Multimerization potential of the cytoplasmic domain of the human cytomegalovirus glycoprotein B

Hanno Scheffczik[a], Ina Kraus[a], Simone Kiermayer[a], Elke Bogner[b], Andreas Holzenburg[c], Wolfgang Garten[a], Markus Eickmann[a,*]

[a] *Institut für Virologie, Robert-Koch-Str. 17, D-35037 Marburg, Germany*
[b] *Institut für Klinische und Molekulare Virologie, D-91052 Erlangen, Germany*
[c] *Electron Microscopy Center and Department of Biology, Texas A&M University, College Station, TX 44783-2155, USA*

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Abstract In the present study the coding sequence of the cytoplasmic tail of the human cytomegalovirus glycoprotein B (gB) was expressed. The secondary structure of the purified recombinant protein was analyzed by circular dichroism, and the quaternary structure was investigated by gel permeation chromatography, and electron microscopy. Our data indicate that the cytoplasmic gB domain contains α-helix structures and assembles into tetramers, suggesting that the authentic gB may represent a homotetramer.

Key words: Human cytomegalovirus glycoprotein; Cytoplasmic domain; Homotetramer

1. Introduction

Glycoprotein B (gB) of the human cytomegalovirus (HCMV) is a major envelope component which elicits a dominant neutralizing response. HCMV gB is required for viral attachment and penetration of the virus into the host cell as well as for syncytium formation [3]. The HCMV gB protein is synthesized as a high molecular weight precursor [19]. Its ectodomain is translocated into the lumen of the rough endoplasmic reticulum and cotranslationally *N*-glycosylated. The gB precursor is a type I membrane protein consisting of 906 amino acids with a molecular mass of 102 kDa in the deglycosylated form, of which 750 amino acids comprise the ectodomain (82 kDa), 22 amino acids the transmembrane domain, and 134 amino acids the cytoplasmic domain (gBcyt, 14.8 kDa) (Fig. 1). gB has been demonstrated as a disulfide-linked homodimer within the envelope of mature virions [4,8,16]. The existence of higher molecular weight forms and their quarternary structure are still unknown.

The glycosylated gB precursor has an apparent molecular mass of 150 kDa and is cleaved during transport along the exocytotic pathway by the subtilisin-like endoprotease furin into disulfide-linked fragments, the luminal gp116 and the membrane-anchored gp55 [6,23]. Characteristically for herpes virus maturation, gB is transported also to the inner nuclear membrane, where it possibly participates in the exit of nucleocapsid through the nuclear envelope [7,13]. The nuclear transport of gB is directed by a recently discovered peptide signal within its cytoplasmic domain [11]. In addition to the nuclear localization signal, the cytoplasmic domain also contains several internalization signals for retrieval from the infected cell surface for virus envelopment [9,14,20,21]. Among the various internalization signals there are the tyrosine motifs Y_{845}QML and Y_{894}RHL, the dileucine motifs LL_{849} and LL_{884}, and an acid sequence containing $serine_{900}$, which may be phosphorylated by casein kinase II and thus modulate the retrieval of gB from the plasma membrane [2,9,21] (Fig. 1).

For a better understanding of the multiple functions, it is indispensable to improve the knowledge of the structural properties of the gB protein. To study its intrinsic conformational potential we expressed gBcyt as a recombinant fusion protein in *Escherichia coli*, and analyzed the secondary and quaternary structures of the purified gBcyt after proteolytic

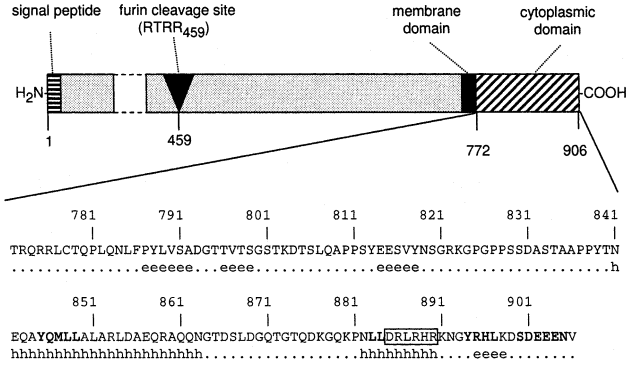

Fig. 1. Schematic representation of the HCMV gB precursor molecule comprising the signal peptide, the truncated gp116 connected by a basic furin recognition peptide with gp55 containing a part of the ectodomain, the membrane anchor domain and the cytoplasmic domain. The amino acid sequence of the cytoplasmic domain is given by amino acids in single letter code. The localization signal for inner nuclear membrane is indicated by a box, the exo- and endocytotic trafficking motifs are indicated by bold faced letters. Helical and β-sheet structures obtained by the secondary structure prediction program 'PhD, B. Rost, EMBL Heidelberg' are notified by h and e, respectively.

*Corresponding author. Fax: (49)-6421-286 8962.
E-mail address: eickmann@mailer.uni-marburg.de (M. Eickmann).

Abbreviations: gB, glycoprotein B; gBcyt, cytoplasmic domain of glycoprotein B; CD, circular dichroism; DTT, dithiothreitol; GST, glutathione *S*-transferase; HCMV, human cytomegalovirus; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HPLC, high performance liquid chromatography; IPTG, isopropyl-β-D-thiogalacto-pyranoside; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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cleavage from the fusion protein. The investigations included circular dichroism (CD) measurements, sizing of monomers and oligomers, and electron microscopic images of single particles of gBcyt. Evidence is provided that the cytoplasmic tail of gB of HCMV by itself is sufficient to assemble into tetramers.

2. Materials and methods

2.1. Construction and expression of the recombinant gBcyt fusion protein

HCMV-gBcyt DNA was amplified by PCR using the pRC/CMV-gB plasmid [15] and the primers gB-tail-*Bam*HI-f, 5′-TAAGGATCC-ACTCGACAGCGGCGTCTGTGCACG-3′; gB-tail-*Xho*I-r, 5′-AAA-CTCGAGTCAGACGTTCTCTTCTTCGTCGAG-3′. The *Bam*HI–*Xho*I DNA fragment was then inserted in the multiple cloning site of the pGEX-6P-1 vector (Amersham Pharmacia Biotech), constructed for the expression of glutathione *S*-transferase (GST) fusion proteins. Cloning of the gene was performed in *E. coli* DH5α and the recombinant plasmid was isolated in large scale (QIAfilter Plasmid Maxi kit, Qiagen). The cloned sequence of the recombinant plasmid was confirmed by DNA sequencing and then transformed into *E. coli* BL-21.

GST fusion protein expression: an overnight culture of *E. coli* BL-21 carrying the plasmid pGEX-6P/gBcyt was diluted 1:100 in LB medium containing 100 μg/ml ampicillin and incubated at 37°C. At an optical density at 600 nm (OD_{600}) of 0.75 expression was induced by 0.1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 2.5 h at 37°C. Sedimented cells were resuspended in 200 ml phosphate-buffered saline (PBS) containing 400 μl lysozyme (10 mg/ml) and were incubated on ice for 30 min. 1 ml DNase I (5 mg/ml), 1 ml RNase I (5 mg/ml) and four tablets Complete™ protease inhibitor cocktail (Boehringer Mannheim) were added and lysis was completed by three freezing and thawing cycles. Undissolved material was sedimented at 5000×*g* at 4°C.

2.2. Affinity purification and proteolytic cleavage

The clarified supernatant of lysed *E. coli* expressing gBcyt was incubated with glutathione Sepharose 4B (Amersham Pharmacia Biotech) for 1 h at room temperature and washed three times with cleavage buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.0), within GST fusion protein was cleaved with 80 U PreScission™ protease, a rhinovirus protease, per ml Sepharose beads overnight at 4°C. gBcyt was eluted with buffer containing 20 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)), 150 mM NaCl, 2 mM DTT, pH 7.6.

2.3. Chromatography

Affinity-purified gBcyt was passed through a Superose-12 (HR 10/30) size exclusion column using an ÄKTA™ Explorer high performance liquid chromatography (HPLC) System (Amersham Pharmacia Biotech) equilibrated with three bed volumes buffer mentioned in Section 2.2. Eluate fractions were monitored at 280 nm.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot

Samples were supplemented with sample buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue), heated for 5 min at 96°C, and proteins were separated on 15% polyacrylamide gels by SDS–PAGE. Proteins were either stained with Coomassie brilliant blue or electrophoretically transferred onto nitrocellulose membranes (Schleicher&Schuell). The membranes were blocked at 4°C overnight with a solution of 3% bovine serum albumin in PBS containing 0.1% Tween 20, incubated for 1 h with monoclonal antibody 58-15-18 [1] diluted 1 to 200 in PBS–Tween (0.1%) followed by an incubation of a 1 to 2000 diluted anti-mouse IgG (rabbit) complexed with horseradish peroxidase (rabbit-α-mouse-HRP; Dako). Protein bands were visualized using Super-Signal chemoluminescence substrate (Pierce).

2.5. CD measurements

CD spectra were recorded on a Jasco J-710 spectrometer calibrated with 20 mM phosphate buffer, pH 7.2 containing 2 mM DTT. The measurement was done at 20°C in 0.1 cm path length quartz cuvettes with a protein concentration of 22.9 μM. Spectra were recorded in the range of 190–250 nm wavelength in 0.2 nm steps and 2 s integration time.

2.6. Electron microscopy of single particles

Negatively stained specimens were prepared essentially according to Valentine et al. [22] using an aqueous solution of uranyl acetate (4% w/v, pH 4.3) and omitting any fixation steps. Specimens were mounted on 400 mesh copper grids and observed in a Zeiss EM T109 operated at an acceleration voltage of 80 kV. Electron micrographs were recorded at a calibrated magnification (26 190×). Selected micrographs were digitized using a Microtex Scan Maker E6. Scanning increments were adjusted so that the final scan step corresponded to 4.1 Å/pixel at the specimen level.

3. Results and discussion

The gene fragment encoding the entire gBcyt (amino acids 773–906) was inserted into the pGEX-6P plasmid downstream of the coding sequence of the GST and that of a rhinovirus protease cleavage site. A fusion protein was generated consisting at the N-terminus of GST connected by a proteolytically fissile peptide to gBcyt as the C-terminal fragment. The GST–gBcyt fusion protein was expressed in *E. coli*. After lysis of the bacterial cells, the soluble fusion protein was specifically bound to glutathione Sepharose beads, gBcyt was cleaved off by treatment with the rhinovirus protease and eluted from the beads. Final purification of gBcyt was carried out by gel permeation chromatography through a Superose-12 column which resulted in a >98% purity of the protein (Fig. 2). The purified protein was used for conformational and oligomerization studies.

Using the programs of PhD [17], the conformational prediction for gBcyt yields an α-helix content of 23% and a β-folded amino acid content of 14% (Fig. 1). In order to prove the α-helix structure of gBcyt, CD was measured (Fig. 3). The CD spectrum exhibited a distinct minimum of 205 nm wavelength, which is characteristic for a considerable portion of α-helices. Further CD measurements of gBcyt at a constant wavelength of 222 nm at different temperatures ranging from 4°C to 90°C showed no discernible protein denaturation below 60°C (data not shown) indicating that the gBcyt represents a rather stable secondary structure. Whether or not this

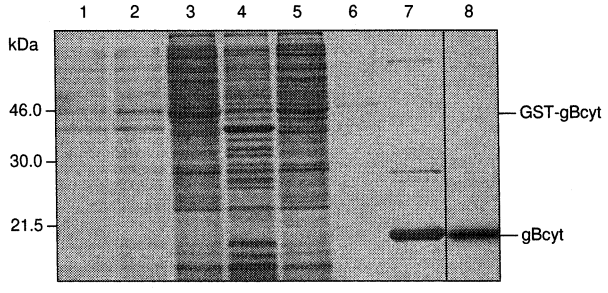

Fig. 2. SDS–PAGE analysis under reduced conditions. The image shows proteins of *E. coli* lysate suspension uninduced (lane 1), IPTG-induced (lane 2), lysate proteins of *E. coli* (lane 3), *E. coli* pellet after lysis (lane 4), supernatant after GST-affinity incubation (lane 5), wash buffer after GST-affinity incubation (lane 6), eluted gBcyt after cleavage of the fusion protein on the glutathione Sepharose beads (lane 7), and purified gBcyt after Superose-12 size exclusion chromatography (lane 8). Protein bands were stained with Coomassie brilliant blue. The GST–gBcyt fusion protein is marked by GST–gBcyt, the cytoplasmic domain of HCMV gB by gBcyt.

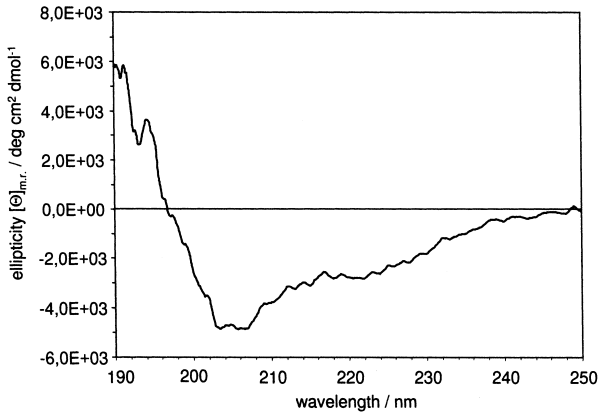

Fig. 3. CD spectra of the cytoplasmic domain of HCMV gB in 20 mM phosphate, 2 mM DTT, pH 7.2 at room temperature. The gBcyt was expressed in *E. coli* as a recombinant protein and purified. The spectrum of gBcyt was obtained as described in Section 2. It is typical for a mainly α-helical protein. Further details are given in the text.

stable secondary structure results in a solid quarternary structure was analyzed by gel filtration analyses. To study the highly ordered multimeric structure of gBcyt, the purified protein and molecular mass markers were analyzed by fast protein liquid chromatography using a Superose-12 column without detergent. The gBcyt eluted from the gel filtration column with an apparent molecular mass of 65 kDa (Fig. 4). When gBcyt peak fractions of the chromatography were analyzed by SDS–PAGE under reducing conditions, gBcyt migrated as a protein band with an apparent molecular mass of about 15 kDa, which perfectly agrees with the formula mass of a gBcyt monomer (Fig. 2). This observation indicated that gBcyt exists as a tetramer under non-denaturing conditions. The minor peaks at 16 ml and 19 ml represent the gBcyt monomer and undefined material, respectively.

Regarding that the cytoplasmic domain contains one cysteine residue at amino acid position 778 (Fig. 1), purified gBcyt was analyzed by SDS–PAGE under non-reducing conditions for inspection of potential dimer formation. The protein pattern on the acrylamide gel revealed only monomers, indicating that gBcyt does not form disulfide-linked dimers (data not shown).

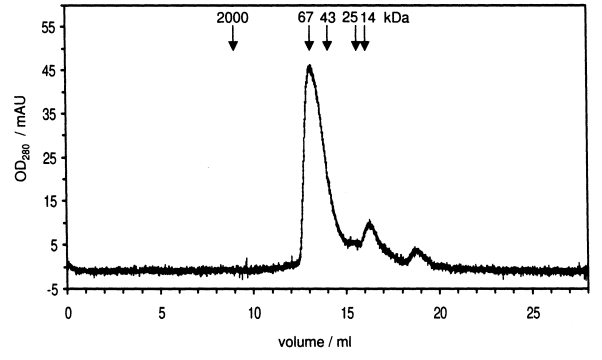

Fig. 4. Size exclusion chromatography of gBcyt of HCMV. GST-affinity-purified and cleaved off recombinant gBcyt was subjected to a column chromatography through a Superose-12 (HR 10/30) column and eluted with a buffer containing 20 mM HEPES, pH 7.6, 150 mM NaCl, and 2 mM DTT at 23°C using an ÄKTA™ Explorer HPLC System (Amersham Pharmacia Biotech). The proteins were recorded by OD_{280}. The column was calibrated with molecular standards of the following sizes: 2000 kDa (dextran blue), 67 kDa (albumin), 43 kDa (ovalbumin), 25 kDa (chymo-trypsinogen), and 14 kDa (ribonuclease A) as indicated by arrows.

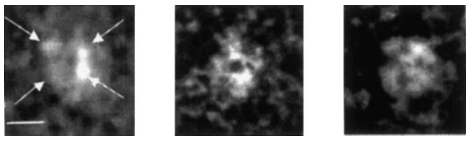

Fig. 5. Purified gBcyt was negatively stained with 4% uranyl acetate. Electron microscopic single particle analysis demonstrated that gBcyt forms tetramers. Note that the quaternary molecules are resulted in ring-like shapes. Arrows indicate the positions of protein monomers. The scale bar corresponds to 10 nm.

In order to directly demonstrate the quaternary structure of purified recombinant gBcyt electron microscopic studies were performed (Fig. 5). When analyzed by negative staining, square-shaped particles representing tetramers of gBcyt were detected. Fig. 5 shows three representative tetramers of gBcyt which exhibit an electronlucent center. The dimension of a tetramer is consistent with the molecular mass of four single gBcyt monomers based on the correlations published [25].

Extensive studies have been conducted to determine the high molecular weight forms of gB, but the reported results are conflicting. Evidence has been presented suggesting that gB high molecular weight forms are gB–annexin II oligomers and gB dimers [18]. However, studies involving oligomerization of viral gB or mammalian expression of mutated gB forms claimed that high molecular weight forms of gB are artifactual aggregates [5,24]. It was shown that the ectodomain of gB forms disulfide-bonded homodimers but higher molecular weight complexes could not be excluded [1,8]. In the present study we were able to show that the cytoplasmic domain of the HCMV gB alone exhibits a stable secondary and quaternary structure, suggesting that gBcyt by itself is sufficient to assemble into homotetramers. This was experimentally demonstrated by different, independent approaches: gel filtration and electron microscopy of *E. coli* recombinant gBcyt. To the best of our knowledge, the present study is the first to investigate the cytoplasmic domain of a viral glycoprotein by ultrastructural analysis of negatively stained single particles. The quaternary structure of gBcyt represents an approximate square and is characterized by four circular protein domains enclosing an electronlucent center similar to the ectodomain of herpes simplex virus-1 gD [12].

Data have accumulated in recent years demonstrating that glycoproteins of several enveloped viruses form homotetrameric spikes. Direct proof for the involvement of cytoplasmic domains with the potential for tetramerization of a viral glycoprotein was recently described for a fusion protein containing the cytoplasmic domain of the transmembrane subunit gp41 of the human immunodeficiency virus (HIV-1) [10]. In this case the lentiviral lytic peptide-1 and peptide-2, with large helical hydrophobic moments, play a critical role in cytoplasmic domain self assembly. For the gB cytoplasmic tail, large helical hydrophobic moments are also predicted, which are located within residues 844–860. It remains to be examined whether or not recombinant gB with deletions in this region can form stable tetramers and it has to be proven if the tetrameric structure of gBcyt indeed exists in the envelope of the infectious virion.

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