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# Sequence Requirements for Proteolytic Processing of Glycoprotein B of Human Cytomegalovirus Strain Towne

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**Truncated versions of the human cytomegalovirus (CMV) strain Towne glycoprotein B (gB) gene were stably expressed in CHO cell lines. The calcium-specific ionophore A23187 inhibited proteolytic cleavage of C-terminal-truncated gB expressed by cell line 67.77. These inhibition studies also showed that the 93-kilodalton cleavage product most likely represents the N-terminal cleavage fragment of gB. The ionophore carboxyl cyanide *m*-chlorophenyl-hydrazone was used to show that proteolytic cleavage of gB did not occur in the endoplasmic reticulum. Two-dimensional polyacrylamide gel electrophoresis demonstrated that the N- and C-terminal cleavage products of gB remained associated by disulfide linkages after cleavage. Expression studies using constructs in which 80% or all of the N terminus was deleted demonstrated that the N terminus was required for secretion of the gB molecule. The amino acid sequence at the site of cleavage was shown to be critical for cleavage by a cellular protease. Our results indicate that an arginine-to-threonine change at either amino acid 457 or 460, a lysine-to-glutamine change at amino acid 459, or all three substitutions together block gB cleavage. The effect on proteolysis of the arginine-to-threonine amino acid change at residue 457 (position −4 relative to the cleavage site) demonstrated that a basic pair of amino acids at the endoproteolytic processing site is not the only requirement in *cis* for gB cleavage.**

Human cytomegalovirus (CMV) is a member of the herpesvirus group and is a significant cause of morbidity and mortality in certain human populations (reviewed by Ho; see reference 24). CMV infections may take a severe course in congenitally infected newborns, in recurrent infection due to reactivation of latent virus, or in generalized infection as a consequence of prolonged immunosuppression (1, 16). Because passively acquired antibodies have been shown to provide some protection against CMV disease (40, 42, 53, 59, 60, 61), a subunit vaccine which would elicit a neutralizing-antibody response against CMV could be useful for prevention of the serious consequences of CMV disease. We and others have previously identified CMV glycoprotein B (gB) as a candidate antigen for inclusion in a subunit vaccine (12, 14, 21, 46, 52, 57).

The gB complex is a major target for virus-neutralizing antibody both in the virus (4, 9, 12, 14, 35, 38, 46, 52, 57) and in complexes isolated from viral membrane preparations (17, 21–23, 27, 41). These reports concur that the polypeptides being described are from the family of glycoproteins originally designated gA by Pereira et al. (45) and now identified as gB (14, 32, 36, 52). The mature 55-kilodalton (kDa) gB glycoprotein, detected on extracellular virions (6, 34, 46), is cleaved from the 130-kDa gB precursor at a specific processing site and represents the C-terminal half of the gp130 precursor (52). This processing was predicted by pulse-chase experiments (10, 14, 46) and by use of λgt11 expression studies (36). However, the identity or the fate of the N-terminal half of gB in CMV-infected cells has not been established.

The consensus sequence at the processing site of gB homologs that are cleaved is Arg-(Thr/Ser/Arg)-(Lys/Arg)-Arg and is conserved among human herpesviruses (30, 52). The basic amino acids near the cleavage sites of many viral glycoproteins appear to contribute to the structural requirements for cleavage (20, 44, 50, 51). In particular, we have previously noted (52) the similarity of the herpesvirus processing sites to the processing sites of human immunodeficiency virus gp160 and the influenza virus hemagglutinin (HA) (18, 28, 29, 39, 58). Endoproteolytic cleavage of CMV gB is mediated by a host cell enzyme (52) whose identity and intracellular location have not yet been established.

In this study, we have used recombinant forms of CMV gB to identify components of the gB complex and to clarify details of the processing of gB. Specifically, we have generated cell lines expressing three forms of gB: C-terminally truncated secreted gB; molecules specifically mutated near the proteolytic-processing site; and N-terminal deletion constructs. These cell lines were treated with various ionophores in experiments designed to examine the structural requirements for cleavage and secretion. Since the proteolytic cleavage of HA can be inhibited by the calcium-specific ionophore A23187 (31), we were interested in knowing whether proteolysis of CMV gB would be inhibited by the ionophore in cell lines expressing a C-terminal-truncated form of gB. These studies have demonstrated that A23187 could inhibit proteolytic cleavage of gB by these cells in a dose-dependent manner, resulting in the secretion of the 110-kDa truncated precursor. We have previously demonstrated that the 31-kDa cleavage product is derived from the C-terminal portion of gB (52), and the studies with A23187 have been used to identify the N terminus as the 93-kDa cleavage product. The ionophore carboxyl cyanide *m*-chlorophenyl-hydrazone (CCCP) inhibits transit of proteins from the endoplasmic reticulum (ER) to the Golgi (3). We have used CCCP to examine the processing of gB and have found

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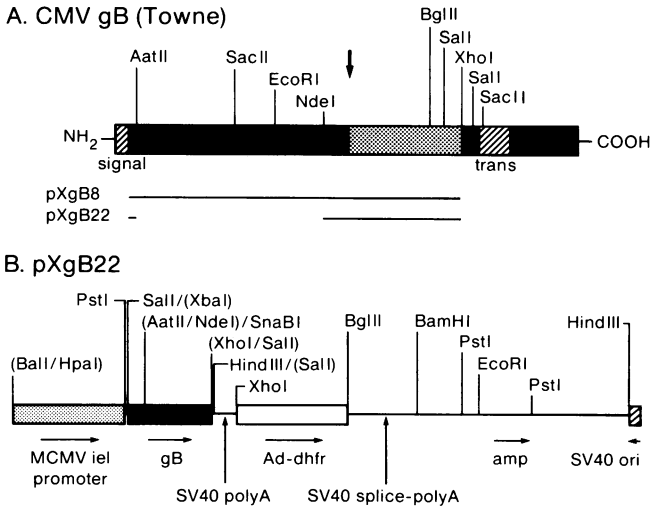


FIG. 1. Schematic representation of CMV gB (Towne) and the constructs used in expression experiments. (A) The 907-amino-acid gB protein from Met-1 to Val-907 (52). Selected restriction sites present in the DNA from this region are indicated above the linear map of the protein. The signal sequence and the transmembrane domain (trans) are indicated ( ▨ ). Arrow denotes proteolytic cleavage site which produces gp55 (i.e., the 55-kDa carboxyl-terminal portion of gB) in virus-infected cells. The 31-kDa portion of gB expressed by cell line 67.77 and detected by MAbs 27-156 and 15D8 ( ▩ ) is indicated. Plasmid constructs which have been stably expressed in CHO cells are shown below. Plasmid pXgB8 is as previously described (52). Plasmid pXgB22 encodes a gB molecule in which 80% of the N-terminal region has been deleted. (B) Schematic drawing of the structure of mammalian cell expression plasmid pXgB22. Plasmid pXgB22 was constructed by deleting a 1,108-bp *Aat*II (nucleotide 126)-to-*Nde*I (nucleotide 1232) fragment from gB. This N-terminal deletion retains the signal sequence and proteolytic processing site (52) but lacks the transmembrane and cytoplasmic domains of gB. Expression of gB is promoted by the murine CMV (MCMV) promoter/enhancer (15). SV40, Simian virus 40.

that cleavage occurs later in the secretory pathway than the ER. Consistent with this observation, we have shown that monensin also blocked gB proteolysis. Finally, we demonstrated that the N-terminal sequences of gB are required to maintain a structure which is capable of being cleaved and secreted. Consistent with the findings in the influenza virus system (28), we also report that for CMV gB the amino acid sequence at the site of cleavage does affect the susceptibility of the molecule to be cleaved by a cellular protease.

## MATERIALS AND METHODS

**gB-expressing cell lines.** Chinese hamster ovary (CHO) cell lines producing recombinant gB derivatives were generated as previously described (43, 52). CHO cell line 67.77 is a 0.25 μM methotrexate-amplified derivative of cell line 9-14 (previously described by Spaete et al. [52]), which was generated by cotransfection of the C-terminal-truncated gB construct pXgB8 (Fig. 1) with a plasmid encoding the selectable *dhfr* gene (54).

CHO cell line 126 was generated by transfection of CHO cells with plasmid pXgB22, a truncated form of gB in which the majority of the N-terminal domain (Thr-43 to Thr-411) has been deleted, as shown in Fig. 1. Briefly, media from 144 lines transfected with pXgB22 were analyzed by enzyme-linked immunosorbent assays (52) by using either monoclonal antibody (MAb) 15D8 or 27-156, and no secreted gB was detected (data not shown). Twelve of these cell lines were also analyzed by indirect immunofluorescence using MAbs 15D8 and 27-156, and three cell lines (61, 84, and 126) were positive by immunofluorescence with both antibodies (data not shown), indicating that gB was being expressed but not secreted.

CHO cell lines 113, 33, 199, and 79, expressing a gB molecule resistant to cleavage, were generated by transfection of CHO cells with plasmids pXgB24*clv*1 (line 113), pXgB24*clv*2 (line 33), pXgB24*clv*3 (line 199), and pXgB24*clv*4 (line 79), respectively. Media from 400 lines transfected with plasmids pXgB24*clv*1, -*clv*2, -*clv*3, or -*clv*4 were analyzed by enzyme-linked immunosorbent assay (52). Positive clones were detected at the frequency of one of every four tested, and selected clones were expanded for further analysis.

CHO cell lines 5-5 and 322 were generated by transfection of CHO cells with plasmids encoding the selectable *dhfr* gene and are *dhfr*-resistant negative control cell lines.

Cells were cultured with Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% dialyzed fetal calf serum and supplemented as described previously (43), except for experiments with the ionophores A23187 and CCCP.

**Plasmid constructions.** The pXgB8 construct (52) encodes a gB molecule lacking 227 amino acids from the transmembrane and C-terminal domains of gB. Plasmid pXgB22 (Fig. 1) was constructed by deleting 1,108 base pairs (bp) of N-terminal gB-coding sequences between the *Aat*II site (nucleotide 126) and the *Nde*I site (nucleotide 1232) from plasmid pXgB6 (52). The *Aat*II and *Nde*I ends were blunted with the Klenow fragment and ligated to create a *Sna*BI site and to preserve the reading frame. This 1,036-bp fragment was cloned into pMCMVAdhfr, a mammalian cell expression vector (Fig. 1B) which encodes the selectable *dhfr* gene, whose expression is promoted by the adenovirus major late promoter (54). Expression of the gB derivative encoded by pXgB22 is driven by the murine CMV immediate early promoter/enhancer (15), which was taken from pON402 (37) as a 1,370-bp *Hpa*I-to-*Pst*I fragment. All the transcriptional units in this construct are terminated by simian virus 40 polyadenylation signals. Plasmid pXgB24 was constructed by excising gB sequences from plasmid pXgB9 containing the full-length gB gene cloned as a 3.12-kilobase-pair *Eag*I fragment. Plasmid pXgB9 is identical to pXgB11 (52) except that the gB sequences are in the reverse orientation. The gB sequences (2,196 bp) were excised from pXgB9 by using the *Bam*HI site in the polylinker and the unique *Xho*I site in gB previously used in the construction of pXgB8. This 2,202-bp *Bam*HI-*Xho*I fragment was cloned into pMCMVAdhfr at *Sal*I to produce a *Sal*I-*Xho*I fusion at the 3′ end of gB, and the 5′ *Bam*HI and *Sal*I sites were filled with Klenow and ligated. This truncated gB molecule extends to Leu-680 (52) and is identical to that described for pXgB8 (52), except that four additional amino acids (Asp-Leu-Asp-Lys) are derived from the polylinker in pMCMVAdhfr before the translation termination codon. Plasmid pXgB24 also differs from pXgB8 by including 153 bp of 5′-untranslated gB leader sequence, whereas pXgB8 contains 84 bp of the 5′-untranslated gB leader.

Plasmids pXgB24*clv*1, -*clv*2, -*clv*3, and -*clv*4 were generated by substituting a 309-bp *Bal*I gB restriction fragment, which encodes mutated sequences specifying alternate amino acids at the proteolytic cleavage site, for the wild-type gB sequences. Polymerase chain reaction (48, 49) was used

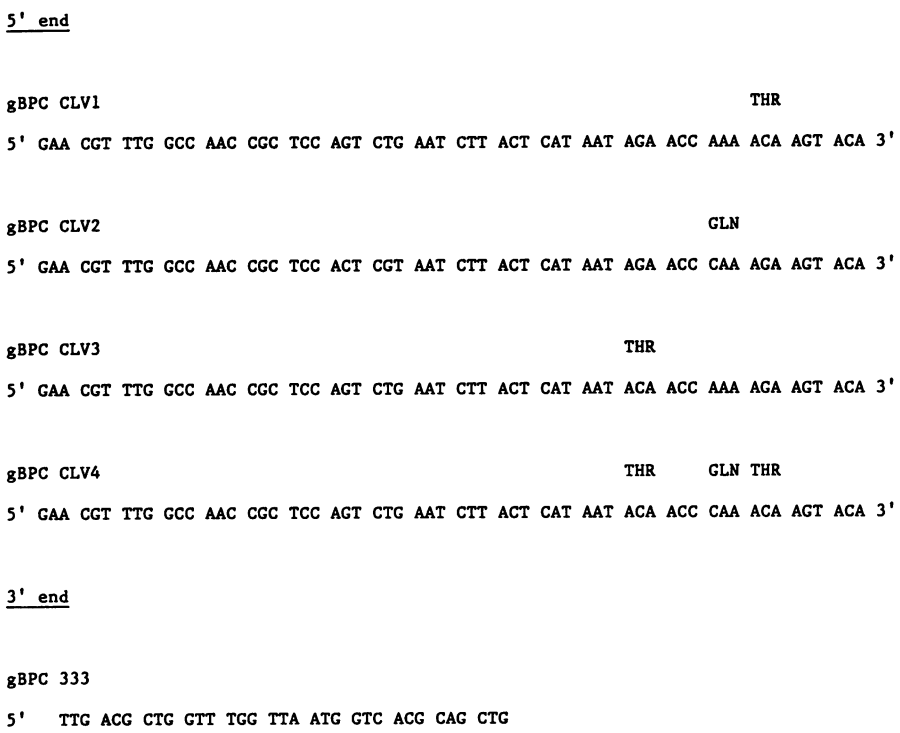


FIG. 2. Polymerase chain reaction primer pairs used to generate the site-specific mutations specifying alternate amino acids at the endoproteolytic processing site of gB. The 5′ oligonucleotide primers specifying mutated amino acids (shown above nucleotide sequence) for plasmids pXgB24*clv*1, -2, -3, and -4 are indicated as gBPC CLV1 through 4. The 3′ primer, gBPC 333, was used in all amplifications. Oligonucleotide primers were synthesized on an Applied Biosystems 380A synthesizer as described previously (52).

to generate the mutated sequences. The 309-bp *Bal*I fragment was enzymatically amplified for 30 cycles by using the primer pairs shown in Fig. 2.

Double-stranded M13mp18 DNA containing a 914-bp *Eco*RI-to-*Bgl*II gB fragment which spans the cleavage site was used as a template. The authenticity of the nucleotide changes was verified by sequencing each of the resulting *clv* mutant plasmids.

**Ionophore studies.** Processing of recombinant gB in the presence of the ionophore A23187 (Calbiochem, La Jolla, Calif.) was examined by growing cells 2 h before labeling in reinforced Eagle medium (REM) (2) lacking calcium chloride but with the ionophore. The cells were pulsed for 2 h with 250 μCi of [$^{35}$S]methionine per ml and incubated in unlabeled medium for 4 h, and the supernatants were analyzed by radioimmunoprecipitation with MAb 15D8 followed by electrophoresis on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels as previously described (43). For studies using the ionophore CCCP, cells were pulsed with 250 μCi of [$^{35}$S]methionine per ml for 15 min in minimal essential medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 0.292 g of L-glutamine per liter and lacking glucose, nucleosides, and $NaHCO_3$, without or with the addition of CCCP. Labeled supernatants were analyzed by radioimmunoprecipitation with MAb 15D8 and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (43).

**Antibodies, electrophoresis, and Western blotting.** Supernatants from cells pulsed with [$^{35}$S]methionine were immunoprecipitated with MAbs 15D8 (46) and 27-156 (52; W. Britt, unpublished data), which are specific for a region on the C terminus of the 31-kDa glycoprotein (Fig. 1A). Western immuno blot analyses were done by the method of Towbin et al. (55) as previously described (52) by using MAb 27-156. SDS-PAGE was done by the method of Laemmli (33). Samples were solubilized by heating for 3 min at 100°C in Laemmli sample buffer with or without the addition of β-mercaptoethanol to obtain reducing or nonreducing conditions, respectively. For two-dimensional SDS-PAGE, medium from [$^{35}$S]methionine-labeled CHO line 67.77 was immunoprecipitated with MAb 15D8, and equal portions were solubilized with or without 10% β-mercaptoethanol and subjected to first-dimension SDS-PAGE in a 10% gel. Both resulting lanes were cut from the first gel, soaked for 10 min in sample buffer containing β-mercaptoethanol, placed horizontally on top of a second 10% gel, and subjected to second-dimension SDS-PAGE. Gels were stained with Coomassie blue to locate unlabeled marker proteins and fluorographed to visualize [$^{35}$S]methionine-labeled proteins (8).

**Glycosidase studies.** The amount of glycosylation on the truncated or C-terminal cleavage fragment of recombinant gB was examined by treating lysates of lines 126 and 67.77 with *N*-glycanase (Genzyme, Boston, Mass.) and/or with *N*-neuraminidase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) followed by endo-α-*N*-acetyl-galactosaminidase (*O*-glycan peptide hydrolase) (Boehringer Mannheim Biochemicals). For *N*-glycanase studies, cell lysates were prepared from CHO lines, and 30 μg of total protein as determined by Bradford reagent (Bio-Rad Laboratories, Richmond, Calif.) was adjusted to 0.17% SDS and 0.03 M β-mercaptoethanol. Samples were boiled for 3 min, cooled on ice for 5 min, and spun briefly at 4°C in a microcentrifuge (Eppendorf). *N*-Glycanase buffer (5×) (6 μl) (1 M sodium phosphate [pH 8.6], 50 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 6.25% Nonidet P-40) was

added to samples; this was followed by the addition of 1.2 μl (0.012 U) of *N*-glycanase. Samples were incubated at 37°C for 60 h, frozen for 1 h at −20°C, suspended in 5× Laemmli sample buffer, and subjected to 12% SDS-PAGE. For studies with endo-α-*N*-acetyl-galactosaminidase (*O*-glycan peptide hydrolase), samples were first treated with neuraminidase. Protein A-Sepharose-antibody-protein complexes were washed twice with 0.1 M sodium acetate (pH 5.5)–2 mM $CaCl_2$. Washed complexes were suspended in 300 μl of 0.1 M sodium acetate (pH 5.5)–2 mM $CaCl_2$–10% (vol/vol) 1,10 Phenanthroline Hydrate (Sigma Chemical Co., St. Louis, Mo.)–0.1 U of neuraminidase. The complex was allowed to digest overnight at 37°C and spun briefly in a microcentrifuge, and the resulting pellet was washed twice with 0.1 M sodium phosphate (pH 6.4). The complex was suspended in 200 μl of 0.1 M sodium phosphate (pH 6.4)–10 mM D-galactonic acid (Sigma, St. Louis, Mo.), and 2 μl (1.0 mU) of endo-α-*N*-acetyl-galactosaminidase was added to the reaction mixture, which was incubated overnight at 37°C. The reactants were pelleted and suspended in Laemmli sample buffer, and the products were separated by SDS-PAGE. Deglycosylated gB molecules were visualized by Western blot analysis using MAb 27-156 as described above and elsewhere (52).

## RESULTS

**The calcium-specific ionophore A23187 inhibits proteolytic cleavage of recombinant gB.** CHO cell line 67.77 is a methotrexate-amplified (0.25 μM) derivative of line 9-14 (52) which expresses a gB molecule truncated by the deletion of amino acids 681 to 907 at the C terminus. As we have described previously for line 9-14, the gB synthesized by line 67.77 is rapidly processed to a 110-kDa precursor which is proteolytically cleaved into 93- and 31-kDa molecules (Fig. 3, lane 1). Cleavage is incomplete, and both the cleaved and uncleaved forms of gB are secreted by this cell line. The calcium-specific ionophore A23187 has been shown to inhibit the proteolytic cleavage of HA (31). To examine the effects of A23187 on the biosynthesis and processing of gB, cell line 67.77 and control cell line 5-5 were grown in REM (2) lacking calcium plus increasing concentrations of A23187 as described by Klenk et al. (31). The cells were pulse-labeled for 2 h in [$^{35}$S]methionine-containing medium and then incubated for another 4 h in medium without radiolabel. The resulting supernatants were radioimmunoprecipitated with MAb 15D8 and analyzed by SDS-PAGE. In the absence of A23187, both the 110-kDa precursor gB and its 93-kDa amino and 31-kDa carboxyl cleavage products were detected (Fig. 3, lanes 1 and 2). With increasing drug concentrations the intensities of the cleavage product bands decreased, until at 0.25 μM essentially all of the label was present in the precursor band (lanes 4, 6, and 8). Precipitates of the control line 5-5 showed no immune reactivity (lanes 3, 5, 7, and 9). Similar analyses of intracellular gB in the presence of A23187 showed the same inhibition of cleavage products, indicating that the results shown in Fig. 3 are not due to selective retention of the cleaved molecule (data not shown). Although cleavage of the gB precursor was not completely blocked by A23187, it was inhibited at least 99% at 0.25 μM A23187. Supernatants of cell line 67.77 and control line 5-5 were also analyzed by Western blotting with human anti-CMV antiserum (Whittaker Bioproducts) after radioimmunoprecipitation with MAb 15D8. The 93-kDa fragment of line 67.77 was specifically immunoreactive with the human anti-CMV antiserum (data not shown). This experiment demonstrates that the 93-kDa fragment is viral in origin and eliminates the possibility that MAb 15D8 has coprecipitated a cellular 93-kDa molecule in the complex. Since we have previously identified the 31-kDa fragment as originating from the C terminus of gB (52), taken together these experiments show that the 93-kDa fragment most probably represents the N terminus of gB.

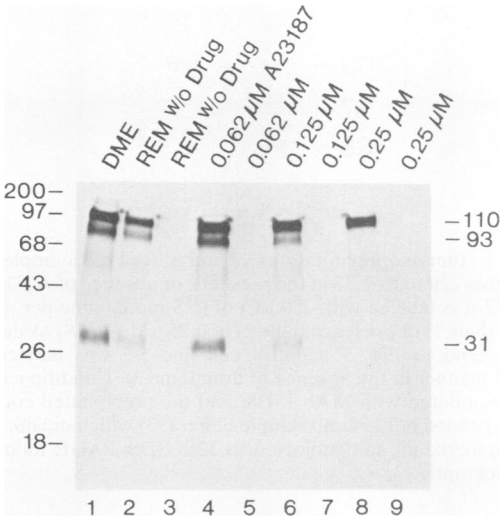


FIG. 3. The calcium-specific ionophore A23187 inhibits proteolysis of CMV glycoprotein B. Cell line 67.77, expressing a C-terminal-truncated form of gB, and control cell line 5-5 (52) were radiolabeled with 250 μCi of [$^{35}$S]methionine per ml for 2 h in Dulbecco modified Eagle (DME) medium or REM lacking calcium (2) without or with the addition of A23187 at the concentrations indicated. The radiolabel was then removed, and the cells were incubated for an additional 4 h in unlabeled medium without or with the previously used concentration of A23187. Cells labeled in the presence of A23187 were preincubated for 2 h before being labeled in REM lacking calcium but with the appropriate concentration of A23187. Medium samples from cell line 67.77 (lanes 1, 2, 4, 6, and 8) or control cell line 5-5 (lanes 3, 5, 7, and 9) were immunoprecipitated with MAb 15D8, subjected to 12% SDS-PAGE, and autoradiographed as described previously (43). Identical results were also obtained with MAb 27-156 (data not shown). The protein molecular mass standards are shown on the left of the autoradiogram in kilodaltons, and the sizes of the gB-specific molecules are indicated on the right.

To study the composition of the gB complex in its unreduced form, supernatants of cell line 67.77 prepared in the presence or absence of A23187 were immunoprecipitated with MAb 15D8 and analyzed without reduction by SDS-PAGE (Fig. 4). Both immunoprecipitates contained a prominent radiolabeled protein with an apparent size of 160 kDa (lanes 1 and 2) which appeared to be noncleaved gB (Fig. 3, lane 8). Untreated cells contained an additional gB band, of 140 kDa (Fig. 4, lane 1), which is composed of the two disulfide-linked 93- and 31-kDa gB cleavage products, as discussed below.

**Cleavage does not occur in the ER.** The results of studies by other laboratories using the ionophore monensin (4, 11, 22, 47), which blocks Golgi transit (26), were conflicting with respect to the ability of monensin to inhibit endoproteolytic processing. Since monensin is known to block the transit of membrane vesicles from the Golgi to the cell surface, we wanted to examine earlier processing events in the endoplasmic reticulum (ER) by using a well-characterized inhibitor of transit, CCCP (3). CCCP is an oxidative phosphorylation

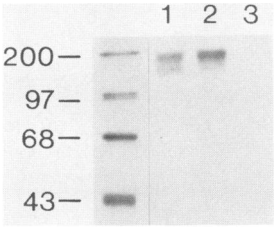


FIG. 4. Immunoprecipitations of unreduced gB complexes secreted from cell line 67.77 in the presence or absence of A23187. Cell line 67.77 was labeled with 250 μCi of [$^{35}$S]methionine per ml in the absence (lane 1) or presence (lane 2) of 0.25 μM A23187 as described in the legend to Fig. 3. Control cell line 5-5 was labeled in an identical manner in the absence of drug (lane 3). Conditioned media were precipitated with MAb 15D8, and the precipitated complexes were suspended in Laemmli sample buffer (33) which did not include β-mercaptoethanol and subjected to 12% SDS–PAGE followed by autoradiography.

inhibitor that blocks all ATP-requiring protein transport from the ER to the Golgi (25). CHO line 67.77 and a control line (5-5) were pulse-labeled for 15 min with [$^{35}$S]methionine, and lysates were immunoprecipitated with MAb 15D8. When transit of gB to the Golgi is blocked by CCCP (Fig. 5), endoproteolytic processing is inhibited, as indicated by the absence of the 93- and 31-kDa cleavage products in lanes 5, 7, and 9. The presence of the 93- and 31-kDa cleavage products in the radioimmunoprecipitated products of cells receiving no drug (Fig. 5, lane 1) or 20 μm CCCP (Fig. 5, lane 3) indicates that the pulse time was sufficient to allow normal

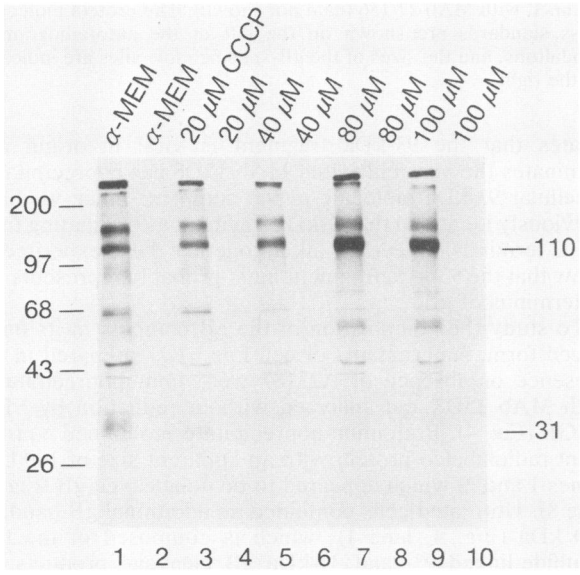


FIG. 5. Effect of CCCP on gB cleavage. Cell line 67.77 (lanes 1, 3, 5, 7, and 9) and a control cell line, 5-5 (lanes 2, 4, 6, 8, and 10), were pulsed with 250 μCi of [$^{35}$S]methionine per ml for 15 min in minimal essential medium containing 25 mM HEPES and 0.292 g of L-glutamine per liter and lacking glucose, nucleosides, and $NaHCO_3$, without or with the addition of CCCP at the concentrations indicated. Lysates were immunoprecipitated with MAb 15D8, electrophoresed in a 12% polyacrylamide gel, and autoradiographed. The protein molecular mass standards are shown on the left of the autoradiogram in kilodaltons, and the sizes of two gB-specific molecules are indicated on the right.

transit of gB through the ER. The band migrating with an apparent mobility of ca. 147 kDa above the 110-kDa fully processed gB (Fig. 5) is gB-related, as judged by Western blot analysis (data not shown). This 147-kDa species most likely represents a nontrimmed, high-mannose form of gB and confirms the intracellular location of the gB molecules as residing very early in the secretory pathway (11, 22, 47; D. M. Benko and W. Gibson, unpublished results). We have also done experiments to assess the effect of monensin on gB secreted by CHO line 67.77 and have found that monensin inhibits cleavage at concentrations between 1 and 25 μM (data not shown). Some residual 31-kDa C-terminal cleavage product was evident at monensin concentrations of 1, 5, 10, and 20 μM, but 25 μM strongly inhibited processing (data not shown). Because we have demonstrated that cleavage does not occur in the ER, the observation that monensin blocks cleavage in a dose-dependent manner can be taken to imply that cleavage most likely occurs very late in the secretory pathway.

**Analysis of recombinant gB complexes.** The relationship of the cleaved and the noncleaved recombinant gB molecules under both reducing and nonreducing conditions was examined by two-dimensional SDS-PAGE. Proteins lacking intermolecular disulfide cross-linkages often have identical relative mobilities ($M_r$s) under reducing and nonreducing conditions and fall on a diagonal line in the second-dimension gel. As expected, noncleaved truncated gB and its amino ($gB_N$) and carboxyl ($gB_C$) cleavage products were all present on the diagonal after reducing-reducing two-dimensional SDS-PAGE (Fig. 6B). The apparent $M_r$s of these proteins separated under reducing conditions were 135 (truncated gB), 97 ($gB_N$), and 35 kDa (truncated $gB_C$). Under nonreducing conditions (Fig. 6A), noncleaved truncated gB had an estimated $M_r$ of 160 kDa and was again present on the diagonal, indicating that it is monomeric and does not have intermolecular disulfide linkages. In contrast, the gB cleavage products $gB_N$ and $gB_C$ were present below the diagonal and moved together in the first-dimension nonreducing gel as a 140-kDa, disulfide-linked complex. The apparent size of this complex is consistent with it being a heterodimer composed of the 97- and 35-kDa fragments. The different relative mobilities of these three forms of gB (i.e., noncleaved nonreduced gB, 160 kDa; cleaved nonreduced gB, 140 kDa; noncleaved reduced gB, 135 kDa) indicate that the conformation of gB strongly influences its migration during SDS-PAGE. This result is consistent with single-dimension separations on polyacrylamide gels (Fig. 4).

**The N terminus of gB is required for cleavage and secretion.** To explore the structural requirements for proteolytic processing and secretion, CHO cell lines expressing a gB construct (pXgB22) in which 80% of the N-terminal domain of gB was deleted were generated in an attempt to isolate cell lines secreting this deleted gB molecule. This N-terminal deletion construct retains the signal sequence and proteolytic processing site (52) but lacks the transmembrane and cytoplasmic domains of gB (Fig. 1). CHO line 126 is a representative of the cell lines generated with the pXgB22 construct which resulted in the highest gB expression levels. Western blot analysis of lysates from three cell lines expressing this gB construct revealed a 43-kDa gB molecule that was reactive with MAb 27-156 (Fig. 7, lanes 1, 2, and 3). No secreted gB from these cell lines was detected by enzyme-linked immunosorbent assay using either MAb 15D8 or 27-156 (data not shown). For comparison, Western blots of lysates of CHO line 67.77 revealed the expected 110-kDa precursor and the 31-kDa cleavage fragment (Fig. 7, lane 4).

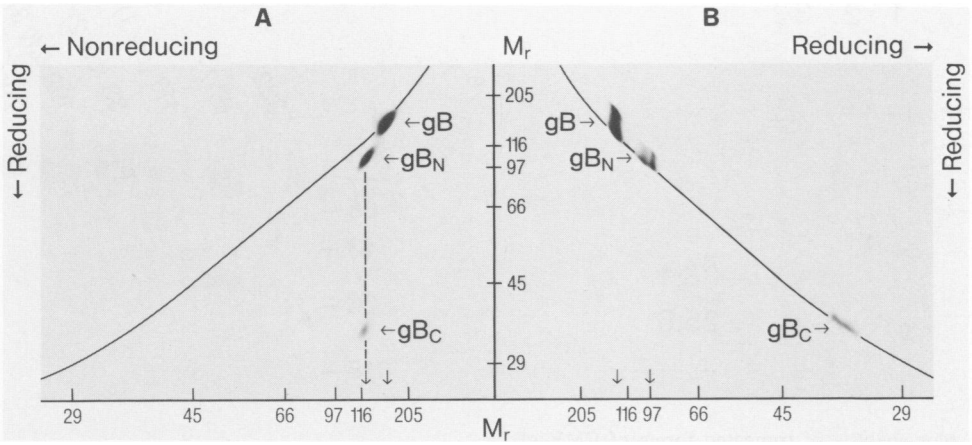


FIG. 6. Two-dimensional SDS-PAGE separation of recombinant gB expressed by CHO cell line 67.77. [$^{35}$S]methionine-labeled gB, immunoprecipitated by MAb 15D8, was combined with unlabeled molecular mass markers (Sigma; SDS 6H) and separated under nonreducing conditions followed by reducing conditions (A) or under reducing conditions in both dimensions (B). The second-dimension gel was stained with Coomassie brilliant blue to visualize the marker proteins and processed for fluorography (8). Shown here is a fluorogram of the resulting gel. A single gel was used to separate the reduced and nonreduced samples in the first dimension; another single gel was used for separation in the second dimension. gB, $gB_N$, and $gB_C$ denote the uncleaved, N-terminal, and C-terminal gB cleavage products, respectively. The relative mobilities ($M_r$s [$10^{-3}$]) of the marker proteins used to determine the diagonal line are shown on the axis for both dimensions. The doublet appearance of $gB_N$ in panel B is due to its comigration with the unlabeled marker, phosphorylase *b*.

Because it appeared that secretion but not expression of the truncated gB derivative was impaired, we examined the reasons for the increased size of the 43-kDa molecule compared with the expected 31-kDa C-terminal cleavage product. The 43-kDa N-terminal-deleted gB molecule is 10 kDa larger than the 33-kDa primary translation product expected with the signal peptide cleaved but with no other proteolytic processing. However, glycosylation at the eight remaining N-linked glycosylation sites encoded in the 43-kDa molecule, as well as the contribution of *O*-linked glycosylation, could account for this discrepancy. Additionally, we did not know whether the expressed gB molecule was cleaved.

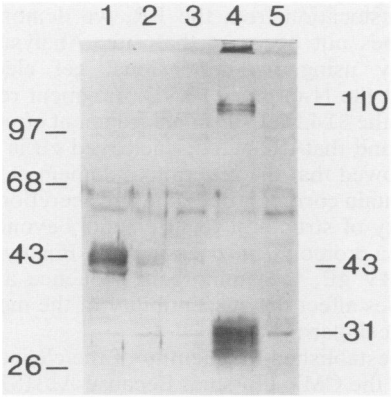


FIG. 7. Western blot analysis of truncated forms of CMV glycoprotein B expressed by CHO cell lines. Cell lysates were prepared from CHO lines 126 (lane 1), 84 (lane 2), and 61 (lane 3), which were generated by transfection of dihydrofolate reductase-deficient CHO cells (56) with gB expression plasmid pXgB22 (Fig. 1), or from CHO cell line 67.77 (lane 4) and control cell line 5-5 (lane 5). Approximately 40 μg of total protein, as determined by Pierce protein assay (Pierce Chemical, Rockford, Ill.), was loaded per lane, and samples were subjected to 12% SDS–PAGE, transferred to a nitrocellulose filter, and probed with MAb 27-156 as described previously (52). The protein molecular mass standards are shown in kilodaltons on the left, and the sizes of gB-specific expressed forms are shown on the right.

First, to examine the amount of glycosylation on the 43-kDa molecules, we treated lysates of lines 126 and 67.77 with *N*-glycanase alone and/or with *N*-neuraminidase and *O*-glycan peptide hydrolase. *N*-Glycanase specifically hydrolyzes asparagine-linked oligosaccharides from glycoproteins. Neuraminidase hydrolyzes terminal *N*- or *O*-acylneuraminic acids, and *O*-glycan peptide hydrolase hydrolyzes the *N*-acetylgalactosamine disaccharide core joined to the glycoprotein in an O-type linkage. After *N*-glycanase treatment, the C-terminal gB fragment migrates at 25 kDa for line 67.77 (Fig. 8, lane 1) and 33 kDa for line 126 (Fig. 8, lane 2), in agreement with the predicted size of the primary translation products. The mobilities of these molecules were not altered by *O*-glycanase (data not shown). Since the gB expressed by line 126 has 20% of the N terminus, its inability, after deglycosylation, to comigrate with the appropriately processed gB of line 67.77 is most probably due to lack of proteolytic processing at the cleavage site. Thus, some structural requirement for cleavage has been altered by the N-terminal deletion in the pXgB22 construct (Fig. 1).

**The amino acid sequence at the proteolytic processing site does affect the susceptibility of gB to be cleaved by the cellular protease.** The processing studies of Kawaoka and Webster (28, 29) have identified critical basic amino acids for cleavage activation of HA. We chose to alter similarly distributed basic amino acids at the site of proteolytic processing to test the effect of selected substitutions on the ability of gB to be cleaved. As described in Materials and Methods and shown in Fig. 9, a series of four constructs were generated by using pXgB24 with the following substitutions: Thr for Arg at −1 (*clv*1) and -4 (*clv*3) relative to the point of cleavage, Gln for Lys at −2 (*clv*2), and all three substitutions simultaneously (*clv*4). These plasmid constructs, designated pXgB24*clv*1 through 4, respectively, were used to generate stable CHO cell lines.

To examine the effect of the amino acid substitutions on

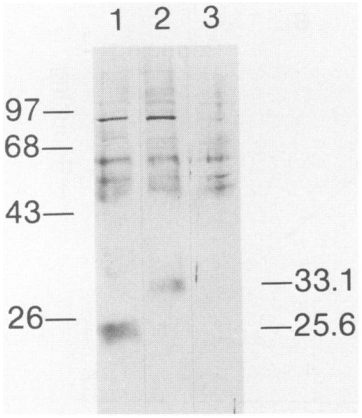


FIG. 8. Western blot analysis of truncated forms of CMV gB after treatment by *N*-glycanase. Cell lysates were prepared from CHO lines 67.77 (lane 1), 126 (lane 2), and control line 5-5 (lane 3), and 30 μg of total protein (as determined by Bradford reagent; Bio-Rad Laboratories) was adjusted to 0.17% SDS and 0.03 M β-mercaptoethanol. Samples were boiled for 3 min, cooled on ice for 5 min, and spun briefly at 4°C in a microcentrifuge (Eppendorf). *N*-glycanase buffer (5×) (6 μl) (1 M sodium phosphate [pH 8.6], 50 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 6.25% Nonidet P-40) was added to samples, followed by the addition of 1.2 μl (0.012 U) of *N*-glycanase (Genzyme). Samples were incubated at 37°C for 60 h, frozen for 1 h at −20°C, suspended in 0.2 volume of 5× Laemmli sample buffer (33), and subjected to 12% SDS–PAGE. The proteins were electroblotted onto nitrocellulose and probed with MAb 27-156 as described previously (52). Protein molecular mass standards are shown in kilodaltons on the left, and the predicted sizes of the deglycosylated gB molecules are shown on the right.

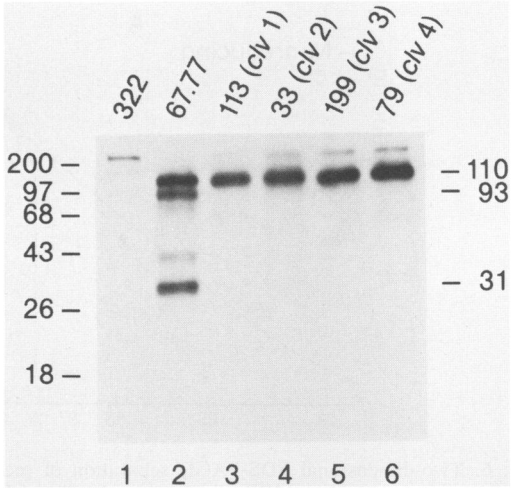


FIG. 10. Immunoprecipitation of gB cleavage mutants expressed by CHO cell lines. Cell lines 113, 13, 199, and 79 expressing pXgB24 *clv*1, -2, -3, or -4, respectively (lanes 3 to 6), were labeled with 250 μCi of [$^{35}$S]methionine for 4 h in Dulbecco modified Eagle medium lacking methionine plus 10% fetal calf serum. Negative control line 322 transfected with pCMVAdhfr (lane 1) and cleaved gB line 67.77 (lane 2) were labeled in an identical manner. Conditioned media were precipitated with MAb 15D8, subjected to 12% SDS–PAGE, and autoradiographed. The protein molecular mass standards are shown on the left of the autoradiogram in kilodaltons, and the sizes of the gB-specific molecules are indicated on the right.

the processing of gB, the four cell lines expressing *clv* mutant gB proteins were analyzed by radioimmunoprecipitation with MAb 15D8. All the *clv* mutant cell lines express a gB molecule which comigrates with the uncleaved form of gB expressed by wild-type CHO line 67.77 (Fig. 10). Longer exposures of the autoradiogram shown in Fig. 10 revealed that a small proportion (less than 1%) of the gB expressed by each mutant line continues to be appropriately processed (data not shown). This indicates that substitutions for basic amino acids at or near the proteolytic processing site result in gB molecules that are almost completely resistant to proteolysis. These data also confirm the A23187 processing data.

| | amino acid sequence | | | | | | | | gB cleavage |
|---|---|---|---|---|---|---|---|---|---|
| CMV(Towne) | $R_{457}$ | T | K | R↓ | S | T | D | $G_{464}$ | + |
| *clv* 1 | R | T | K | T* | S | T | D | G | - |
| *clv* 2 | R | T | Q* | R | S | T | D | G | - |
| *clv* 3 | T* | T | K | R | S | T | D | G | - |
| *clv* 4 | T* | T | Q* | T* | S | T | D | G | - |

FIG. 9. The effect of mutations near the gB cleavage site on the endoproteolytic processing of gB. Row 1 shows four amino acids on either side of the Towne gB endoproteolytic cleavage site as previously depicted (52). Rows 2 to 5 show the comparable regions of cleavage (*clv*) mutant expression plasmids. The ability of the molecule to be cleaved by the cellular protease is indicated by + or − (see Fig. 10). The subscript numbers refer to the amino acid position within the gB protein sequence (52). Arrow indicates the known proteolytic processing site. Asterisks denote amino acid sequence changes from the wild-type sequence shown in row 1.

## DISCUSSION

In this study, we have shown that the calcium-specific ionophore A23187 inhibits the endoproteolytic processing of CMV gB. Moreover, by using the ionophore CCCP, which inhibits translocation from the ER, we demonstrated that cleavage does not occur at that site. Analysis of the gB complex by using two-dimensional gel electrophoresis showed that the N-terminal 93-kDa fragment remains associated with the 31-kDa C-terminal fragment after proteolytic processing and that truncated, uncleaved gB is monomeric. We also showed that the N-terminal domain of gB is necessary to maintain correct processing and secretion, suggesting the necessity of structural requirements beyond the simple presence of a proteolytic processing site. Finally, we showed that for CMV gB, the amino acid sequence at the site of cleavage does affect the susceptibility of the molecule to be cleaved by cellular protease.

We have established the identity of the cleavage products encoded by the CMV gB gene. Because A23187 inhibits the proteolytic cleavage of gB in a dose-dependent manner, we were able to use the ionophore to establish the relationship of the cleavage products to the precursor and thus establish the identity of the N terminus of the processed gB molecule as the 93-kDa cleavage product. This cleavage fragment is immunoprecipitated by MAbs 15D8 and 27-156 as part of a complex which includes the truncated C-terminal cleavage fragment, gp31, which is analogous to gp55 in virus-infected cells (52). The ionophore A23187 may also prove to be a valuable reagent in assessing the requirements of a processed gB for viral infectivity. The inhibition of gB cleavage by the ionophore A23187 suggests that a calcium-dependent

cellular protease is responsible for gB processing. We have hypothesized (52) that a mammalian cell cleavage enzyme of this specificity may be related to the previously identified yeast KEX2 protease (5, 19). The calcium dependence of KEX2 (19) and the cellular protease which cleaves gB also supports this hypothesis. Earlier work in the influenza virus system with A23187 (31) established the neutral pH optimum and the nonlysosomal origin of the cellular cleavage enzyme in MDBK cells.

Previous results from other laboratories which examined the effects of monensin on processing (4, 11, 22, 47) had left open to question the formal possibility that processing occurs even earlier than the *cis* Golgi. Our result obtained with the ionophore CCCP showed that proteolytic processing occurs subsequent to the ER. Recently, Bennet et al. (7) have indicated that cleavage of HA appears to be initiated either during transport vesicle formation in the *trans* Golgi network or during transit of the *trans* Golgi vesicle. Our results using various ionophores and our results showing that specific amino acid sequence changes near the processing site interfere with cleavage suggest that these features of the processing of CMV gB are shared with HA in the influenza virus system and that the cellular location where processing occurs is probably the same.

Two-dimensional gel electrophoresis was used to show that the noncleaved truncated gB molecule does not have intermolecular disulfide linkages and that the N-terminal and C-terminal cleavage products remain associated by disulfide linkages, implying that cleavage occurs after the molecule has folded. These findings are compatible with earlier interpretations of the structure of gB complexes (6, 10, 17, 22, 46).

We generated a CHO cell line expressing a construct (pXgB22) in which 80% of the N terminus is deleted in order to express the C-terminal region of the gB molecule encoding a majority of the currently recognized neutralizing epitopes (4, 52, 57). We have also constructed a eucaryotic expression vector in which the 660-base-pair C-terminal gB fragment was fused to the tissue plasminogen activator signal sequence, which has been shown to facilitate the synthesis and secretion of human immunodeficiency virus type 1 gp120 (N. Haigwood, personal communication) and fragments of Factor VIIIc (13). When tested in transient expression assays in COS-7 cells, secretion but not expression was impaired, in agreement with the results described above for the pXgB22 construct (data not shown). These results suggest a block in the secretion pathway which correlates with the absence of N-terminal sequences in both the constructs.

The inability to obtain secretion of truncated gB with two different N-terminal deletion constructs tested indicates that the N terminus provides some essential structural requirement for secretion. The results obtained with the ionophore A23187 demonstrate that blocking proteolytic cleavage is not an impediment to secretion. This result is germane to the case of the pXgB22 construct expressed in CHO line 126, in which 80% of the N terminus is deleted and the expressed, nonsecreted protein is not cleaved. More probably, inappropriate folding or deletion of 7 of the 11 cysteine residues remaining after signal peptidase cleavage has altered the structure of this version of gB.

Finally, following the lead provided by Kawaoka and Webster (28, 29) in their studies of the sequence requirements for cleavage activation of influenza virus HA, we showed that CMV gB has similar sequence requirements for cleavage. As discussed above and previously (52), the possibility that a KEX2-like cellular enzyme is responsible for cleavage suggested that changes in the dibasic residues (KR) at the site of proteolysis would have an effect on processing. Changing either of these residues did inhibit cleavage. In contrast to this finding, it has recently been shown that the basic pair of amino acids is not absolutely required for glycoprotein processing of human immunodeficiency virus type 1 gp160 (18). Surprisingly, for CMV gB a single amino acid change at −4 (R→T) also inhibited proteolytic processing. In addition, processing of the gB glycoprotein expressed by the *clv3* mutant was the most completely inhibited of all the gB molecules expressed by the *clv* mutants (data not shown). It is possible that the substitution has altered the conformation of the region such that the N-linked glycosylation sites at or near the change are no longer used. Alternatively, a conformational change might render the proteolytic processing site inaccessible to the cellular protease. The effects of proteolytic cleavage of gB on infectivity and pathogenicity for the herpesviruses have not yet been addressed. The results reported here offer important first clues as to which amino acids should be altered in addressing this question.

This study has examined several of the structural and sequence requirements for gB processing and secretion. The study has identified the N terminus of the processed gB molecule. Recent work has demonstrated that the gB molecule expressed by CHO line 67.77 is capable of eliciting a potent virus-neutralizing immune response (unpublished observations). Additional studies will be required to determine whether the uncleaved molecules we have engineered also elicit an antiviral immune response.

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