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Calnexin Acts as a Molecular Chaperone during the Folding of Glycoprotein B of Human Cytomegalovirus

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Human cytomegalovirus glycoprotein B (gB) is synthesized as a 105-kDa nonglycosylated polypeptide and cotranslationally modified by addition of N-linked oligosaccharides to a 160-kDa precursor in the endoplasmic reticulum (ER). It is then transported to the Golgi complex, where it is endoproteolytically cleaved to form the disulfide-linked mature gp55-116 complex. Pulse-chase experiments demonstrate that the 160-kDa gB precursor was transiently associated with calnexin, a membrane-bound chaperone, in the ER. The association was maximal immediately after synthesis, and they dissociated with a half-time of 15 min. Complete inhibition of binding by tunicamycin or castanospermine indicates the importance of N-linked oligosaccharides for it. Nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that during an initial stage in the biogenesis, the 160-kDa gB precursor was first synthesized as a fully reduced form and rapidly converted to an oxidized form, with a half-time of 18 min. Both forms of the gB precursor could bind to calnexin. The kinetics of the conversion from the fully reduced to the oxidized form coincided with that of dissociation of the 160-kDa gB precursor from calnexin, suggesting that the two steps are closely related.

Human cytomegalovirus (HCMV) is a member of the herpesvirus group and a common pathogen that persists in the host. The infection is generally asymptomatic; however, it can cause severe diseases in immunocompromised individuals and in congenitally infected newborns (1, 20). Little is known about protective immunologic responses which limit HCMV infection in vivo, but it is likely that both cellular and humoral immune responses against HCMV proteins play an important role in curtailing the severity of infection with HCMV (9, 44, 46, 47).

The 229-kb genome of HCMV encodes more than 200 proteins, of which at least 35 are thought to be virion associated (26). These virion proteins include some envelope glycoproteins (4, 43, 51). Among these, the most widely studied and most abundant is the gp55-116 complex, a disulfide-linked heterodimer consisting of two glycosylated proteins with estimated molecular masses of 55,000 and 116,000 Da (10). The glycoprotein complex is now termed glycoprotein B (gB), since it has sequence homology to the herpes simplex virus gB (13). The HCMV gB is a type I glycoprotein, with gp116 representing the surface component and gp55 being the transmembrane portion of the complex. As deduced from the nucleotide sequence, gB of HCMV strain AD is a 906-amino-acid polypeptide containing 16 potential N-linked glycosylation sites (13), and it has been shown to be synthesized as an approximately 105,000-Da nonglycosylated polypeptide which is cotranslationally modified by addition of N-linked oligosaccharides to a 150,000- to 160,000-Da precursor protein (referred to as the 160-kDa gB precursor in the text) in the endoplasmic reticulum (ER) (11, 25). The precursor is then transported from the ER to the Golgi complex, where terminal sugar modifications occur, and further endoproteolytically cleaved to form the mature gp55 and gp116, which are linked by a disulfide bond (25).

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Fidelity in the expression of proteins in the cell is ensured at many levels, from transcription of the gene to intracellular transport of the final products. The ER also possesses a so-called "quality control" system, acting at the posttranslational level, that permits properly folded and assembled proteins to leave the ER and proceed to the Golgi complex and beyond while simultaneously retaining incompletely folded, misfolded, and unassembled proteins (34). Recent studies suggested that molecular chaperones play important roles in the quality control system (24, 31). They transiently recognize and stabilize partially folded intermediates and misfolded polypeptides. Along with other folding enzymes, such as a protein disulfide isomerase (21) and prolyl *cis-trans*-isomerases (50), the ER contains chaperones, including immunoglobulin heavy-chain binding protein BiP/GRP78 (an HSP70 analog) and GRP94 (an HSP90 analog) (24). It has recently been shown that another ER resident protein, calnexin, also acts as a chaperone that preferentially associates with glycoproteins (5, 27, 28, 30, 42).

Calnexin (previously known as p88 or IP90) was originally identified by virtue of its transient association with the nascent heavy chain of class I major histocompatibility complex molecules (16, 17). Unlike other chaperones, such as BiP/GRP78 and GRP94, it is an integral membrane protein, with a molecular mass of approximately 90 kDa for human cells (52). Subsequently, calnexin has been found associated with folding and assembly intermediates of a wide array of soluble and membrane proteins (5). These include subunits of T-cell receptor (15, 32), membrane immunoglobulin (23, 32), class II major histocompatibility complex molecules (3), and many secretory glycoproteins, such as α_1-antitrypsin, transferrin, complement 3 (38, 42), and thyroglobulin (35, 36). In addition to these cellular proteins, some viral proteins, including influenza virus hemagglutinin and vesicular stomatitis virus (VSV) G protein, have been reported to utilize the host protein calnexin as a chaperone (27–29).

In this study, we tried to determine the role of calnexin in the processing of gB of HCMV by pulse-chase experiments and

obtained the following results. Immediately after synthesis, the nascent 160-kDa gB precursor bound to calnexin transiently and dissociated from it with a half-time of 18 min. We also demonstrated that the 160-kDa gB precursor was distinguished into a fully reduced and an oxidized form on a sodium dodecyl sulfate (SDS)-polyacrylamide gel under nonreducing conditions. The gB precursor was first synthesized as the fully reduced form and rapidly processed to the oxidized form. Both the fully reduced and oxidized forms were found to be associated with calnexin. The kinetics of dissociation coincided with that of disappearance of the fully reduced form of the 160-kDa gB precursor, indicating that disulfide bonding might bring about the dissociation of the gB precursor from calnexin. These data suggest that HCMV has evolved to utilize a host chaperone, calnexin, to ensure fidelity in the expression of its proteins.

MATERIALS AND METHODS

Specific reagents. Castanospermine was purchased from Wako Pure Chemical Industries (Osaka, Japan). 1-Deoxymannojirimycin was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The following reagents were from Sigma Chemical Co. (St. Louis, Mo.): tunicamycin, dithiothreitol (DTT), and *N*-ethylmaleimide (NEM).

Antibodies. Monoclonal antibody (MAb) TI-23, directed against the gB of HCMV, was purchased from Fujisawa Pharmaceutical Co., Osaka, Japan. A polyclonal rabbit antiserum against a synthetic peptide corresponding to amino acids 487 to 505 of canine calnexin was a generous gift of J. M. Bergeron (McGill University, Montreal, Canada).

Viruses and cells. HCMV strain AD169 and human embryonic lung fibroblasts (HEL) were used throughout this study. HEL were propagated in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and used between passages 15 and 25. The virus stock was prepared in HEL by infection at a low multiplicity as described previously (41).

Virus infection. Confluent monolayers of HEL were infected with HCMV strain AD169 at a multiplicity of 3 PFU per cell. After a 2-h adsorption period, the cells were washed twice with MEM and incubated at 37°C with MEM containing 10% FCS. Unless indicated otherwise, infection was allowed to proceed for 3 days, and then the cells were subjected to pulse-chase study.

Metabolic labeling and chase. Metabolic labeling was performed as follows. After two washes with methionine-free MEM, confluent monolayers of HEL were incubated for 30 min at 37°C in methionine-free MEM containing 10% FCS and labeled for 10 min with 0.2 mCi of [^{35}S]methionine per ml. The labeled cells were chased in complete medium containing an excess of unlabeled methionine (5 mM).

In some experiments, double labeling of the infected cells with $^{32}P_i$ and [^{35}S]methionine was performed to increase the incorporation of radioactivity into calnexin. On the second day of infection, the medium was exchanged with MEM containing 10% FCS and $^{32}P_i$ (750 μCi/ml), and infection was allowed to proceed for further 12 h for steady-state labeling. Then the cells were washed with methionine-free MEM twice, preincubated with methionine-free MEM containing 10% FCS for methionine starvation, and pulse-labeled for 10 min with [^{35}S]methionine (0.1 mCi/ml). The labeled cells were chased in complete medium containing an excess of unlabeled methionine (5 mM). After the chase, the cells were subjected to immunoprecipitation as described below.

Immunoprecipitation and electrophoresis. At the end of the chase, the cells were quickly chilled by being placed them on ice and treated with ice-cold phosphate-buffered saline (PBS) containing 20 mM NEM to alkylate free sulfhydryl groups (14). Generally, immunoprecipitation was performed under nondenaturing conditions. In brief, the radiolabeled cells were lysed in ice-cold 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.5% Nonidet P-40 (NP-40) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg of aprotinin per ml, 20 mM NEM, 0.1% NaN_3, and bovine serum albumin (BSA; 1 mg/ml). The lysate was precleared with normal rabbit serum and protein A-Sepharose 4 FF (Pharmacia LKB Biotechnology, Uppsala, Sweden) twice and immunoprecipitated with either a polyclonal rabbit antiserum against calnexin or MAb TI-23 and protein G-Sepharose 4 FF (Pharmacia LKB Biotechnology). The Sepharose beads were washed in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.5% NP-40 seven times to remove nonspecifically adsorbed proteins. Immunoprecipitates were eluted from the beads by boiling in 50 μl of 2× SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS) for nonreducing conditions or in 50 μl of 2× SDS sample buffer containing 10% 2-mercaptoethanol for reducing conditions. Then antigens were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with the buffer system of Laemmli (37). After the gels were fixed with 10% acetic acid and 10% methanol and dried, protein bands were visualized, and in some experiments, the band intensities were determined with the Fujix Bio-Imaging Analyzer BAS2000 System (Fuji Photo Film Co., Ltd., Tokyo, Japan) to quantify radioactivity incorporated into the protein.

When indicated, immunoprecipitation was performed under denaturing conditions. The radiolabeled cells were lysed in ice-cold 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–1% NP-40–0.5% sodium deoxycholate–0.1% SDS (RIPA buffer) containing 1 mM PMSF, aprotinin (10 μg/ml), 20 mM NEM, 0.1% NaN_3, and BSA (1 mg/ml). In these conditions, all the buffers used were replaced with RIPA buffer except that the last three washes of the Sepharose bead-bound immune complexes were done in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.5% NP-40. Then, the antigens were subjected to SDS-PAGE as described above.

In some experiments, immunoprecipitates were analyzed by SDS-PAGE first under nonreducing conditions and then under reducing conditions. Briefly, after the immunoisolated gB was separated on an SDS-polyacrylamide gel under nonreducing conditions as described above, the gel was dried without fixation, and the bands were visualized with the Fujix Bio-Imaging Analyzer BAS2000 System. Then the desired bands were excised from the gel and pushed into the appropriate well of the second-round SDS-polyacrylamide gel. For the second-round gel, all the gel mixes contained 1 mM EDTA and the stacking gel had about twice the normal depth. The gel slices were reswelled with 125 mM Tris-HCl (pH 6.8)–0.1% SDS–1 mM EDTA–2.5 mM DTT and overlaid with 30 μl of 2× SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS) containing 2.5 mM DTT and 1 mM EDTA, and the gel was run until the gel front migrated about two-thirds of the distance into the stacking gel. The power supply was turned off, and the reduction of the samples was allowed to proceed for 30 min. Then the gel was run thoroughly, fixed, and dried, and protein bands were visualized with the Fujix Bio-Imaging Analyzer BAS2000 System.

Isolation of the protein from the gel and reimmunoprecipitation. Proteins precipitated with anticalnexin antibody from the infected-cell lysates pulsed with [^{35}S]methionine for 10 min were analyzed by SDS-PAGE under reducing conditions. The gel was dried without fixation, the protein band was visualized with the Fujix Bio-Imaging Analyzer BAS2000 System, and the 160-kDa protein that coprecipitated with calnexin was excised from the gel. The protein was electro-eluted with the Bio-Rad model 422 electroeluter according to the manufacturer's protocol and reimmunoprecipitated with the second-line antibody, MAb TI-23 or anticalnexin antibody. Then, the immune complexes were subjected to SDS-PAGE as described above.

Glycosylation inhibitor treatment. In some experiments, infected cells were treated for 30 min with tunicamycin (10 μg/ml), 1 mM castanospermine or 1 mM 1-deoxymannojirimycin in methionine-free medium and then pulse-labeled with [^{35}S]methionine for 10 min and chased in the presence of the inhibitor. After the chase, the cells were subjected to immunoprecipitation with either anti-gB MAb TI-23 or anticalnexin as described above.

RESULTS

Calnexin associates with HCMV-encoded proteins immediately after synthesis. To determine whether there are any HCMV proteins transiently associated with calnexin, we compared the profiles of proteins coprecipitated with calnexin from HCMV-infected cell lysates with those from mock-infected cell lysates. Confluent monolayers of HEL were mock infected or infected with HCMV at a multiplicity of 3 PFU per cell. On the third day of infection, the cells were pulsed with [^{35}S]methionine for 10 min in methionine-free medium and chased for 180 min in complete medium containing 5 mM unlabeled methionine. At the indicated times, the cells were lysed with denaturing lysis buffer containing SDS as described in Materials and Methods, and the lysates were immunoprecipitated with an anticalnexin antibody. When the precipitates were analyzed by SDS-PAGE under reducing conditions, a labeled calnexin band at 90 kDa could be observed for both the mock-infected and HCMV-infected cell lysate immediately after the pulse, and this calnexin band was still seen with its radioactivity unaltered even after 180 min of chase (Fig. 1). The amounts of calnexin precipitated were not significantly different between mock-infected and HCMV-infected cells immediately after the pulse and later in the chase, indicating that HCMV infection did not affect either the biosynthesis or turnover of calnexin.

When the cells were lysed with the nondenaturing lysis buffer described in Materials and Methods, several proteins were coprecipitated from the mock-infected cell lysate immediately after the pulse, including proteins of 191, 182, 141 (the most prominent), 85, 70, and 25 kDa (Fig. 1). After 180 min of chase, most of the coprecipitated proteins disappeared except the 141-kDa protein, which was still seen as a faint band. For

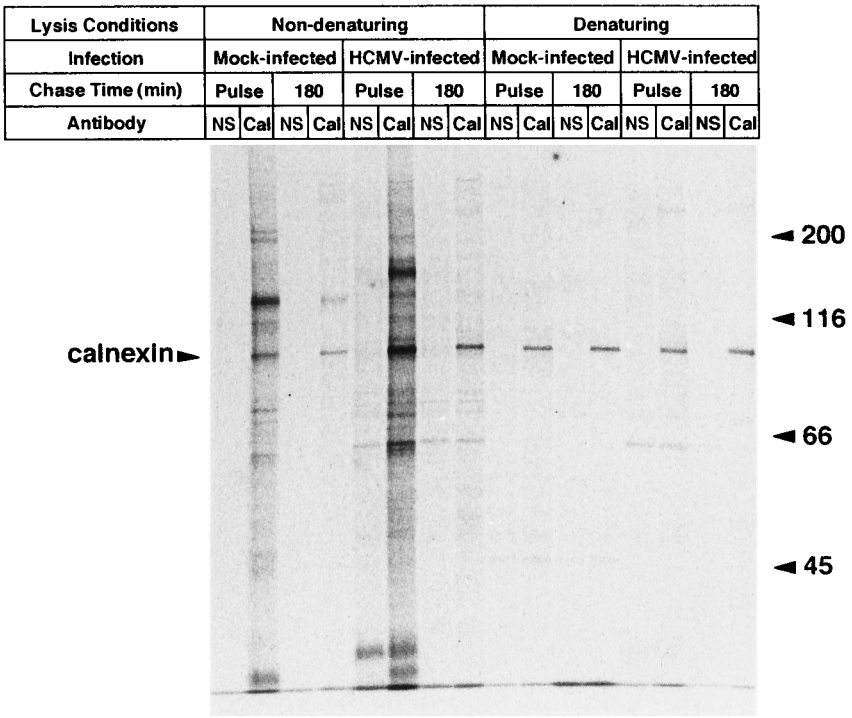

FIG. 1. Association of newly synthesized proteins with calnexin in HCMV-infected HEL. Mock-infected and HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 10 min and chased for 0 or 180 min. The cells were lysed under denaturing or nondenaturing conditions, and the lysates were precipitated with anticalnexin (Cal) or normal rabbit serum (NS). The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions. The position of calnexin is indicated. Molecular mass markers are also shown to the right of the gel (in kilodaltons).

HCMV-infected cells, some proteins specific for the infected cells were coprecipitated with calnexin along with the host proteins seen in the mock-infected cell lysate. These included 166-kDa, 156-kDa, and 116-kDa proteins. Again in the infected cells, these coprecipitated proteins were fading after 180 min of chase. This transient association between calnexin and viral proteins suggests that some proteins encoded by HCMV utilized calnexin as a molecular chaperone (27–29).

Calnexin binds preferentially to the nascent glycoprotein B precursor of 160 kDa. Among the proteins that coprecipitated with calnexin in the infected cells, we chose the most abundant protein of 160 kDa and tried to identify it. It is well known that calnexin prefers to associate transiently with a folding intermediate of glycoproteins in the ER (5, 27). Among the HCMV-encoded glycoproteins, one of the most abundant is gB, which is synthesized as a 160-kDa precursor, and we thought it most likely that the 160-kDa protein coprecipitated with calnexin only immediately after the pulse was the 160-kDa gB precursor. To determine whether it was, we next immunoisolated gB from the infected cells and compared its mobility with that of the calnexin-associated proteins on the same SDS-polyacrylamide gel under reducing conditions. Nascent gB is cotranslationally glycosylated in the ER and then transported to the Golgi complex, where it undergoes endoproteolytic cleavage into a disulfide-linked 116-kDa and a 55-kDa component (11, 25). When the processing of gB was analyzed on an SDS-polyacrylamide gel under reducing conditions, the 160-kDa gB precursor diminished, and a fuzzy, broad band with a molecular mass of 116 kDa and a distinct 55-kDa band appeared along with the chase (Fig. 2). Again, the 160-kDa band was coprecipitated with calnexin from the lysates prepared under nondenaturing conditions (Fig. 2, lower panel), and it was likely to be the 160-kDa gB precursor because they had identical electrophoretic mobilities. In this experiment, a band that was migrating slightly faster than the 160-kDa gB precursor was seen in the anti-gB precipitates early in the chase, and this was also likely some gB precursor. However, we have no precise explanation for this band.

To further confirm that the calnexin-associated 160-kDa

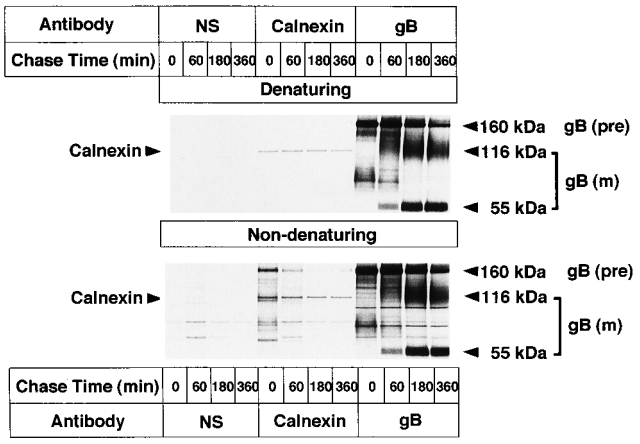

FIG. 2. Transient association of the 160-kDa gB precursor with calnexin. HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 10 min and chased for 0 to 360 min. The cells were lysed under denaturing or nondenaturing conditions, and the lysates were precipitated with anticalnexin (Calnexin), anti-gB (gB), or normal rabbit serum (NS). The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions. The positions of calnexin, gB precursor [gB(pre)], and mature gB [gB(m)] are indicated.

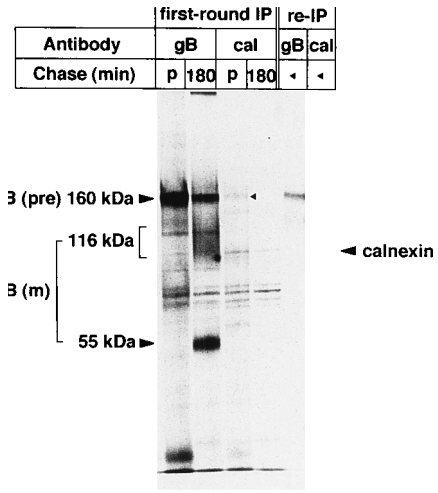

FIG. 3. Identification of the 160-kDa protein coprecipitated with calnexin as the 160-kDa gB precursor. HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 10 min and chased for 0 or 180 min. The cells were lysed under nondenaturing conditions, and the lysates were precipitated with anticalnexin (cal) or anti-gB (gB). The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions (first-round IP). The 160-kDa band coprecipitated with calnexin from the lysates immediately after the pulse (solid triangle) was eluted from the unfixed gel. The eluted protein was subjected to second-round immunoprecipitation with either anti-gB (gB) or anticalnexin (cal). The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions (re-IP). The positions of calnexin, gB precursor [gB(pre)], and mature gB [gB(m)] are indicated.

protein was the 160-kDa gB precursor, we next determined whether anti-gB antibody recognized the 160-kDa protein that coprecipitated with calnexin. Infected cells were pulse labeled, lysed under nondenaturing conditions, and subjected to immunoprecipitation with anticalnexin, and the precipitates were analyzed on an SDS-polyacrylamide gel under reducing conditions. The band migrating at 160 kDa was excised, eluted, and subjected to second-round immunoprecipitation with either anti-gB antibody or anticalnexin antibody. Figure 3 summarizes the results. The calnexin-associated 160-kDa protein reacted with anti-gB antibody and migrated at the position of the 160-kDa gB precursor (compare the band for the second-round immunoprecipitation with anticalnexin with that for the first round before band excision). Moreover, anticalnexin antibody did not recognize the eluted antigen. These results indicated that the calnexin-associated 160-kDa protein was the authentic gB precursor, and the coprecipitation was not attributed to cross-reactivity of anticalnexin antibody with the 160-kDa gB precursor.

It should be noted that in the experiments mentioned above, no precipitation of radiolabeled calnexin was observed with the 160-kDa gB precursor from the infected-cell lysates under nondenaturing conditions, although the reverse did occur. However, it may be not strange that calnexin might turn over slowly and not be labeled efficiently by the short time of pulse used in these experiments. To address this point, we used a double labeling protocol with $^{32}P_i$ for steady-state labeling and [^{35}S]methionine for pulse labeling, since calnexin is a phosphoprotein (52). HCMV-infected HEL were labeled with $^{32}P_i$ under steady-state conditions for 12 h to prelabel phosphoproteins that turn over slowly. These cells were then pulse labeled with [^{35}S]methionine for 10 min to label the newly synthesized gB precursor, chased for 180 min, and lysed under nondenaturing conditions. Calnexin and the gB were immunoprecipitated and subjected to SDS-PAGE under reducing conditions. For comparison, SDS-PAGE profiles for the immunoprecipitates with either pulse labeling only or steady-state labeling only are also shown in Fig. 4A and B; the gels in both were given equal exposure times. It was apparent that calnexin, detected at 90 kDa, was more efficiently labeled by steady-state labeling with $^{32}P_i$ than by a [^{35}S]methionine pulse. In this experiment, we could detect 160-kDa gB precursor-bound calnexin in the lysates immediately after the pulse when the double labeling protocol was introduced, confirming the association between calnexin and the 160-kDa gB precursor.

As shown in Fig. 4A, the 160-kDa gB precursor was also efficiently labeled with $^{32}P_i$ although gB had not been known to be a phosphoprotein. Since one component of mature gB, gp55, was detected in the $^{32}P_i$-labeled cells while the other, gp116, was not, the site for phosphorylation of mature gB was apparently the C-terminal half of the protein.

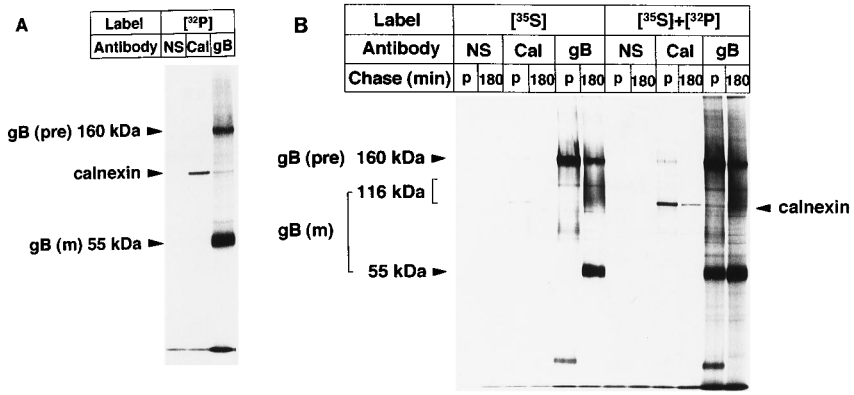

FIG. 4. In vivo metabolic phosphorylation of calnexin and gB in HCMV-infected HEL. (A) HCMV-infected HEL were metabolically radiolabeled with $^{32}P_i$ for 12 h. The cells were lysed under nondenaturing conditions, and the lysates were precipitated with anticalnexin (Cal), anti-gB (gB), or normal rabbit serum (NS). The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions. (B) Prior to pulse-labeling with [^{35}S]methionine, HCMV-infected HEL were metabolically radiolabeled with $^{32}P_i$ for 12 h for steady-state labeling. Then the cells were pulse-labeled with [^{35}S]methionine for 10 min and chased for 0 (p) or 180 min ([^{35}S]+[^{32}P]). The lysates prepared under nondenaturing conditions were precipitated with anticalnexin (Cal), anti-gB (gB), or normal rabbit serum (NS). The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions. For comparison, SDS-PAGE profiles of the immunoprecipitates from the lysates processed in parallel except that the steady-state labeling with $^{32}P_i$ was omitted are also shown to the left of the gel ([^{35}S]). The positions of calnexin, gB precursor [gB(pre)], and mature gB [gB(m)] are indicated.

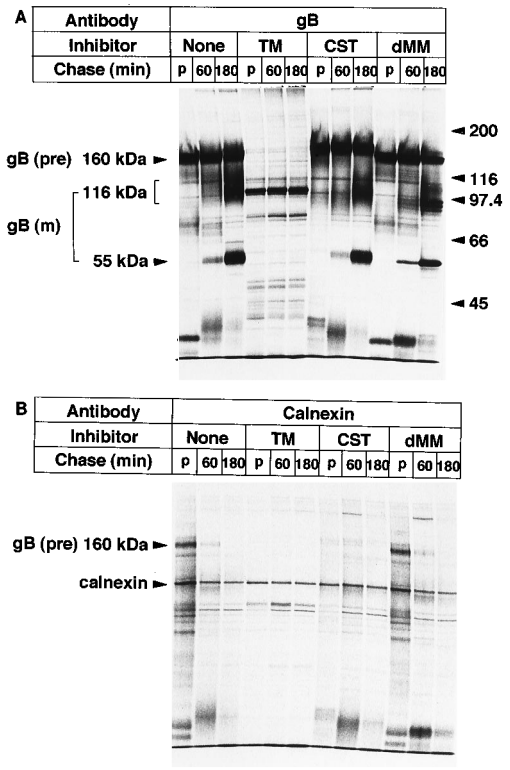

FIG. 5. Effects of inhibitors of N-linked oligosaccharide processing on the association of the 160-kDa gB precursor with calnexin. HCMV-infected HEL were pretreated for 30 min with tunicamycin (TM) (10 μg/ml), 1 mM castanospermine (CST), or 1 mM 1-deoxymannojirimycin (dMM), pulse-labeled with [^{35}S]methionine for 10 min, and chased for 0 or 180 min in the continued presence of the inhibitor. The cells were lysed under nondenaturing conditions, and the lysates were precipitated with anti-gB (A) or anticalnexin (B). The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions. The positions of gB precursor [gB(pre)], mature gB [gB(m)], and calnexin are indicated to the left of the gels. Molecular mass markers are also shown to the right of the gel (in kilodaltons) in panel A.

Effects of inhibitors of N-linked carbohydrates processing on association of the glycoprotein B precursor with calnexin. Previous studies have shown that the N-linked oligosaccharide side chain is important for glycoproteins to bind calnexin (5, 27, 28, 30, 42), although some exceptions that can form stable complexes with calnexin in spite of the absence of N-linked oligosaccharides have been reported (39, 45). To explore whether that holds true for the gB precursor of HCMV, we tested the effects of the glycosylation inhibitors tunicamycin, castanospermine, and 1-deoxymannojirimycin on the association of the gB precursor with calnexin. Infected HEL were pulsed with [^{35}S]methionine and chased in the presence of inhibitors. Figure 5A shows the SDS-PAGE profile of precipitates with anti-gB MAb, and Fig. 5B shows that of the anticalnexin precipitates. The mobility differences displayed by the gB bands in inhibitor-free controls and inhibitor-treated infected cells showed that tunicamycin and castanospermine inhibited the processing of N-linked sugars. Because tunicamycin inhibits a membrane-bound oligosaccharide transferase and thus blocks core glycosylation of nascent glycoprotein precursors (18), the nascent gB in tunicamycin-treated infected cells never acquired N-linked oligosaccharides, and its molecular mass was reduced to 105 kDa. The subsequent processing of the nascent gB in the tunicamycin-treated cells was profoundly impaired, and the molecular mass of gB remained unaltered even after 180 min of chase, when the fully processed form of gB, a disulfide-linked gp55-116, was detected in the untreated cells. In the tunicamycin-treated cells, no coprecipitation of the nascent gB precursor was observed immediately after the pulse through later in the chase, which was consistent with the previous findings that N-linked oligosaccharide is important for binding of glycoproteins to calnexin (5, 27, 28, 30, 42).

In castanospermine-treated cells, the gB precursor was detected as a molecule migrating slightly slower than that in the untreated cells. In contrast to results with tunicamycin, the nascent gB in the castanospermine-treated cells was processed to a disulfide-linked form during the chase, although each component was migrating more slowly than in the untreated cells. In these cells, we could not detect the calnexin-associated gB precursor at all throughout the chase period, which was compatible with the previous findings that castanospermine inhibits the association of calnexin with a variety of nascent glycoproteins (28). Castanospermine inhibits glucosidases I and II and thus prevents the trimming of the three terminal glucoses from the core oligosaccharide, resulting in a decrease in the amount of glycoproteins bearing partially deglucosylated core oligosaccharides (19). Since calnexin preferentially binds to monoglucosylated N-linked oligosaccharides (30, 53), castanospermine treatment leads to abolishment of association between calnexin and nascent glycoproteins.

In 1-deoxymannojirimycin-treated cells, gB was synthesized as a molecule which had the same molecular weight as in untreated cells. 1-Deoxymannojirimycin acts on the later processing step by inhibiting the *cis*-Golgi mannosidase I (19). As has been reported for some glycoproteins (12), 1-deoxymannojirimycin treatment did not affect the processing of gB, and mature gB became detectable during the chase, although the rate of processing was somewhat slowed. In contrast to tunicamycin and castanospermine treatment, 1-deoxymannojirimycin treatment did not abolish the association between calnexin and the 160-kDa gB precursor.

Taken together, the selective sensitivity of the association between calnexin and the gB precursor to the glycosylation inhibitors indicated that this association was likely to be functional rather than nonspecific.

Kinetics of the association between calnexin and the gB precursor. To determine in greater detail the kinetics of the association between calnexin and the gB precursor, we performed pulse-chase experiments with shorter pulse and chase intervals. Infected cells were pulsed with [^{35}S]methionine for 2 min. At different chase times, the radiolabeled gB or calnexin was immunoisolated under nondenaturing conditions and analyzed by SDS-PAGE under reducing conditions (Fig. 6A for calnexin, and Fig. 6B for gB). The gB precursor was first seen as a discrete band with a molecular mass of 160 kDa. As the chase progressed, the band intensity decreased, and a part of the gB precursor came to migrate slightly more slowly, resulting in the band's becoming fuzzy and broad. The mature gB components, gp116 and gp55, were detected 40 to 60 min after synthesis and increased during the chase (Fig. 6A). The radioactivity incorporated into the 160-kDa gB precursor coprecipitated with calnexin (Fig. 6B) was quantified (Fig. 6C). The maximum association between the two molecules was observed immediately after the pulse, and during the chase, the amount of the coprecipitated gB precursor decreased rapidly in an exponential manner, with a half-time of 15 min. Figure 6C also illustrates the disappearance rate of the gB precursor, which was expected to reflect the transport rate of the gB precursor from the ER to the Golgi complex, since it is endoproteolytically cleaved into a disulfide-linked gp55-116 complex in the

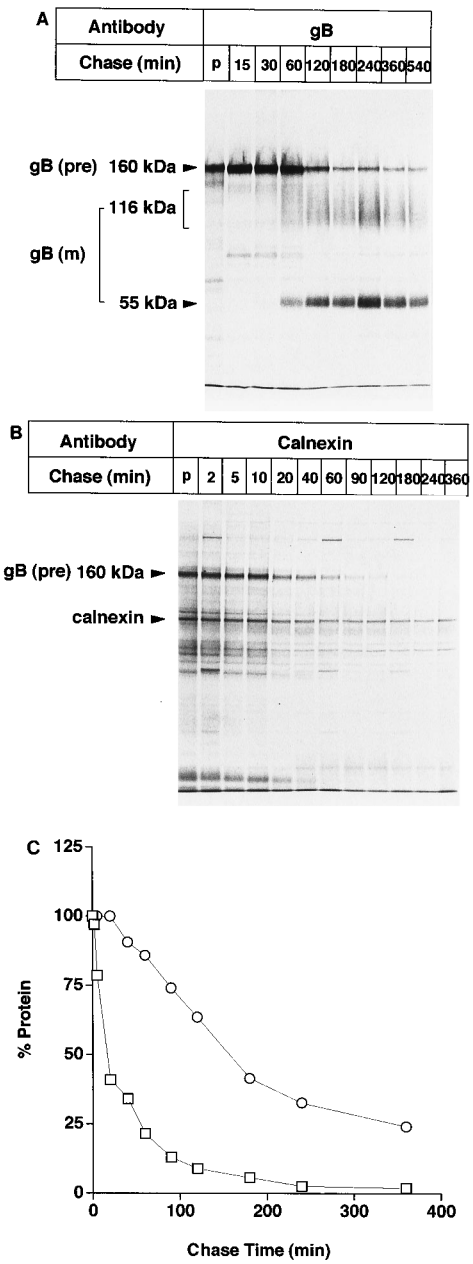

FIG. 6. Kinetics of the association of the 160-kDa gB precursor with calnexin. (A) HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 2 min and chased for 0 to 540 min. The cells were lysed under denaturing conditions, and the lysates were precipitated with anti-gB (gB). The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions. The positions of gB precursor [gB(pre)] and mature gB [gB(m)] are indicated to the left of the gel. (B) HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 2 min and chased for 0 to 360 min. The cells were lysed under nondenaturing conditions, and the lysates were precipitated with anticalnexin. The immunoprecipitates were analyzed by SDS–7.5% PAGE under reducing conditions. The positions of gB precursor [gB(pre)] and calnexin are indicated to the left of the gel. (C) The bands of the 160-kDa gB precursor coprecipitated with calnexin were quantitated with the Fujix Bio-Imaging Analyzer BAS2000 System, and the incorporated radioactivity at each time point was plotted as a percentage of the maximum coprecipitation found (open squares). The bands of the 160-kDa gB precursor that were not cleaved to the mature gp55 and gp116 forms under reducing conditions were quantitated in the same way. The incorporated radioactivity was plotted as a percentage of the total gB at each time point (open circles), reflecting the rate of transport of the 160-kDa gB precursor from the ER to the Golgi complex.

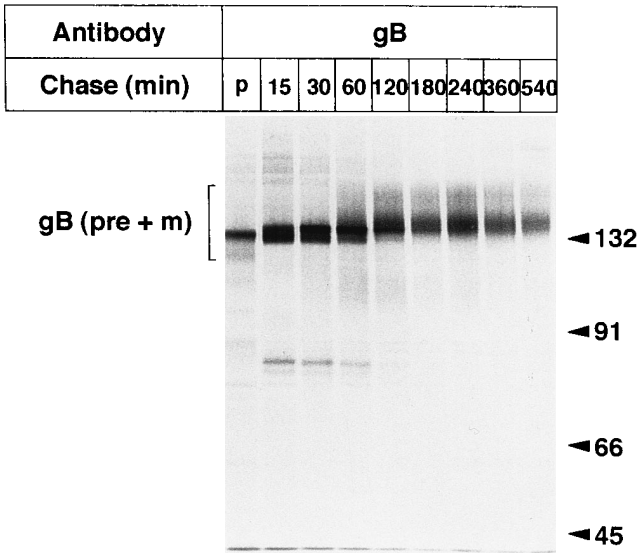

FIG. 7. Processing of gB of HCMV analyzed on a nonreducing SDS-polyacrylamide gel. HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 10 min and chased for 0 to 540 min. The cells were lysed under denaturing conditions, and the lysates were precipitated with anti-gB (gB). The immunoprecipitates were analyzed by SDS–7.5% PAGE under nonreducing conditions. The positions of gB precursor and mature gB [gB(pre+m)] are indicated. Molecular mass markers are also shown to the right of the gel (in kilodaltons).

Golgi apparatus (25). The half-time of the transport of gB from the ER to the Golgi apparatus was approximately 160 min. These results indicated that the association and dissociation between calnexin and the nascent gB precursor occurred at very early steps of its processing in the ER.

Glycoprotein B precursor of 160 kDa can be distinguished as the fully reduced form and the oxidized form. Many proteins synthesized in the ER sequentially form disulfide bonds, which are a prerequisite for normal folding and subsequent transport to the Golgi beyond. To allow disulfide formation, the ER provides a more oxidizing environment than the cytosol and contains a redox enzyme, a protein disulfide isomerase (22). As previously reported for some proteins (2, 6–8, 49), folding intermediates in the ER can be distinguished by their redox state by SDS-PAGE under nonreducing conditions. We thus examined the formation of intramolecular disulfide bonds in the gB precursor molecule by this method. Infected cells were pulsed with [^{35}S]methionine for 10 min and chased for periods of up to 540 min. At the end of each chase time, the cells were treated with a membrane-permeating alkylating agent, NEM, to block free sulfhydryl groups. The cells were then lysed in the presence of NEM, and the radiolabeled gB was immunoprecipitated and analyzed by SDS-PAGE under nonreducing conditions.

As shown in Fig. 7, gB was first detected as a single distinct band, migrating at an apparent molecular mass of 160 kDa. Again under nonreducing conditions, a fuzzy and thin band migrating slightly faster was seen, as mentioned above for SDS-PAGE under reducing conditions. At 15 min of chase, the gB precursor was detected as two discrete bands, one of which migrated faster and the other of which migrated slower than that initially seen. When these two bands were excised from the gel and subjected to second-round SDS-PAGE under reducing conditions as described in Materials and Methods, they had the same electrophoretic mobilities under reducing conditions (Fig. 8A and B). This indicated that these two bands, seen from

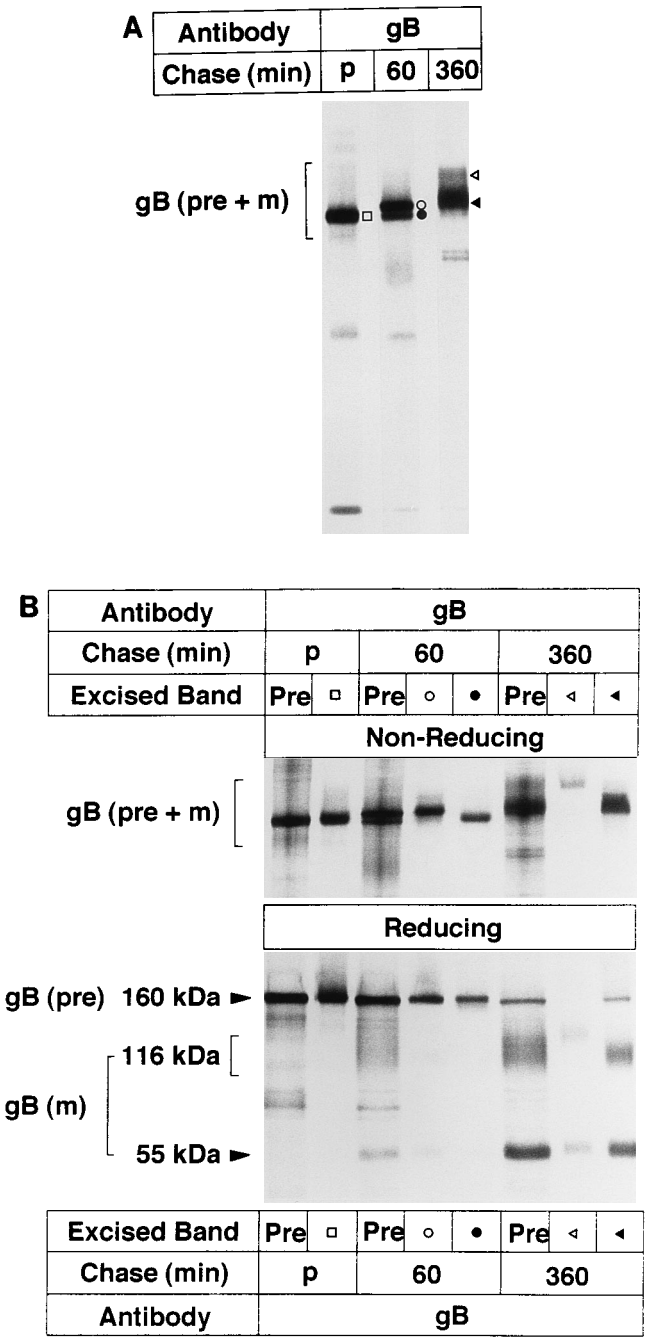

FIG. 8. Identification of the fully reduced and oxidized forms of 160-kDa gB precursor. (A) HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 10 min and chased for 0 to 360 min. The cells were lysed under denaturing conditions, and the lysates were precipitated with anti-gB (gB). The immunoprecipitates were analyzed by SDS–7.5% PAGE under nonreducing conditions. The positions of gB precursor and mature gB [gB(pre+m)] are indicated to the left of the gel. (B) At each time point, the gB precursor and mature gB bands shown in panel A were excised from the unfixed first-round SDS-polyacrylamide gel. The excised bands are indicated as follows in panel A to the right of the lanes: immediately after the pulse (open square), 60 min of chase (faster, solid circle; slower, open circle), and 360 min of chase (faster, solid triangle; slower, open triangle). These bands were subjected to second-round SDS–7.5% PAGE under nonreducing (upper panel) or reducing (lower panel) conditions. For comparison, the immunoprecipitates at each time point were analyzed as for the first round in the same gel (Pre). The positions of gB precursor [gB(pre)] and mature gB [gB(m)] are indicated to the left of the gels.

15 to 60 min after the pulse, were distinct folding intermediates of the 160-kDa gB precursor, whose redox state might be different, reflecting the extent of intramolecular disulfide bond formation.

As deduced from the nucleotide sequence, gB of HCMV strain AD169 has 16 cysteine residues in its amino acid sequence, and 11 of them are present in the ectodomain (13). Ten of 11 cysteine residues are conserved among HCMV, Epstein-Barr virus, and herpes simplex virus type 1, suggesting that some of them may be involved in the formation of intramolecular disulfide bonds and contribute to the secondary structure of the molecule, although their precise positions have not yet been defined. The ER provides and therefore can promote the formation of disulfide bonds (6, 22). Among these, the band that migrated faster was the fully reduced gB precursor, since the gB precursor isolated from infected cells incubated in the presence of DTT migrated at the same point as the molecule (data not shown). DTT is a membrane-permeating reducing agent, and treatment of viable cells with it gives fully reduced molecules. Upon chase, the amount of the fully reduced 160-kDa gB precursor diminished, and the oxidized 160-kDa gB precursor, which migrated more slowly, became predominant, indicating the completion of disulfide bond formation (Fig. 7). As both the gB precursors vanished, a fuzzy broad band which migrated far more slowly appeared after 60 min of chase and gradually became thick. Apparently, this band corresponded to mature gB, since it could be reduced and cleaved into the two components of the molecule, gp116 and gp55, under reducing conditions (Fig. 8B). It is noted that immediately following the pulse, nascent gB precursor migrat-

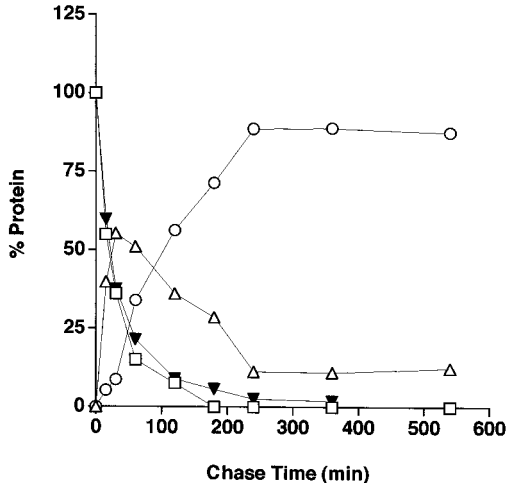

FIG. 9. Processing of the 160-kDa gB precursor from the fully reduced to the oxidized form. HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 10 min and chased for 0 to 540 min. The cells were lysed under denaturing conditions, and the lysates were precipitated with anti-gB (gB). The immunoprecipitates were analyzed by SDS–7.5% PAGE under nonreducing conditions. The bands of the fully reduced (open squares) and the oxidized (open triangles) forms of the 160-kDa gB precursor were quantitated with the Fujix Bio-Imaging Analyzer BAS2000 System, and the incorporated radioactivity was plotted as a percentage of the total gB at each time point. In parallel, HCMV-infected HEL pulse-labeled with [^{35}S]methionine for 10 min were lysed under denaturing conditions at the indicated times, and the radiolabeled gB was isolated with anti-gB and analyzed by SDS–7.5% PAGE under reducing conditions. The amount of radioactivity incorporated into the mature gB which could be cleaved into gp55 and gp116 under reducing conditions was quantitated in the same way and plotted as a percentage of the total gB at each time point (open circles), reflecting the transport rate of gB from the ER to the Golgi. The kinetics of the disappearance of the 160-kDa gB precursor associated with calnexin shown in Fig. 6C was again plotted in the same graph (solid triangles).

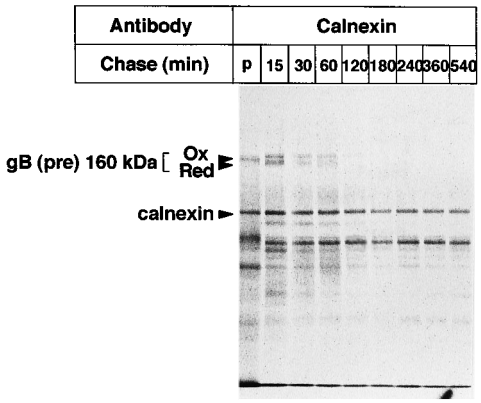

FIG. 10. Both the fully reduced and oxidized forms of the 160-kDa gB precursor associate with calnexin. HCMV-infected HEL were pulse-labeled with [^{35}S]methionine for 10 min and chased for 0 to 540 min. The cells were lysed under nondenaturing conditions, and the lysates were precipitated with anticalnexin. The immunoprecipitates were analyzed by SDS–7.5% PAGE under nonreducing conditions. The positions of the fully reduced (Red) and oxidized (Ox) forms of the 160-kDa gB precursor and calnexin are indicated to the left of the gel.

ing between the positions of the reduced and oxidized forms was seen at 15 min of chase. Two possible explanations can be proposed. One is the presence of some short-lived aberrant disulfide bonds in the nascent gB precursor, and the other is removal of one or two mannose residues from it by the ER mannosidases. The former is more likely, since the small shift in mobility was not seen when reducing conditions were employed (see Fig. 6A).

Note that both the fully reduced and oxidized forms of the 160-kDa gB precursor were not cleaved into gp116 and gp55 by reduction (Fig. 8B). This indicated that these two folding intermediates did not undergo endoproteolytic cleavage in the Golgi complex, and thus it was likely that the conversion from the fully reduced form to the oxidized form occurred in the ER. The radioactivities incorporated into the fully reduced and oxidized gB precursors were quantified and are plotted in Fig. 9. The reduced 160-kDa gB precursor level decreased in an exponential manner, with a half-time of 18 min. The amount of the oxidized 160-kDa gB precursor peaked 30 min after the pulse and thereafter dropped slowly, with a half-time of about 100 min. The transport rate of the gB precursor from the ER to the Golgi, estimated from the appearance of the cleavable 160-kDa gB precursor under reducing conditions, is also shown in Fig. 9 (see Fig. 6C). The half-time of the transport was 160 min. These data indicated that the completion of disulfide bond formation of the gB precursor was a far earlier process in the ER, before it was transported to the Golgi complex. Again, the amount of the calnexin-associated gB precursor was quantified and overlaid on the data in Fig. 9. A comparison of the curve for the disappearance of the complex with that for the fully reduced 160-kDa gB precursor revealed that the two processes were closely linked kinetically.

We next determined which gB precursor, the fully reduced or oxidized one, was associated with calnexin. Radiolabeled calnexin was immunoisolated from the infected lysates prepared under nondenaturing conditions in the presence of NEM and subjected to SDS-PAGE under nonreducing conditions (Fig. 10). It was apparent that both the fully reduced and the oxidized gB precursors were associated with calnexin. The calnexin-associated fully reduced gB precursor was first detected immediately after the pulse and became undetectable at 120 min of chase, when it was all processed to the oxidized form. The oxidized form was coprecipitated with calnexin from 15 min of chase as the fully reduced form, and its association with calnexin lasted until 120 min of chase. Although the amount of the oxidized gB precursor peaked at 30 min of chase (Fig. 9), its maximum association with calnexin was observed at 15 min of chase. The discrepancy indicated that some process(es) other than disulfide bond formation might promote the dissociation of the gB precursor from calnexin.

DISCUSSION

The ER has a so-called quality control system that permits properly folded and assembled proteins to leave the ER while simultaneously retaining incompletely folded, misfolded, and unassembled proteins to ensure fidelity in the expression of proteins in the cell (33). Growing evidence has suggested that molecular chaperones such as BiP/GRP78 as well as folding enzymes play an important role in the quality control system in the ER (24, 31). Calnexin, a recently identified integral membrane protein in the ER, also acts as a chaperone in the ER (5). It associates transiently with the folding intermediates of a wide variety of glycoproteins during normal processing but also binds to malfolded glycoproteins and retains them in the ER (34, 42, 45). Not only many glycoproteins of mammalian cells but also some viral glycoproteins, such as influenza virus hemagglutinin and VSV G protein, utilize calnexin as a molecular chaperone during processing (27–29). Unlike influenza virus and VSV, HCMV, a member of the herpesvirus group, is a complicated DNA virus, whose genome encodes at least 200 proteins (26). We were interested in determining whether such a complicated virus also utilized calnexin, a key molecule of the host quality control system, as a chaperone for its glycoproteins and explored it in this study.

We could detect some HCMV-specific proteins coprecipitated with calnexin under nondenaturing lysis conditions along with several host proteins (Fig. 1). Calnexin associated with these proteins only immediately after the pulse and dissociated from those later in the chase. Among those seen in HCMV-infected cells, we chose the most abundant species, with an estimated molecular mass of 156 kDa, and characterized its association with calnexin.

First, we identified the 156-kDa protein coprecipitated with calnexin as the 160-kDa gB precursor, since they had the same electrophoretic mobility (Fig. 2). This was further confirmed by the results shown in Fig. 3. The 156-kDa molecule was recognized by anti-gB antibody but not by anticalnexin antibody, indicating that the protein was authentically the 160-kDa gB precursor. Calnexin was not coprecipitated with the 160-kDa gB precursor with an anti-gB antibody (Fig. 2). However, we could settle this problem by using the double labeling protocol with a [^{35}S]methionine pulse and $^{32}P_i$ steady-state labeling. In these experiments, calnexin was efficiently labeled with $^{32}P_i$ and we clearly detected calnexin coprecipitated with the 160-kDa gB precursor with an anti-gB antibody (Fig. 4B), confirming the association between calnexin and the 160-kDa gB precursor.

Calnexin is a unique chaperone with respect to its preferential binding to glycoproteins (5, 27, 30). At present, calnexin is thought to act like a lectin with specificity for N-linked oligosaccharides, since most of the proteins proven to associate with calnexin are glycoproteins with N-linked oligosaccharides. Moreover, in HepG2 cells, treatment with tunicamycin, which inhibits core N-linked glycosylation of nascent glycoproteins, completely abolishes the binding between calnexin and glycoproteins (42). For VSV G protein, binding to calnexin is prevented not only by tunicamycin but also by a glucosidase in-

hibitor, castanospermine (27, 28). This apparent requirement for N-linked oligosaccharides for binding to calnexin was also confirmed by our system. As shown in Fig. 5B, treatment with tunicamycin or castanospermine completely inhibited the binding of calnexin to the 160-kDa gB precursor, whereas 1-deoxymannojirimycin, an inhibitor of *cis*-Golgi mannosidase I, did not have such an effect. Recent studies indicate that the monoglucosylated form of N-linked oligosaccharides supports optimal attachment of glycoproteins to calnexin (53).

For the majority of the proteins with which calnexin transiently interacts as a chaperone, calnexin binding seems to be restricted to a period of 0 to 60 min immediately after chain termination, during which individual proteins acquire disulfide bonds and assemble into oligomeric complexes (27, 28, 42). As shown in Fig. 6C, the 160-kDa gB precursor bound maximally to calnexin immediately after the pulse, and after that, it dissociated from calnexin in an exponential manner (half-time of dissociation, 15 min). These data are compatible with previous reports. The dissociation half-times that have been reported are 5 min for α_1-antitrypsin, ~35 min for transferrin, ~25 min for complement 3, ~25 min for apoB-100, and 10 to 12 min for VSV G protein.

As reported for some proteins, such as influenza virus hemagglutinin (7, 8), VSV G protein (29, 49), the immunoglobulin light chains (40), and tissue-type plasminogen activator (2), nascent folding intermediates of polypeptides can be separated on an SDS-polyacrylamide gel under nonreducing conditions, depending on their redox state. As for the nascent gB precursor with a molecular mass of 160 kDa, the fully reduced and oxidized forms could be also distinguished (Fig. 7, 8A, and 8B). Nascent gB was first synthesized as the fully reduced form and rapidly processed to the oxidized form, with a half-time of 18 min, probably as a result of formation of disulfide bonds catalyzed by protein disulfide isomerase (Fig. 9). Once processed to the oxidized form, the 160-kDa gB precursor stayed in the ER for a relatively long time, during which it might undergo additional modifications prerequisite for transport from the ER to the Golgi, since the rate for ER-to-Golgi transport roughly coincided with that for the disappearance of the oxidized form in the ER (Fig. 9).

As shown in Fig. 10, calnexin bound to both the fully reduced and the oxidized forms of the 160-kDa gB precursor. Furthermore, a comparison of the half-time for the disappearance of the calnexin-gB precursor complex with that for the disappearance of the fully reduced 160-kDa gB precursor revealed that the two processes were closely linked kinetically. With these data, it is possible that the oxidation of the precursor took place while it was associated with calnexin and that the accomplishment of the oxidation of the molecule, in other words, the acquisition of the correct intramolecular disulfide bonds, predisposed the molecule to dissociate from calnexin. This hypothesis is reminiscent of a model of the quality control system for glycoproteins in the ER proposed by Hammond et al. (27). According to the model, calnexin serves as a retention factor for incompletely folded glycoproteins and binds to their monoglucosylated N-linked oligosaccharides. They hypothesize that when conformationally defective glycoproteins are released from calnexin, the UDP-glucose:glycoprotein glucosyltransferase recognizes and specifically reglucosylates them, resulting in the continued association and ER retention of incompletely folded glycoproteins. Escape from the de- and reglucosylation cycle occurs when a glycoprotein reaches a folded conformation and is no longer a substrate for the glucosyltransferase. For the nascent gB precursor, the acquisition of correct intramolecular disulfide bonds may be one of the prerequisites for escape from the cycle.

In most cases previously reported, maximal binding to calnexin appears only after 2 to 10 min of chase (28, 30, 42), which does not reconcile with our findings that maximal binding between calnexin and the 160-kDa gB precursor was seen immediately after the pulse (Fig. 6C and 9). However, Kim and Arvan (35) recently reported that after in vivo reduction with DTT, thyroglobulin binds maximally to calnexin immediately after the pulse and dissociates progressively thereafter. The kinetics of association between calnexin and nascent folding intermediates might be different from protein to protein. In the case of in vivo reduced thyroglobulin, sequential chaperoning of calnexin and BiP/GRP78 was also reported (35). That is, after thyroglobulin is released from calnexin, it subsequently binds to BiP/GRP78. The ER contains many chaperones, such as BiP/GRP78 and GRP94, other than calnexin (24), and processing of nascent polypeptides in the ER is not a simple step but is composed of many steps, including glycosylation, disulfide bond formation, isomerization, and oligomerization (48). So it is reasonable to assume that these chaperones work together to facilitate correct folding of polypeptides. Indeed, increasing evidence of sequential chaperoning has been reported, although the identities of the chaperones and the order in which they operate have not been determined. Melnick et al. (40) demonstrated that BiP/GRP78 preferentially binds an early disulfide intermediate of the immunoglobulin light chain and dissociates within a few minutes, whereas GRP94 exclusively binds fully oxidized molecules and dissociates with a half-time of 50 min. As for a viral glycoprotein, BiP binds maximally to early folding intermediates of VSV G protein, whereas calnexin binds after a short lag to more folded molecules (28). In the case of HCMV gB, since it took a relatively longer time for the 160-kDa gB precursor to be transported from the ER to the Golgi apparatus (half-time of 160 min [Fig. 6C]), it is likely that after release from calnexin, the subsequent interaction of other chaperones such as BiP/GRP78 and GRP94 might occur. However, we could demonstrate the association of BiP with neither the reduced nor the oxidized form of the 160-kDa gB precursor (data not shown). Considering that the association of BiP and folding intermediates previously reported is delicate and quite transient (the dissociation half-times of the VSV G protein and the immunoglobulin light chain from BiP are 3 to 4 min and less than 10 min, respectively) (28, 40), milder lysis conditions or cross-linking might be required to detect the association of BiP with the gB precursor. Alternatively, it is possible that some other chaperones, such as GRP94 and presently unknown ones, might work together with calnexin.

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