor characterization studies (Keay & Baldwin, 1992) are located in other regions of the receptor protein. Furthermore, neither an AUG translation initiation codon nor a polyadenylation signal was present, indicating that the 5’ and 3’ ends may not be present in the cDNA of the partial receptor. Throughout the sequence, numerous cysteines were evident, suggesting many possible disulfide bonds.

Database searches performed by use of the Pearson and Lipman Blastn, Blastp and Blastx programs (Altschul et al., 1990) indicated that the receptor gene may be interrupted by an intronic sequence and also localized on human chromosome.
Partial receptor protein

Transfection of MOLT-4 cells with cDNA encoding CMVFR had a noticeable similarity (51–53%) to human DNA-

CMVFR appears to be novel, 52 aa from the 5

tagged genomic clone (STS4-411, 374 bp). Even though

4 since 100% homology over 261 bp was found to a sequence-

Transfection of MOLT-4 cells with cDNA encoding partial receptor protein

Previously, we had showed that expressed proteins FR131 and FR611 could block both HCMV/cell fusion and HCMV

infectivity (Baldwin et al., 1996). To confirm that the protein encoded by the cDNA could mediate virus fusion, MOLT-4

cells were transfected with 611-pSecTagA (in-frame), 611-
pSecTagB or 611-pSecTagC (out-of-frame) or pSecTagA

vector control. An immunofluorescence assay using MAb 4-3-
to show detail of cell membrane binding.

To further verify that the 611-pSecTagA-expressed protein

is the gH fusion receptor, fusion blocking experiments were performed on MOLT-4 cells transfected with 611-pSecTagA

using either MAbs directed against rgH or purified viral

gH:gL, or rgH. As shown in Fig. 4, HCMV

inoculation, HCMV

fusion with HCMV

mapping of the functional region of the receptor for fusion with HCMV

The polypeptide containing the epitope to which MAb 4-3-5 binds was first determined by Western blot. MAb 4-3-5

bound specifically to polypeptide B–GST (aa 204–345, 42 kDa) and D–GST (aa 146–345, 48 kDa), while BALB/c IgM and

secondary control antibodies did not bind to these two polypeptides (blots not shown). In comparison, 4-3-5 did not

\(0.24 \pm 0.04\), whereas cells transfected with 611-pSecTagB, 611-pSecTagC or pSecTagA alone had no enhancement of

fluorescent signal as compared to non-transfected cells (\(0.23 \pm 0.04, \ P > 0.20; \ 0.23 \pm 0.03, \ P > 0.20; \ 0.23 \pm 0.03, \ P > 0.20\), respectively).

Mapping of the functional region of the receptor for fusion with HCMV

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Fig. 3. Fusion assay of MOLT-4 cells and R18-labelled HCMV. (a) Fluorescence of cells due to relief of self-quenching of R18-labelled HCMV on (A) non-transfected MOLT-4 cells, or cells transfected with (B) 611-pSecTagA, (C) 611-pSecTagB, (D) 611-pSecTagC or (E) pSecTagA (magnification ×90); and (b) quantification of fusion determined by image analysis; asterisk indicates a significant increase (P < 0.05) in fluorescent signal as compared to controls.

Fig. 4. Inhibition of HCMV fusion with CMVFR transfected MOLT-4 cells by MAbs specific for rgH or purified gH:gl. Fusion assays were performed in which MAbs [1G6 (■); 11-1-1 (□)] or control antibody IgG2a (▲) were preincubated with R18-labelled HCMV for 30 min at 37 °C prior to the inoculation of 611-pSecTagA-transfected MOLT-4 cells. Bars indicate standard error of the mean values from 2–3 high-power fields; asterisk indicates a significant decrease (P < 0.05) in fluorescent signal as compared to control.

bind to polypeptide A–GST (aa 1–203, 48 kDa) or C–GST (aa 1–145, 42 kDa), indicating that the epitope to which 4-3-5 binds is located within aa 204–234. Two peptides from the 5' end of this region were then synthesized [peptides 1 (aa 204–224) and 2 (aa 225–244) (Fig. 5a)] to further define the epitopes for the MAbs. Dot blot analysis indicated strong binding of the MAbb3 (both 4-3-5 and 6-5-1) specifically to peptide 1 in a dose-dependent manner (Fig. 5b). Two smaller peptides from this region (1–11A and 1–10B) were also synthesized; one of which (1–11A, aa 204–214) was specifically recognized by both 4-3-5 and 6-5-1 antibodies in a dose-dependent manner (data shown for 4-3-5, Fig. 5b). These findings indicate that the epitopes to which both 4-3-5 and 6-5-1 bind are located within aa 204–214.

To determine if the same peptides that contained the MAbb3-binding epitope were also able to block HCMV fusion, R18-labelled virus was preincubated with equimolar concentrations of peptide 1 or 2 for 1 h at 37 °C prior to inoculation of HEL cells. As shown in Fig. 6, peptide 1 blocked fusion in a dose-dependent manner [relative fluorescence: 100 µg (0.22 ± 0.09, P = 0.01); 60 µg (0.54 ± 0.15, P = 0.05); or 30 µg (0.88 ± 0.08, P > 0.20) as compared to 0 µg control (0.95 ± 0.11)] whereas the same concentrations of peptide 2 did not inhibit fusion [0.83 ± 0.07, P > 0.20; 0.90 ± 0.07, P > 0.20; 0.92 ± 0.12, P > 0.20; as compared to control (0.95 ± 0.11), respectively]. Like peptide 2, equimolar concentrations of the smallest segments of 1 [11A and 10B, data not shown] were not able to inhibit HCMV/HEL cell fusion; however, when R18-labelled HCMV was preincubated with larger segments of 1 [1–15A (aa 204–218), 1–18A (aa 204–221), 1[1–3]–18A (aa 207–221) or 1[–6]–18A (aa 210–221)] at 30, 60 or 100 µg (0 µg for controls), there was a significant decrease in fusion [values for each peptide, respectively: 0.69 ± 0.01 (P = 0.01), 0.89 ± 0.02, (P > 0.20), 0.90 ± 0.02 (P > 0.20) as compared to control of 0.90 ± 0.02; 0.31 ± 0.02 (P = 0.01), 0.64 ± 0.03 (P = 0.01), 0.75 ± 0.01 (P = 0.01), as compared to 0.90 ± 0.02; 0.24 ± 0.02 (P = 0.01), 0.45 ± 0.02 (P = 0.01), 0.57 ± 0.03 (P = 0.01), as compared to 0.92 ± 0.01; and 0.22 ± 0.01 (P = 0.01), 0.38 ± 0.01 (P = 0.01), 0.51 ± 0.02 (P = 0.01), as compared to 0.92 ± 0.01] (Fig. 6).

Preincubation of HCMV with 10, 30 or 100 µg peptide 1 also significantly decreased plaque formation in a dose-dependent manner [33 ± 4%, P = 0.10; 47 ± 4%, P = 0.02; 64 ± 4%, P = 0.01, respectively] whereas preincubation with equimolar concentrations of peptide 2 [12 ± 1%, P > 0.20; 4 ± 7%, P > 0.20; 9 ± 6%, P > 0.20, respectively] had no
Discussion

In this paper, we report the sequencing and functional mapping of a partial cell membrane fusion receptor for HCMV gH. Although the cDNA clones did not contain the entire receptor, they were capable of mediating virus fusion in fusion-incompetent MOLT-4 cells. In addition, fusion was specifically blocked when these same transfected MOLT-4 cells were preincubated with recombinant HCMV gH, or when the R18-labelled HCMV was preincubated with anti-rgH or anti-gH:gL antibodies, providing strong evidence that these clones encode a functional gH fusion receptor protein. The functional region for HCMV fusion was found to be within aa 210–221, a hydrophilic surface domain of CMVFR. In addition, HCMV infectivity was inhibited in a dose-dependent manner by a peptide from within this region. Mutagenic analysis may help to identify additional functional determinants in the partial receptor protein not identified by the peptide mapping studies.

The predicted amino acid sequence for the receptor protein indicates three putative nuclear localization signals. An emerging body of data indicates that ligand-induced cell membrane protein translocation to the nucleus can occur for certain polypeptide hormones and their receptors, including epidermal growth factor and growth hormone and their receptors (Holt et al., 1994; Lobie et al., 1994). It is conceivable that enveloped viruses similarly couple the nuclear translocation of viral proteins and their cell membrane receptors. For example, the binding of Epstein–Barr virus to its receptor, CR2, may result in the translocation of CR2 which has been shown to be present in the nuclei of Raji cells (Gauffre et al., 1992). In addition, matrix proteins of several enveloped viruses, appreciable effect on plaque formation (Fig. 7). Peptides 1–18A, 1–15A, 1–11A and 1–10B also caused a significant decrease in plaque formation at 100 μg (47–60 μmol) (51 ± 4%, 58 ± 1%, 48 ± 6% and 31 ± 2%, respectively; \( P = 0.01 \) for all four peptides) (Fig. 7), although the smaller peptides 1–11A or 1–10B had no appreciable effect.
including CMV, influenza virus, Newcastle disease virus and human immunodeficiency virus (HIV) (Coleman & Peeples, 1993; Gallay et al., 1995; Mocarski, 1993; Ye et al., 1995), have been shown to undergo nuclear translocation within a short period of time following exposure to virus, as have the gB envelope glycoproteins from both herpes simplex virus-1 and HCMV (Radsak et al., 1990; Raviprakash et al., 1990). Although it is not known whether the translocation of viral proteins (Liu & Stinski, 1992; Roizman & Sears, 1993), some of which appear to be important for transactivation of viral genes, is cell membrane receptor-mediated, it is likely that cellular proteins are involved in the translocation process. The similarity between a region of CMVFR and protamine is therefore of particular interest since protamine, which is a nuclear translocating DNA-binding protein that is concentrated in the acrosomal region of the head of mature sperm (Lyman et al., 1993), also appears to be involved in a membrane fusion event during fertilization (Liu et al., 1996).

Two possible transmembrane domains were also identified in the partial fusion receptor protein. It is notable that several cell membrane proteins that mediate fusion with enveloped viruses, including HIV-1 coreceptors (Deng et al., 1996; Feng et al., 1996), measles virus substance P receptor (Schroeder, 1986) and canine distemper virus CD9 receptor (Loffler et al., 1997), also have been shown to be multispans transmembrane proteins, suggesting that this configuration may be common to virus fusion receptor proteins.

The cellular function and normal ligand for the 92.5 kDa protein are currently unknown. Recently, several genomic clones for the gH receptor have been isolated in our laboratory. Analysis of these clones will undoubtedly provide critical insight into the structure and function of this cell membrane protein.

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References


membrane receptor for HCMV gp86.


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