Short Communication

Excretion of herpes simplex virus type 2 glycoprotein D into the culture medium

Takayuki Murata, Fumi Goshima, Hiroki Takakuwa and Yukihiro Nishiyama

Laboratory of Virology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

Glycoprotein D (gD) of herpes simplex virus type 2 (HSV-2) was excreted from infected cells into the medium. Peptide mapping analysis and lectin binding assays suggested that the gD in the medium is secreted after full glycosylation and cleavage at its C terminus. Release of HSV-2 gD was inhibited by addition of either tunicamycin or brefeldin A, suggesting that the gD in the medium was secreted through the endoplasmic reticulum and Golgi apparatus.

Herpes simplex virus (HSV) is a large DNA virus that causes a variety of diseases ranging from mild skin disorders to fatal encephalitis. HSV encodes at least 10 glycoproteins, among which glycoprotein D (gD), essential for replication in cultured cells, is the most extensively studied. Deletion mutants of the virus for the gD gene cannot penetrate into cells (Ligas & Johnson, 1988) and neutralizing antibodies against the glycoprotein inhibit virus entry (Fullar & Spear, 1987; Highlander et al., 1987). Studies with antibodies raised against HSV gD indicate that the glycoprotein plays a role in the cell-to-cell fusion process (Noble et al., 1983). Expression of gD protein in cells results in resistance to superinfection of the virus (Campadelli-Fiume et al., 1988). Johnson et al. (1990) demonstrated that soluble forms of HSV gD inhibit virus entry into cells. These reports all indicate that HSV gD plays an important role in cell penetration. Indeed, recent studies have shown that HSV gD interacts physically with entry receptors such as herpesvirus entry mediator A (HveA), members of the nectin family or 3-O-sulphated heparan sulphate, and that these interactions enhance the entry step of the virus (reviewed in Campadelli-Fiume et al., 2000).

In addition to its function as an entry mediator, gD elicits strong humoral and cell-mediated immunological responses in vivo (Zarling et al., 1986; Rooney et al., 1989). Furthermore, Zhou et al. (2000) showed that gD is involved in prevention of apoptotic cell death.

Like other glycoproteins, gD is synthesized in the rough endoplasmic reticulum (ER), where it immediately acquires high-mannose oligosaccharides, N-linked to the asparagine residues of the consensus sequence. After trimming of the oligosaccharides in the Golgi apparatus, the glycoprotein is transported to the plasma membrane (Serafini-Cessi & Campadelli-Fiume, 1981; Johnson & Spear, 1982).

In spite of a large number of studies on HSV proteins, little is known about proteins secreted from infected cells (Randall et al., 1980). We have examined whether any proteins are selectively released into the medium of HSV-infected cells. HSV- or mock-infected HEp2 cells were washed at 3 h post-infection (p.i.) and incubated with methionine-free MEM containing 0.2 mCi/ml of [35S]methionine and cysteine (redivue Pro-mix [35S] in vitro cell-labelling mix; Amersham Pharmacia) from 3–12 h p.i. At 12 h p.i., the medium was harvested and the cell debris was discarded by centrifugation at 3000 r.p.m. for 5 min and 15000 r.p.m. for 5 min. Proteins in the medium were precipitated with 10% trichloroacetic acid (TCA) and subjected to SDS–PAGE as described previously (Murata et al., 2000), followed by autoradiography using a Fuji Bio-Imaging Analyser BAS2000 System (Fuji). Whole-cell extracts were also electrophoresed in order to confirm successful production of virus proteins. As shown in Fig. 1(A), at least one strong signal at about 50 kDa was detected in the medium from HSV-2 (186)-infected cells. When cells were infected with HSV-1 (KOS), a band at about 70 kDa was predominant, but the 50 kDa protein was not found.

The 50 kDa protein appeared in 186-infected medium, becoming detectable from 6 to 9 h.p.i. and did not disappear until 15 h.p.i. (Fig. 1B). Although intracellular infectious virus accumulated up to approximately 6·7 × 106 p.f.u./ml, increases in extracellular infectious virus were not observed until at least 15 h.p.i. (Fig. 1C), suggesting that the proteins released into the medium were not virion-associated.

Judging from its molecular mass and the broad pattern of the band, we presumed the 50 kDa protein to be gD. We thus compared the molecular mass of the intracellular gD and the protein in the medium of the HSV-2-infected culture (Fig. 1D). The figure shows that the electrophoretic mobility of the 50 kDa band in the medium was almost the same as that of premature gD (pgD) from infected cells, although the amount...
Fig. 1. Secretion of HSV-2 gD. (A) HEp2 cells were mock-infected (lanes 1, 2) or infected with HSV-2 (186; lanes 3, 4) or HSV-1 (KOS; lanes 5, 6) at an m.o.i. of 3 for 3 h. Cells were then washed with PBS and medium containing [35S]methionine and cysteine was added. At 12 h.p.i., proteins in the medium (lanes 2, 4, 6) were collected by TCA precipitation. Proteins in the cells (1, 3, 5) were also collected and subjected to SDS–PAGE, followed by autoradiography. (B) Time-course of protein release. HEp2 cells were mock-infected (lanes 1, 7) or infected with HSV-2 (186; lanes 3–6, 8) at an m.o.i. of 3 for 3 h. Cells were washed and medium containing [35S]methionine and cysteine was added. At 3 (lane 2), 6 (lane 3), 9 (lanes 1, 4, 7, 8), 12 (lane) and 15 (lane 6) h.p.i., proteins in the medium (lanes 1–6) or in the cells (lanes 7, 8) were harvested and subjected to SDS–PAGE, followed by autoradiography. (C) One-step growth curve of HSV-2 (186) in HEp2 cells. Cells were infected with the virus at an m.o.i. of 3 for 3 h. Cells were washed extensively, added to normal medium and incubated. At indicated times p.i., virus in the medium (black boxes) or in the cells (black circles) was harvested and frozen at −80 °C, followed by titration. (D) Comparison of molecular masses. HEp2 cells were mock-infected (lane 1) or infected with HSV-2 (186; lanes 2, 3) at an m.o.i. of 3 for 3 h. Cells were washed and medium containing [35S]methionine and cysteine was added. At 10 h.p.i., proteins in the medium (lane 3) were harvested by TCA precipitation and the proteins in the cells (lanes 1, 2) were collected by immunoprecipitation with anti-gD antibody. Samples were separated by SDS–PAGE, followed by autoradiography. (E) Immunoprecipitation of the released protein with anti-gD antibody. HEp2 cells were mock-infected (lanes 1, 3) or infected with HSV-2 (186; lanes 2, 4) at an m.o.i. of 3 for 3 h. Cells were washed and medium containing [35S]methionine and cysteine was added. At 10 h.p.i., proteins in the medium were harvested by TCA precipitation (lanes 1, 2) or immunoprecipitation with anti-gD antibody (lanes 3, 4). Samples were separated by SDS–PAGE, followed by autoradiography. (F) Immunoprecipitation of the released protein with anti-gD antibody. HEp2 cells were mock-infected (lanes 1, 3) or infected with HSV-2 (186; lanes 2, 4) at an m.o.i. of 3 for 3 h. Cells were washed and medium containing [35S]methionine and cysteine was added. At 10 h.p.i., the protein in the medium (lanes 3, 4) or in the cells (lanes 1, 2) was collected by immunoprecipitation with anti-gD antibody. Proteins were separated by SDS–PAGE, followed by autoradiography.

of gD in the medium was much less than that of cellular gD. We also found that the 50 kDa protein in the medium could be immunoprecipitated with an anti-gD monoclonal antibody (Fig. 1E). These results indicated that the 50 kDa protein secreted into the HSV-2-infected culture medium was of gD origin. The electrophoretic mobility of the proteins suggested that the protein in the medium might be pgD (Fig. 1D, F). However, the protein formed a broader band than cellular pgD,
suggesting that the gD in the medium was a truncated form of fully glycosylated mature gD (Fig. 1F).

In order to clarify which kind of gD was excreted into the medium, peptide footprinting analysis of the radio-labelled proteins was performed. HEp2 cells were infected with HSV-2 (186) and labelled with $\text{^{35}S}$methionine and cysteine. At 10 h.p.i., proteins in the medium and in the cells were harvested separately and subjected to SDS–PAGE. Cellular and medium gDs were excised from the gel and again subjected to SDS–PAGE. While the second electrophoresis was running, the proteins were digested in-gel with S. aureus V8 protease. Although there were several peptide bands that were not detected in the counterpart (Fig. 2A, arrowheads), the profile of the 50 kDa protein was very similar to that of fully glycosylated mature gD, confirming the previous conclusion that the protein released into the medium is of gD origin.

To investigate the route of gD secretion into the medium, the effect of tunicamycin (TM) and brefeldin A (BFA) was examined. TM inhibits the transfer of N-acetylglucosamine 1-phosphate and thereby blocks the formation of protein N-glycosidic linkages in the ER (Mahoney & Duksin, 1979). BFA blocks protein translocation from the ER to the Golgi apparatus (Strous et al., 1993). Fig. 3(B) shows that addition of either TM or BFA blocked the secretion of gD. It is clear in Fig. 3(A) that the drugs were effective; TM inhibited the production of pgD, which carries high-mannose oligosaccharides, by blocking the proteins in the medium or in the cells were harvested, subjected to SDS–PAGE and electro-blotted onto a membrane. The membrane was treated with peroxidase-conjugated concanavalin A and visualized using the ECL Western blotting detection system (C). The same membrane was subjected to autoradiography (B).
linkage of the oligosaccharides, and BFA affected the maturation of N-linked oligosaccharides. These results suggested that gD is secreted through the ER and Golgi apparatus.

The present study demonstrates that HSV-2 gD is excreted from cells into the medium, and that gD is secreted through the ER and Golgi apparatus. The sugars in the secreted gD are fully trimmed, although the molecular mass of the glycoprotein secreted in cells is smaller than that of mature gD. Therefore, we suggest that the protein is excreted after full glycosylation and cleavage. Because gD has a hydrophobic transmembrane region at its C terminus, it may be cleaved at the C-terminal part and thereby secreted into the medium as a soluble form.

The glycoprotein was secreted from human HEp2 cells or monkey Cos-1 cells when infected with HSV-2, but not from Vero cells (not shown). This result strongly suggests that gD is cleaved by a cellular factor that is missing in Vero cells. However, we cannot exclude the possibility that gD is truncated by a viral factor, because many viruses, including HSV (Liu & Roizman, 1992), have proteases. Generally speaking, mammalian proteases comprise both exopeptidases (also referred to as exopeptidases), which act at the N- or C-terminal ends of polypeptides, and endopeptidases (endopeptidases), which are capable of cleaving peptide bonds in the central regions of polypeptides (Barrett, 1980). Most herpesvirus gB homologues are processed during maturation in the Golgi apparatus (Hampf et al., 1984; Montalvo & Grose, 1987; Sapa et al., 1988), although HSV gB is not (Claesson-Welsh & Spear, 1986). These gB homologues, except for HSV gB, carry the specific cleavage motif RXK/RR, which is the recognition motif for proteolytic processing by the cellular endoprotease furin, and they are processed by this enzyme (Vey et al., 1995). Other viruses, such as respiratory syncytial virus (Bolt et al., 2000) and influenza virus (Jankovics, 1996), also require furin for the processing of fusion or haemagglutinin proteins. The HSV gD, however, does not carry the specific motif or a similar sequence for the endoprotease, suggesting that gD is not processed by furin. A surface glycoprotein of Lassa virus is post-translationally cleaved in the ER by the cellular subtilase SKI-1/Lassa virus is post-translationally cleaved in the ER by the cellular subtilase SKI-1.

What is the physiological significance of excretion of gD into the medium? One possibility is that the protein functions as a decoy for immune responses. Extracellular gD was immunoprecipitated with monoclonal gD-specific antibody (Fig. 1). Inactivation of antibodies against gD by binding to extracellular gD would be beneficial for the virus in vivo. First, extracellular virions could evade neutralization by gD-specific antibodies. Secondly, cells infected with HSV, which express viral proteins, could escape binding of antibodies, thereby eluding complement-dependent cytolsis, and escape from killer cells that have Fc receptors, such as macrophages or natural killer cells.

HSVgs have evolved several ingenious mechanisms to evade immune systems. Viral glycoproteins gE and gI bind to IgG Fc and protect infected cells from antibody-dependent cell cytotoxicity (Johnson et al., 1988). gC plays a role in blocking complement-dependent cell lysis by binding to C3b and factor H (Huemer et al., 1993). ICP47 inhibits surface expression of class I MHC antigens together with antigenic peptides by binding to TAP (transporter associated with antigen processing; Hill et al., 1995). Excretion of gD could be another example of evasion of host immune systems.

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