Proteolytic Processing of Human Cytomegalovirus Glycoprotein B (gpUL55) Is Mediated by the Human Endoprotease Furin

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Inhibition of endoproteolytic cleavage of glycoprotein B (gB; gpUL55) of human cytomegalovirus was achieved by treatment of infected fibroblasts with decanoyl peptidyl chloromethyl ketone (decRVKR-CMK), which inhibits the action of cellular subtilisin-like endoproteases with the amino acid recognition motif R X K/R R. Uncleaved gB precusor molecules of 160 kDa that were accumulated were endoglycosidase H resistant, suggesting that correct cellular transport occurred in the presence of the drug. The inhibitor also prevented endoproteolytic gB processing in CV-1 cells infected with a recombinant vaccinia virus-gB construct (VVgB). Evidence for direct involvement of the ubiquitous subtilisin-like endoprotease furin in gB cleavage was obtained from the observation that coinfection of CV-1 cells with VVgB and a recombinant vaccinia-human furin construct reestablished endoproteolytic activity which was normally absent late after infection with VVgB alone.
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Glycoprotein B, gB (gpUL55), of human cytomegalovirus (HCMV) represents the abundant and immunodominant component of the viral envelope (1). Glycoprotein B homologs are highly conserved within the herpesvirus family (1), an observation that reflects their essential biological function, most likely in membrane fusion processes during viral entry and cell-to-cell spread (2). Interestingly, maturation of most of the herpesvirus gB proteins, e.g., that of HCMV, includes post-translational processing of the glycosylated precursor by proteolytic cleavage into two products that remain linked by disulfide-bonds (3), whereas in some species, e.g., in the case of herpes simplex viruses gB (4), there is no cleavage step involved in processing. The biological significance of herpesvirus gB cleavage does apparently not consist in endoproteolytic activation of a fusogenic domain of the molecule, as demonstrated for the membrane glycoproteins of, e.g., orthomyxoviruses (5; see below).

Endoproteolytic cleavage of a 160-kDa precursor of HCMV gB yields an amino-terminal product of about 100 kDa and a carboxy-terminal product of about 58 kDa (6; Fig. 1A, lanes pulse and chase, minus inhibitor). Regarding the enzyme involved in cleavage, the conclusion was drawn from the consistent observation of its correct endoproteolytic processing after expression of the solitary gB gene in a variety of cells, that gB cleavage is executed by an ubiquituous cellular endoprotease. The deduced amino acid sequence at the cleavage site of HCMV gB,

R T K/R R↓ (7), represents the recognition motif of the subtilisin-like serine endoprotease furin recently discovered in animal cells (8).

In order to characterize the enzyme mediating cleavage of HCMV gB, two sets of experiments were performed. In the first set, parallel cultures of human fibroblasts (1.5 × 10⁶ cells each) were infected with the AD169 strain of HCMV with a multiplicity of infection (m.o.i.) of approximately 3. At 72 hr postinfection (p.i.) the cultures were pulse labeled for 1 hr with [^{35}S]methionine (50 µCi/ml), thoroughly washed with PBS, and subsequently chased for 3 hr in the presence of excess unlabeled methionine and various concentrations of the inhibitor decanoyl-peptidyl (R V K R)-chloro-methyl ketone (decRVKR-CMK; 9), which contains the consensus sequence for recognition by furin. To test for cytotoxicity and reversibility of a possible inhibitory effect, an infected culture was labeled in the presence of and chased without inhibitor. After pulse or pulse and chase, cell extracts were prepared and immunoprecipitated with gB-specific monoclonal antibody (mAb 27–156; 7) prior to electrophorectic separation of the precipitates and fluorography as described previously (10). Under these conditions, inhibitor concentrations as little as 5 µM efficiently prevented appearance of the cleavage products (Fig. 1A, lanes chase, plus inhibitor), at the same time a slow migrating gB-specific product of about 160 kDa accumulated which most likely represented a normally short-lived processing intermediate carrying complex carbohydrate side chains (6). This type of modification is thought to occur in the trans-Golgi-network prior to endoproteo-
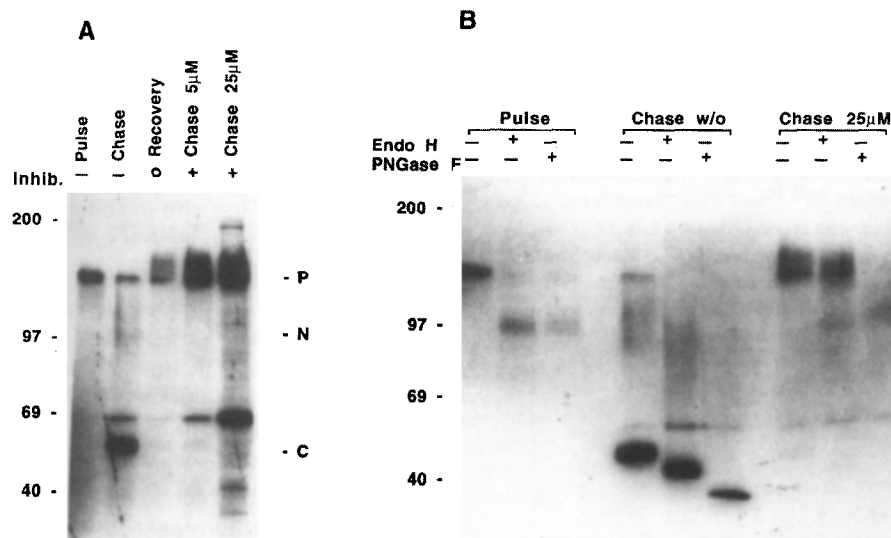
FIG. 1. Inhibition of endoproteolytic cleavage of HCMV gB in decRVKR-CMK-treated infected human fibroblasts and glycosidase sensitivity of gB from inhibitor-treated cells. (A) Parallel cultures (1.5 × 10⁶ cells each) of HCMV (strain AD169)-infected fibroblasts were pulse labeled at 72 hr p.i. with [³⁵S]methionine (50 μCi/ml) for 1 hr prior to a chase interval of 3 hr. The inhibitor was either absent (Inhib. −) during pulse and chase (lanes pulse and chase), present (25 μM) only during the pulse (Inhib. o, lane Recovery), or present (Inhib. +) at various concentrations only during the chase (lanes Chase 5 μM and o, lanes Chase 25 μM). After 168. Pulse and chase cell extracts were subjected to immunoprecipitation with mab untreated cells (Pulse and Chase w/o) and inhibitor-treated cells (Chase 25 μM) were digested with endo H or PNGase F (lanes endo H or PNGase F + or −) prior to analysis by SDS-PAGE and fluorography. The relative migration of the marker proteins myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (69 kDa), and aldolase (40 kDa) is indicated on the left of (A) and (B), that of gB precursor (P), aminoterminal (N), and carboxyterminal (C) cleavage products is indicated on the right of (A). Coprecipitation of a polypeptide of about 65 kDa was frequently observed independently of inhibitor use (see text).

lytic cleavage in the same cellular compartment (6). When decRVKR-CMK (25 μM) was present during the pulse interval, incorporation of methionine was not prevented showing that the inhibitor was not toxic. Also, the inhibitory action of the drug was not reversed during the drug-free chase period because there was no recognition of gB cleavage products, but again accumulation of the processing intermediate occurred under these conditions (Fig. 1A, lane recovery). In precipitates from untreated as well as inhibitor-treated chase samples (Fig. 1A, particularly pronounced in chase 25 μM), a polypeptide of about 65 kDa was frequently seen which is known to be coprecipitated from infected cells by the gB-specific monoclonal antibody used here (11).

To directly address the question of endo H-resistance of the 160-kDa gB processing intermediate, immunoprecipitates of chase samples of drug-treated cells were digested with endoH or endoglycosidase F (PNGase F) and analyzed (10; Fig. 1B). Pulse and chase samples from untreated cells served as controls. This comparative analysis revealed sensitivity for either glycosidase of gB precursor and partial endo H resistance of cleavage products from untreated cells (Fig. 1B, panels pulse and chase w/o) and clearly endo H resistance of the 160 kDa gB intermediate (Fig. 1B, panel chase 25 μM). This observation suggested that the uncleaved gB molecule was correctly transported in inhibitor-treated cells. It is noteworthy in this context that virus titers determined

from the chase media showed that about equal amounts of viral progeny were released from untreated and drug-treated cultures during the chase interval (about 2 × 10⁴ infectious units/ml), indicating again absence of interference with cellular transport and of cytotoxicity. Previously it was demonstrated for palmitoyl-peptidyl-CMK-treated (palFAKR-CMK) infected cells that uncleaved gB precursor may become associated with extracellular virions (11). This observation was extended for decRVKR-CMK-treated infected cells used here by immunoprecipitation with mAb 27−156 of uncleaved gB from extracellular virions (data not shown). Regarding our previous observation that palFAKR-CMK, which does not contain the complete recognition motif for cellular furin (11), also inhibited HCMV gB cleavage may have been due to the higher near-toxic concentrations needed as compared to those used here with the new inhibitor decRVKR which was formerly not available.

In the second set of experiments recombinant HCMV-gB in a vaccinia virus vector was used (12). Radiolabeling with [³⁵S]methionine (50 μCi/ml at 4.5−5.5 hr p.i. prior to 2 hr chase) early after infection of cultures of CV-1 cells (m.o.i. of approximately 10; 1 × 10⁶ cells each) with this construct and subsequent analysis of the immunoprecipitates revealed correct endoproteolytic processing of the gB molecule (Fig. 2A, lane VVgB w/o). Presence of decRVKR-CMK in the medium efficiently prevented endoproteolytic cleavage of the recombinant gB and again
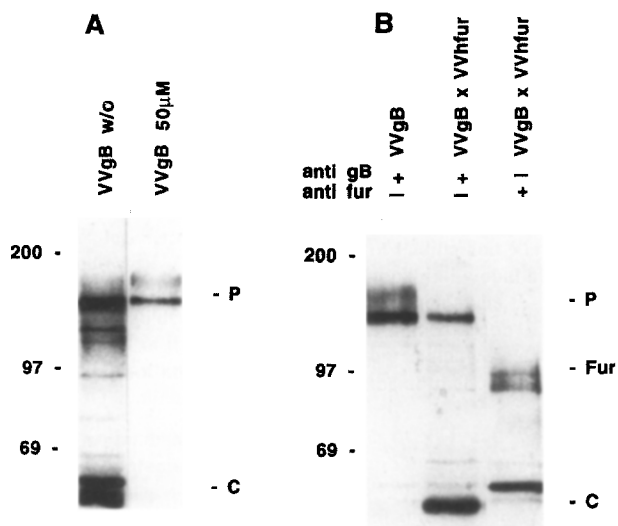
FIG. 2. (A) Inhibition of endoproteolytic cleavage of recombinant vaccinia virus gB by decRVKR-CMK. Parallel cultures of CV-1 cells (1 × 10^6 cells each) were infected with a recombinant vaccinia virus gB construct at a m.o.i. of 10, without (VVgB w/o) or in the presence of decRVKR-CMK (VVgB 50 μM) and pulse labeled with [^{35}S]methionine (50 μCi/ml) from 4.5–5.5 hr p.i. prior to immunoprecipitation of cell extracts with mab 27–156 and analysis of the precipitates by SDS–PAGE and fluorography. (B) Reestablishment of endoproteolytic activity late after VVgB infection by coexpression with VVhfur. Parallel cultures of CV-1 cells were infected either with VVgB alone (VVgB) as under (A) or coinfected at equal m.o.i. with VVhfur (VVgB × VVhfur) and pulse labeled with [^{35}S]methionine (50 μCi/ml) from 17–18 hr p.i. prior to immunoprecipitation with mab 27–156 (anti gB+) or with polyclonal antifurin (anti fur) and analysis of the precipitates by SDS–PAGE and fluorography. The relative migration of the identical marker proteins listed in the legend of Fig. 1 is indicated on the left, that of gB precursor (P), carboxyterminal cleavage product (C), and furin (Fur) is indicated on the right.

led to accumulation of the above-mentioned 160-kDa gB intermediate (Fig. 2A, lane VVgB 50 μM). In an additional experimental approach advantage was taken of the observation that late after infection with vaccinia virus (18 hr p.i., 1 hr pulse prior to 2 hr chase) endogenous cellular endoprotease activity is apparently depleted (13); by coinfection with recombinant vaccinia virus carrying the human furin gene (VVhfur), endoproteolytic activity may be reestablished (9, 13). Therefore, CV-1 cell cultures were coinfected with both vaccinia virus gB (VVgB) and VVhfur and radiolabeled as described above with [^{35}S]-methionine for 1 hr at 18 hr p.i. prior to immunuprecipitation of gB; cells infected with VVgB alone served as a control. Under these conditions precipitates obtained with mAb 27–156 from VVgB-infected cells contained uncleaved gB precursor as well as 160-kDa intermediate (Fig. 2B, lane VVgB, + anti gB), and those from coinfected cells contained in addition to uncleaved gB precursor cleavage products of gB (Fig. 2B, lane VVgB × VVhfur, + anti gB). Immunoprecipitation with antifurin verified for the coinfected cultures correct expression also of recombinant furin (Fig. 2B, lane VVgB × VVhfur, + anti-

fur), which obviously reestablished the endoproteolytic activity in this system.

The experiments presented indicate that the cellular endoprotease furin is a prime candidate for proteolytic cleavage during maturational processing of HCMV gB in infected cells. This conclusion is based on the following observations: First, the inhibitor decRVKR-CMK with the furin recognition motif R V K/R R prevented cleavage of HCMV gB in different systems. In addition to the experimental setups described here, inhibition of gB cleavage by the drug was also observed in HCMV-infected astrocytoma cells and in transfected astrocytoma cell lines stably expressing gB (10; data not shown). Interestingly, in the presence of the inhibitor consistent accumulation was noticed of a normally short-lived, endo H-resistant gB intermediate which is most likely the immediate substrate of the endoprotease. Second, coexpression experiments showed that recombinant human furin promoted directly endoproteolytic cleavage of recombinant HCMV gB in the absence of endogenous cellular proteolytic activity.

Recently, a cell line, LoVo, was described lacking a functional furin gene (14). Interestingly, two viral membrane glyoproteins, gp 160 of human immunodeficiuency virus (HIV) and the fusion glycoprotein of Newcastle disease virus which were both thought to be proteolytically cleaved by furin, are differently processed in LoVo cells (15). Unexpectedly, HIV gp160 was found to be correctly processed in spite of the absence of functional furin. This observation indicated that endoproteases other than furin may function at the R × K/R R-recognition motif. It is noteworthy in this context that two other ubiquitous endoproteases, PACE 4 and PC6, that resemble furin in their cleavage specificities (16, 17) have been recently identified. Thus, it cannot be excluded that furin may not be the only cleavage enzyme of HCMV gB.

Regarding the biological significance of the herpesvirus gB cleavage step, it has been demonstrated that gB of bovine herpes virus 1 (BHV-1), which was rendered uncleavable by mutagenesis, could functionally substitute for the cleavable counterparts of BHV-1 as well as of pseudorabies virus (18). Cleavage of the gB molecule appears thus not to be essential for its function under the in vitro test conditions. However, it may well be that the conformational alteration resulting from cleavage at the conserved recognition site may be necessary, e.g., for improvement of viral infectious potential and propagation in certain cell types in vivo. This aspect should be amenable to experimental examination. With respect to the structure of herpesvirus gB cleavage products, there is no obvious fusogenic peptide which is proteolytically activated like in the case of influenza virus hemagglutinin or human immunodeficiency virus glycoprotein 160 kDa (for review see 19). An intramolecular hydrophobic domain neighboring the actual membrane anchor domain with striking sequence similarity to fusogenic peptides

has recently been recognized for herpesvirus gB (20). So far the structural conception for the fusogenic activity attributed to herpesvirus gB-type molecules is still largely enigmatic. Precise structural analysis of the gB molecule is needed to eventually elucidate its functional structure.

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